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DETECTION AND PRELIMINARY IDENTIFICATION OF OCHRATOXINS AND AFLATOXINS PRODUCED BY *ASPERGILLUS* SPECIES ISOLATED FROM COFFEE

Moussaoui Mohammed[1], Ziane Mohammed[2,3*], Ben Braïek Olfa[4],Yezli Wassim[5,6] and Moussaoui Adellah[1]

[1]Laboratoire de valorisation des ressources végétales et *sécurité alimentaire* dans les *zones* semi *aride du Sud Ouest Algérien, Université de Bechar, Algérie*

[2]Université de Ain Témouchent, route de Sidi bel abbès, N101, Ain Témouchent 46000, Algérie.

[3]Laboratoire de Microbiologie Appliquée à l'Agroalimentaire, au Biomédical et à l'Environnement (LAMAABE), Faculté des SNV/STU, Université de, Tlemcen 13000 Tlemcen, Algérie

[4]Laboratory of Transmissible Diseases and Biologically Active Substances (LR99ES27), Faculty of Pharmacy, University of Monastir, Tunisia

[5]Laboratory of Applied Microbiology, Department of Biology, Faculty of Nature and Life Sciences, University Oran 1 Ahmed Ben Bella, Oran, Algeria.

[6]Faculty of Nature and Life Sciences, Ibn Khaldoun University, Tiaret, Algeria.

*Corresponding author : Tel. : 00213553869293. E mail : ziane.mohammed@yahoo.fr

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ABSTRACT

The presence of mycotoxins in food, such as agricultural products (e.g. coffee, rice, wheat…), is one of the important health issues that threaten the safety consumers. This study aimed to detect and identify mycotoxigenic molds, detect their produced mycotoxins in coffee. Therefore, coffee samples were taken from various locations to carry out the methodology needed to achieve the cited objectives.

The mycological analysis revealed strong contamination dominated by *Aspergillus* with values ranged from 3.4 log CFU/g to 3.6 log UFC / g. Thus, the occurrence of this genus could be linked to a lack of hygiene. Otherwise, other genera have been found to be less abundant in the analyzed coffee samples such as *Alternaria*, *Fusarium*, *Rhizopus*, *Sporobolomyces* and *Ulocladium* are the least abundant in the different samples of the analyzed coffees. In addition, our results showed that all isolated *Aspergillus* species were able to produce mycotoxins.

Thin layer chromatography analysis reported the contamination of various samples by AFB1, AFG1 and OTA. Therefore, this finding was important to conduct the preliminary exposure assessment in the served coffee cups in the cafeterias in Algeria.

***Keywords*:** Molds, Mycotoxins, Aflatoxins B1, Ochratoxins A, Aflatoxins G1, Ground coffee, Algeria.

INTRODUCTION

Coffee is the very famous black sour resulted from roasting coffee seeds grown in more than 70 countries in the world. The majority of global output comes from the top five producers: Brazil, Vietnam, Colombia, Indonesia, and Ethiopia (USDA, 2020). It is the world's second product in circulation after oil, with annual production of around 171 millions of 60 kg bags as for 2019 (Shahbandeh, 2020). Hence, a high number of daily drinking coffee cups could be estimated in the world. Algeria, as other countries, will never be an exception in this issue, but on the contrary, it occupies the first Arab place in coffee consumption at a rate of 135 tons per year, which is equivalent to 3.1 kg per person. The coffee beans are produced in tropical countries, where climatic conditions are favorable for the development of toxigenic fungi (Teferi and Ayano, 2019).

Because of the dry nature of coffee beets and the small amount of water activity (≥12%) (JORAD N°15, 2017, page 9), they are more conducive to fungal growth. Thus, the risk of fungal contamination is higher than that of bacteria. Their growth is depending also to transport, storage time and conditions; especially when Algeria supplied its coffee from different far countries as Côte d'Ivoire, Indonesia, Brazil, Italy and Vietnam (Shahbandeh, 2020). The fungal growth is accompanied with the secretion of mycotoxins, which are silent and secondary compounds released during their development stages and posing a direct threat to the consumer's health. In fact, mycotoxins are known as causative agents of cancerous lesions in several human body parts (Marquardt, 1996). Among the widespread mycotoxins that cause serious outcomes are the Aflatoxin-B1 (AF) and Ochratoxin (OTA) which are mainly secreted by *Aspergillus flavus-parasitucus, A. ochraceus* and rarely *A. niger* (Silva *et al*., 2000; Pandey *et al*., 2000; Noonim *et al*., 2008; Nakajima *et al*., 1997; Djossou *et al*., 2015, Smaoui *et al*., 2020).

During the coffee cup preparation, the heating treatment (100°C) is insufficient to eliminate the mycotoxins previously present in the coffee beans thus making it unfit for

consumption. Indeed, this fact was observed in the work of Karlovsky *et al*. (2016) in which they reported that the mycotoxins are very resistant to heat used during processing and cup coffee preparation. Therefore, the consumers could easily be exposed to these mycotoxins at different concentrations leading to several illnesses ranging from simple to more severe complications.

In this context, the aims of this study were (1) research and identification of potential fungi producing mycotoxins such as Aflatoxin-B1 and Ochratoxin, especially *A. flavus-parasitucus* and *A. ochraceus,* and (2) evaluation of their ability to produce mycotoxins.

MATERIAL AND METHODS

Sampling of coffee beans

In this study, 66 samples were taken from 6 batches of green coffee marketed from different Algerian origins (Bechar 31°37′N 2°13′W, Mascara 35.4°N 0.133333°E, Tlemcen 34°52′58″N 01°19′00″W, Macheria 33°33′N 0°17′W, Sidi Belabess 35°11′38″N 0°38′29″W). These samples include *Robusta* and *Arabica* varieties. Then, they are subjected to microbiological and mycotoxin analyses.

Physicochemical analysis of coffee beans

The physicochemical parameters that were assessed in this study are the moisture and the pH of coffee beans which are the main factors affecting the fungal growth.

(i) Determination of moisture

The water content is determined by drying the sample in an oven set at 105 ± 2°C until obtaining a constant mass (W_1). To prevent moisture recovery, tare vessels were operated in a desiccators (Multon, 1982). The method consists of putting 5 g of coffee sample (W_0) in a glass Petri dish with tared flat bottom (W_t). The relative humidity (RH) of a sample was given by the following formula:

$$\% HR = (((W_0-W_t)-(W_1-W_t))/(W_0-W_t))\times 100$$

HR= relative humidity (%);

W_0 = Initial weight of the sample of coffee beans (g);

W_1 = constant weight (g) after drying.

(ii) pH measurement

For pH determination, 5 g of the sample was added to 45 mL of distilled water, with continuous stirring and then left to stand for one hour. The pH measurement was performed using the pH meter as described by Multon (1982).

Fungal enumeration, isolation and identification

(i) Total fungal enumeration

This indirect method was used to count the external and internal flora. It consists of adding 5g of each sample to 45 mL of sterile physiological water, then adding 2 to 3 drops of Tween 80. Three Petri dishes were surface spread with 1 mL of each dilution, then incubated at 25°C for 5-7 days. Two media were used for the enumeration: PDAac (Potato Dextrose Agar acidified) and PDArb (PDA with red Bengal). The PDAac medium was acidified to a pH between 4.5 and 5 by adding 1 mL of lactic acid at 25%. On the other hand, the PDArb medium was prepared according to Larpent (1990) by adding 2% of red Bengal to PDA medium in order to inhibit bacterial growth and limit the fungal size in the Petri dish thus making the counting easier.

(ii) Ulster method

As shown by Cahagnier *et al*. (1998), this method did not give any indication on the contamination identity. It allows the identification of low spore forming genera, such as *Alternaria* or *Fusarium*, even in the presence of the very spore forming genera such as *Penicillium* or *Aspergillus*.

It consists of deposing randomly 100 g of each sample at a rate of 10 beans per Petri dish containing PDAac or PDArb medium. The dishes were incubated at 25°C for 5 to 7 days.

(iii) Modified Ulster method

It is a method that detects depth fungi. It consists of beans surface disinfection using sodium hypochlorite ($NaCl_2O_3$) marketed at 4°, to avoid possible contamination by the outside environment germs. Indeed, 100 beans of each sample were introduced into the bleach for 2 min, then washed with distilled water 2 to 3 times. After that, they were deposited in Petri dishes containing PDAac and PDArb media, at a rate of 10 beans per dish. Dishes were then incubated at 25°C for 5 to 7 days.

(iv) Purification and conservation of fungal isolates

Purification consists of aseptically transferring the different fungal strains isolated by the different methods (dilution method, Ulster and Ulster modified method), on Petri dishes containing PDA medium. Pure strains were stored in PDAac tubes that were cooled to 4°C to maintain viability, limit variation and facilitate subsequent identification.

(v) Genius identification by micro-culture technique

As described by Haris (1989), the micro-culture technique consists of inoculating fungal spores on a strip of small squares of red Bengal PDA medium and covering them with a strip. The spores were seeded at the periphery limits of the medium to provide them a high oxygen potential needed to germinate. The whole is conditioned in a sterile and humid chamber and incubated at 25°C for 3 to 5 days.

After incubation, the lamellae, to which the mycelium adheres, were transferred to other sterile slides containing drops of cotton blue to allow mycelium swelling and facilitate microscopic observation.

Observations were made at magnifications of 10X, 40X and 100X. The cultural and microscopic characters as previously described by Leslie and Summarell (2006) determined the genera.

(vi) Identification of potential mycotoxin-producing *Aspergillus* species

The identification of *Aspergillus* species was carried out by the method of Pitt (1973) and Ramirez (1982). This method is called "Single Spore" and is based on the relationship between the water activity of the culture medium and the incubation temperature. It consists of the inoculation of few spores of a young culture in hemolysis tubes containing a semi-solid suspension based on 0.2% agar and few drops of Tween 80. From this suspension, different culture media were selected.

In the *Aspergillus flavus* and *parasiticus* agar (AFPA), *A. flavus-parasiticus* strain shows a yellow-orange to orange color on the reverse side of the colony. This coloration is due to the production of aspergillic acid which reacts with ferrous

ammonium citrate to form a colored complex after incubation at 25°C for 5 days.

(vii) Production of mycotoxin in YES liquid media

To test the production of Aflatoxin and Ochratoxin, all identified strains as *Aspergillus* strains were inoculated in 50 mL of the YES liquid medium, in sterile 250 mL vials and incubated at 25°C for 14 days.

After incubation, the culture media were filtered through Wathman filter papers n°1 (11µm, 110mm), then, the filtrates were recovered and disposed for the fungal biomass.

Afterward, 50 mL of filtrate was added to 100 mL of chloroform with energetic stirring for 30 min to separate the aqueous phase from the chloroformic phase with a separating funnel. The experiment was repeated by adding 50 mL successively and 30 mL of chloroform in the aqueous phase that was collected each time with a stirring for 15 and 10 min.

The chloroformic phase was concentrated by vacuum evaporation using a rotavapor in 50 mL conical bottom flasks, placed in a water bath at 60°C, and anhydrous sodium sulfate (Na_2 SO_4) was added to absorb the residual water molecules. The concentration was made until 2 to 3 mL of volume was obtained.

(viii) Detection of mycotoxins in coffee beans

(a) Mycotoxins extraction

Fifteen gram of each crushed green coffee sample was added to 100 mL of a solvent mixture (chloroform – methanol (v/v)). The mixture was stirred for 10 min and the liquid phase was separated from the base by filtration. This operation was repeated, each time after filtration, by adding 50 and 30 mL of solvents to the recovered base. The filtrate was then concentrated to a volume of 2 to 3 mL by evaporation with rotavapor.

(b) Mycotoxins purification

The obtained extract was spread on a 2% agar gel previously cast on Petri dishes and then solidified. The Petri dishes were left ajar to allow evaporation of the extraction solvent, and then kept at 4°C for 24 hours.

After mycotoxin diffusion inside the agar, the surface was wiped several times with hexane-soaked filter paper to remove the macromolecules from the organic matter. The agar gel was then cut into small squares and mixed with 100 mL of chloroform. It was stirred for 10 minutes and then filtered. The obtained liquid was added to 50 and 30 mL of chloroform and stirred each time it was recovered after filtration. The obtained filtrates were also mixed and concentrated with a rotavapor to a volume of 2 to 3 mL.

(ix) Mycotoxin detection by Thin-Layer Chromatography (TLC)

The TLC is a classical method which allows the separation of the constituent compounds of a sample between two phases, static and mobile, according to their affinities.

On a ready-to-use TLC plate of silica gel (20 cm, 20 cm), two lines were drawn parallelly to the start and the finish, 1.5 cm at the bottom and 1 cm at the top of the plate. In the starting line and in the first position, the spots of the concentrated extracts of the isolated strains were deposited on YES medium and the extracts obtained from selected samples of green coffee, in the order of 60 µl per spot, were deposited at intervals of 1 cm.

The TLC plate was deposited vertically in the migration vessel filled to a height of 0.5 cm, with the migration solvent composed of toluene/ethyl acetate/formic acid (5v/4v/1v). After saturation, the sample constituents were eluted by the mobile phase, which migrate by capillarity to the top of the plate.

The reading of the TLC plate was realized in a dark room under UV lamp at 366 nm, and the revelation of Ochratoxin A results in a blue-green fluorescence and the presence of Aflatoxin B1 is indicated by a blue fluorescence.

Statistic analysis

A one-way variance analysis (ANOVA) was performed by the Minitab software for each parameter (Relative humidity, pH, mycotoxins detection). Means and standard errors were calculated and probability level of $P<0.05$ was used to test the statistical significance of all experimental data. The significance of mean values in multiple comparison was performed using Tukey's post hoc test at $P<0.05$.

RESULTS AND DISCUSSION

Physicochemical analysis

(i) Relative humidity (RH)

Relative humidity is an essential factor for fungi development and mycotoxins production, especially in poorly hydrated foodstuffs. The RH for all green coffee samples ranged between 9.1% and 11.6%. According to ANOVA analysis, no significant ($P> 0.05$) differences were observed between the samples. The RH for all green coffee samples did not exceed 12%, which is in agreement with the Algerian legislation (JORAD N°15, 2017, page 9) and Codex Alimentarius (CX/CF 09/3/8 add 1, 2009). Effectively, these standards recommended RH values no higher than 12.50%. Regarding the roasted coffee samples, the RH levels ranged from 6.21% to 9.44%. In this line, the ANOVA analysis showed a significant difference ($P<0.05$) between both type green and roasted samples. This difference was due to the roasting conditions in terms of the time and temperature factors and the storage conditions.

(ii) pH values

The pH results indicated that all samples were slightly acidic with pH values ranging from 5.21 to 5.98 and 5.3 to 5.9, for the green and the roasted coffee bean samples, respectively; no samples and beans type effect was highlighted according to ANOVA analysis ($P>0.05$). These values were in accordance with pH values of Brazilian samples (5.10±0.02), whereas were higher than those observed in Ethiopian-Ardi ones (4.85±0.09) (Rao and Fuller, 2018). Therefore, in these physicochemical conditions (low RH and slight acidity), the fungi could grow and produce their secondary metabolites, especially mycotoxins as shown by Le bars and Le bars (1987). In fact, this kind of products was characterized by a dominance of fungi, especially xerotolerant types that could be found in cereals too (Cahagnier, 1998).

Mycological analysis

(i) Total fungi enumeration

The fungi enumeration by dilution method revealed the contamination of all bean samples (green and roasted) ranging from 1.04 log CFU/g to 3.66 log CFU/g. No significant differences (P>0.05) in contamination were shown between green and roasted beans of coffee when using the PDA medium. On the other hand, fungi counting on PDAac medium revealed contamination levels ranging from 1.2 to 3.63 log CFU/g and from 1.04 to 3.66 log CFU/g, for the green and roasted coffee beans, respectively. Otherwise, counting on PDAab showed fungal contaminations of 1.95 to 3.64 log CFU/g and 1.9 to 3.56 log CFU/g, for green and roasted coffee bean samples, respectively.

The fungal genera have been identified as *Aspergillus* (A), *Penicillium* (B), *Rhizopus* (D), and *Ulocladium* (C) (Figure 1). The identification of the fungal flora in the analyzed samples indicated that the genera of the storage flora were the most frequent which were represented by *Aspergillus* particularly *A. ochraceus* and *A. flavus-parasitucus*.

In this study, the detected fungi were similar to those found by Bokhari and Aly (2009). Nganou *et al*. (2014) showed a high level of infection by fungi, in all processes, after drying of coffee beans, particularly, *Penicillium*, *Mucor*, *Fusarium*, *Rhizopous*, *Scopulariopsis* and *Aspergillus* spp., notably the well-known OTA-producing fungal species (*A. ochraceus*, *A. niger* and *A. carbonarius*). In general, the presence of some filamentous fungi gives an indication about the safety of food products. Some of the species identified are related to mycotoxin production such as *Aspergillus niger*, *A. carbonarius*, *A. ochraceus*, *A. flavus*, and *Penicillium citrinum* (Nganou *et al*., 2014).

(ii) *Aspergillus* identification

The fungal estimation, from green coffee beans on PDAac medium, showed the dominance of *Aspergillus*, including *A. niger*. The contamination rates of *A. niger* were 7.20% and 83.60% for samples 9 and 2, respectively. The contamination levels of *A. flavus-parasiticus* were 1.13% and 47.60% for samples 2 and 10, respectively. The values of *A. ochraceus* were 1% and 7.14% for samples 2 and 7, respectively.

Otherwise, PDArb medium counting results also indicated the dominance of *Aspergillus* particularly *A. niger*. This species presented contamination values of 1.11% and 73.70% for samples 11 and 2, respectively. For *A. flavus-parasiticus* species, the values were 0.33% and 22.10% for samples 11 and 1, respectively. *A. ochraceus* species indicated respective values of 0.26% and 57.10% for samples 9 and 5.

The fungal estimation on PDAac medium, from roasted coffee beans, revealed the dominance of *Aspergillus* species especially *A. niger*. The contamination rates of A. *niger* were 1.25% and 75% for samples 9 and sample 1, respectively. Regarding the *A. flavus-parasiticus* rates, they were 1% and 36% for samples 4 and 10, respectively. The values of *A. ochraceus* were 1% and 5.20% for samples 6 and 5, respectively. Hence, it could be noticed that the same *Aspergillus* species dominance was observed with PDArb medium. *A. niger* species presented contamination values of 1% and 62% for samples 6 and 2, respectively. For *A. flavus-parasiticus* species, the contamination values were 1.1% and 15.10% for samples 10 and 9, respectively. *A. ochraceus* species appeared with values of 0.5% and 67% for samples 1 and 5, respectively. According to the contamination rates of our green coffee bean samples analyzed by the Ulster method, the values were 82% for sample 7 and 100% for each of samples 1, 2, 4 and 10. However, by the modified Ulster method, the proportions were 10% and 97% (P<0.05), respectively for samples 9 and 2.

From both methods (Ulster method and modified Ulster), it was stated that *Aspergillus* was the dominant genus, particularly *A. niger* species with a rate of 100%. All green coffee beans revealed the presence of *A. niger*. *A. ochraceus* was present with percentages between 12% for sample 11 and 70% for sample 10, and *A. flavus-parasiticus* occurred with 7% and 87%, respectively for sample 5 and sample 1.

As shown by Perrone *et al*. (2007), fungal contamination by *Aspergillus* notably *A. niger* has been detected in several countries. In fact, Kuntawee and Akarapisan (2015) reported a contamination of 63.9%. Otherwise, Perrone *et al.* (2007) indicated that 75% of Arabica coffee beans samples were contaminated by black Aspergilli in the North Thailand. Other authors reported a dominance of *A. ochraceus* in Brazilinan coffee (Taniwaki *et al*., 2003). In addition, they reported in coffee beans samples (n=408) collected from the states of São Paulo and Minas Gerais the presence of *A. niger* as the most common contaminant species. Nega (2014) showed that the occurrence of these fungal species was somewhat heterogeneous in coffee beans and *Aspergillus* spp. had the highest contamination level (49.375%).

In this study, the results showed no dependent relationship between *Aspergillus* species contamination and the coffee types (green or roasted). Similarly, several researchers showed this fact with green and roasted coffee bean types (Perrone *et al*., 2007; Leong *et al*., 2007; Taniwaki *et al*., 2003 and Pardo *et al*., 2004). Otherwise, some species were absent in the samples. This lack could be explained by the difficulty of identification between *A. westerdijkiae* and *A. ochraceus*. Contrary to this study, other species were found in samples collected from different supermarkets located in the Riyadh city (Saudi Arabia), such as *A. flavus* (50%), as well as *A. carbonarius* and *A. flavus* strains (80%) with Ochratoxin (OTA) production (Moslem *et al*., 2010).

Finally, the results of the three enumeration methods revealed the presence of potentially mycotoxin-producing species in all studied isolates, among which *A. niger* was the most frequent species detected (50%), followed by *A. ochraceus* (30%), then *A. flavus-parasiticus*, *A. versicolor*, *A. wentii*, and other *Aspergillus* spp. Among the identified species, only *A. ochraceus* and *A. flavus-parasiticus* produced the Aflatoxins (AF) and Ochratoxins (OTA) as main mycotoxins (Diener *et al*., 1987).

Several researchers reported a fungal growth and mycotoxins production in coffee beans at different stages of its consumption. The genus *Aspergillus* could grow at different media with low humidity. Indeed, according to El-khoury (2007), the temperature and humidity are among the factors that limit its growth and its toxinogenic activity. The fungi could grow and produce mycotoxins with optimum

temperatures ranging from 20°C to 30°C. These temperatures were often the storage temperatures of coffee beans.

(iii) Search for mycotoxinogenic strains

Chromatographic separation of extracts from the culture of four *A. flavus-parasiticus* and four *A. ochraceus* strains isolated from coffee worms and grown on YES medium, showed that all isolated strains (*A. flavus-parasitucus* and *A. ochraceus*) were mycotoxin producers. *Aspergillus* species are potential producers of OTA and AF. The mycotoxigenic species, especially OA producers, were found in 82% of the geographical regions examined by Djossou *et al.* (2015). However, Magnani *et al.* (2005) had found only 3% of studied isolates (*A. ochraceus* and *A. carbonarius*) that were capable of ATO production. In fact, *A. ochraceus* is able to produce OTA in coffee beans (Batista *et al*., 2003; Batista *et al*., 2009; Santini *et al*., 2011; Taniwaki *et al*., 2003; Velmourougane *et al*., 2011; Logrieco *et al*., 2003). OTA is a mycotoxin with nephrotoxic, immunosuppressive, teratogenic and carcinogenic effects. Otherwise, Aflatoxins are hepatotoxic carcinogens with a genotoxic mechanism of action (Tsubouti *et al*., 1987).

Indeed, the mycotoxins are high stable compounds during storage and roasting treatment. They have heat resistance to technological treatments during roasting and preparation. Hence, the mycotoxins remain in the coffee at consumption time even after removing fungi (Cast, 2003). Several researchers found fungi and their produced mycotoxins in the coffee (Ozden *et al*., 2012; Battacone *et al*., 2010).

Qualitative detection of AF and OTA in coffee beans

Chromatographic assay revealed the presence of Aflatoxin B1 in all analyzed samples e.g. 1, 2, 3, 4, 5, 6 and 7 (Figure 2). Otherwise, OTA results found in all analyzed coffee beans samples (Figure 2) were similar to those reported by Nganou *et al.* (2014). No significant difference was reported with ANOVA analysis ($P > 0.05$).

Other reports have previously demonstrated that a reduction in the OTA content occurs following the roasting process, although additional factors should be considered, including the heterogeneity of coffee beans contamination, natural contamination versus artificial contamination, the performance of analytical methods and the roasting conditions (Scott, 1996).

Faced with the food risks caused by these toxic molecules and the emergence of a feeling of insecurity, consumers are increasingly demanding healthy products with guaranteed food quality and safety.

The implementation of preventive measures (good agricultural practices including the choice of varieties, monitoring throughout the food chain, etc.) can sometimes prove to be insufficient leading to the need of finding means of deactivation after technological treatments.

The most frequent mycotoxins found at highest concentrations were Ochratoxins with maximum levels stated at 12.2µg/kg. The contamination with Aflatoxin B1 was sporadic with a maximum level of 1.2µg/kg (Bessaire *et al*., 2019). It has been demonstrated that all studied mycotoxins were detected in samples with mean concentrations ranging from 0.69 µg/kg to 282.89 µg/kg (García-Moraleja *et al*., 2015).

CONCLUSION

The results of this work considered among a preliminary finding to carry out an advance possible works to the exposure assessment especially linked to mycotoxins contamination in coffee. Results showed a mycotoxigenic fungi contamination especially *Aspergillus* species with its metabolites mycotoxins in the coffee for Aflatoxins and Ochratoxins detected by TLC method. The level of fungi contamination and mycotoxins production are strongly affected by the lack of hygiene and poor storage. In the field of food safety, our work participates in such an analysis on research into the exposure assessment associated with contamination of coffee by mycotoxins that consist to assess the probability of the individual exposure to mycotoxins residues in the coffee cup at time of consumption.

The results of this present work are encouraging and confirm the interest of the study of coffee molds and mycotoxins contaminations. For this and in perspective we propose: (1) monitoring a storage condition of coffee beans at all consumptions steps, (2) the need for further studies of contamination by other mycotoxins in coffee during storage, (3) quantify the mycotoxins concentrations in coffee beans, (4) monitoring some hygiene regulations in cafeteria, and (5). Establish a exposure assessment model for coffee consumption in Algeria.

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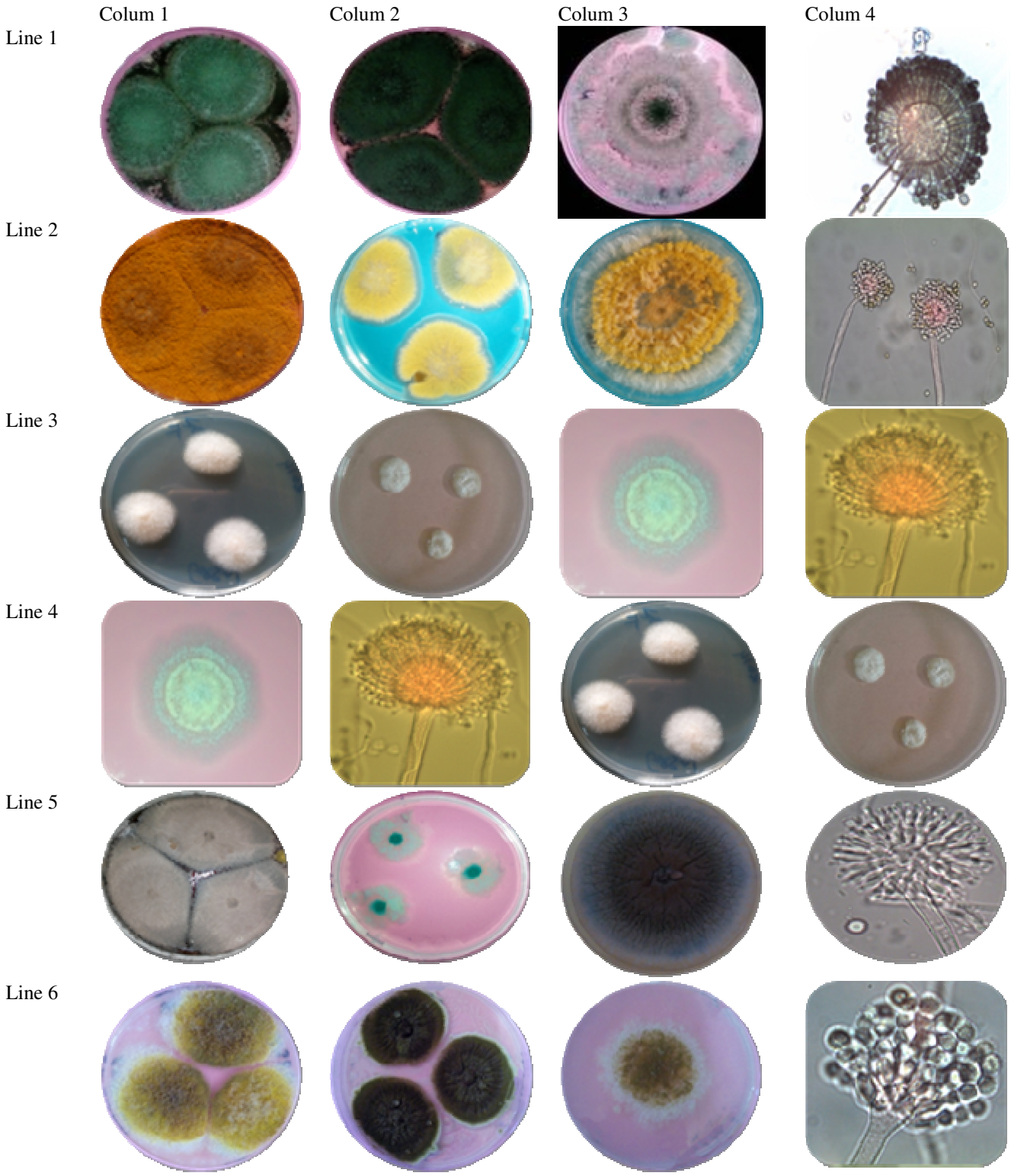

Fig. 1 : Macroscopic observation of isolated fungi by « Single Spore »method. Line : (1) *Aspergillus niger*, (2) *A. flavus-parasiticus*, (*3) A. ochraceus*, (*4) A. ochraceus*, (5) *A. versicolor* and (6) *A. wentii.* Colum : 1 G25N medium, 2 : 2 CYA (37°) medium, 3: CDA medium and 4 microscopic observation 1000x.

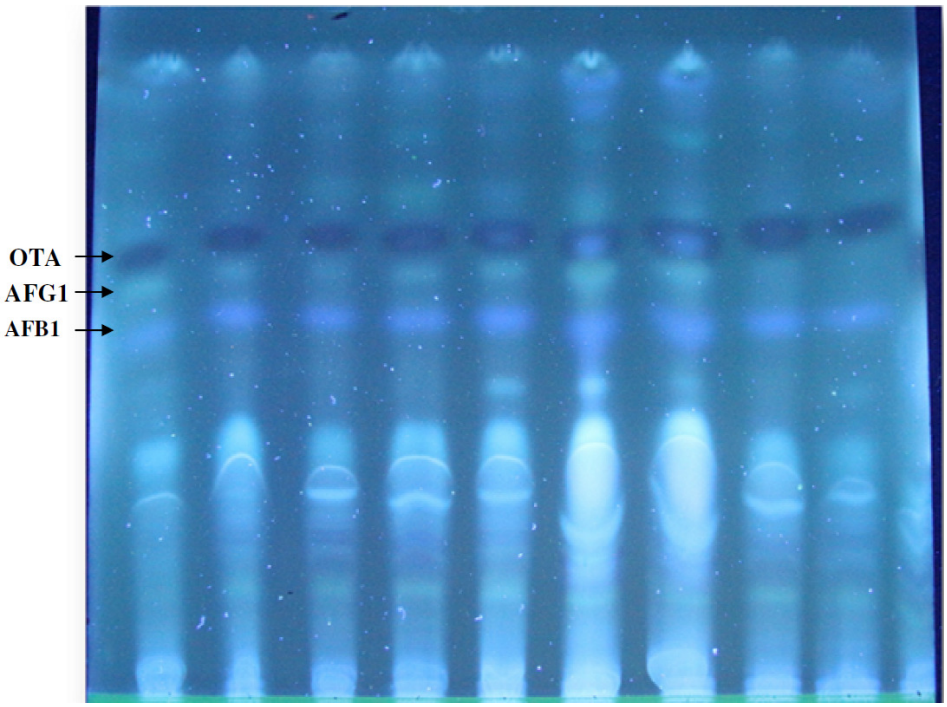

Fig. 2 : TLC profile of different analysed samples of green coffee.