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AFLATOXIN AND OCHRATOXIN PRODUCTION IN GROUND COFFEE DURING STORAGE

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ABSTRACT

Coffee beans can be affected by storage microorganisms, the most notable of those are the fungi that cause severe deterioration of the infected beans. The objective of this study was to investigate the existence of fungi in the samples of Arabica and Robusta ground coffee, as it was observed that they were contaminated with fungi, based on the degree of roasting, humidity and storage duration. The results show the capability of the fungi to produce aflatoxin and ochratoxin A in ground coffee of harari and barri as well as Robusta coffee stored in various temperatures and moisture conditions. Aflatoxin and ochratoxin A were produced in most samples in various amounts. Furthermore, there was not any correlation between the number of existing fungi and the concentration and types of these toxins. For the samples stored in 10% humidity, the total of aflatoxin in barri and dark roasted coffee was 4.387 µg/g, but cappuccino was free of aflatoxin. For the samples under relative humidity of 25%, the french coffee was the most contaminated with toxins with Aflatoxin concentration of 10.436 µg/g, whereas Barri mild roasted coffee was the least contaminated with total Aflatoxin concentration of 0.547 µg/g. In the samples of the relative humidity of 45% stored for 30 days at room temperature, the Barri white roasted coffee was the most contaminated samples, as the total toxins 4.604 µg/g, whereas there wasn't Aflatoxin in Nescafe samples. Regarding Ochratoxins, it has been observed that in twelve samples out of thirty three samples, the fungi couldn't produce ochratoxins. That means that 63.64% of the total samples have produced such toxin, and Cappuccino samples stored for 30 days in 10% humidity have been contaminated with the highest rate of Ochratoxin with 1.40µg/g.

Keywords: Ochratoxin A, Aflatoxin, ground coffee, storage, *Aspergillus*

INTRODUCTION

Ochratoxin A (OTA) can be found in various food products, including the green coffee beans, roasted coffee and instant coffee. It has been found that such toxins are genotoxic, carcinogenic, teratogenic, and immune suppressive, thus causing harm to animals including human beings as stated by Schlatter *et al.* (1996) and Holzhauser *et al*. (2003). Many studies have confirmed the ability of stored fungi to produce toxins, mainly Ochratoxins in the green coffee beans (Nakajima *et al.*, 1997; Romani *et al.*, 2000; Otteneder and Majerus, 2001; Pittet and Royer, 2002; Batista *et al.*, 2002). Surveys conducted on green coffee beans stored from different origins established that African coffee samples contained the highest concentration of Ochratoxins compared to samples of American and Asian origin (Pardo *et al.*, 2004). Filamentous fungi can affect the quality and safety of the final product due to production of mycotoxins during preparation and storage (Batista *et al.*, 2002; Taniwaki *et al.*, 2003; Quiroz *et al.*, 2005; Taniwaki, 2006). Ochratoxin A (OTA) is a mycotoxin produced by secondary metabolism of many filamentous species belonging to the genera *Aspergillus* and *Penicillium* (Keeper-Goodman and Scott, 1989; Miller and Trenholm, 1994; Bredenkamp *et al*., 1989; Budavari, 1989).

Little information is available on the existence of toxins of Ochratoxin A in coffee beans during hydration and mechanical treatment and the impact of these operations on the existence and production of Ochratoxin A. The data published so far indicates that the process of removing pulp reduces the risk of contamination by these toxins through fermentation during drying and there is a need to study the microbiological processes (Frank, 2001), and to protect coffee from contamination by Ochratoxin A, there is a need to know what fungi are capable of producing these toxins and their relationship with drying of coffee grains and process of removing pulp, It was found that there are low concentrations of these toxins in the coffee produced by many countries (Buchcli *et al*., 1998; Pardo *et al*., 2004). Humans are inevitably exposed to a certain levels of these toxins. The pollution with this toxin can happen during the process of drying (Urbano *et al*., 2001), where they noticed the presence of these toxins in the beans prior to storage and pointed out the possibility that the harvest and post-harvest stage may cause the pollution. The frequent occurrence of pollution was confirmed by Le-Bars and Le-Bars (2000) in crops all over the world, which is the

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main fungal poison tainting coffee beans. It has been suggested that *A. niger* and closely related species such as *A.carbonarius* can produce Ochratoxin A in the coffee beans (Bucheli *et al*., 1998), while the *Aspergillus ochraceus* Fungus is the most significant producer of Ochratoxin A in coffee beans (Pitt, 2000).

Heenan *et al*. (1998) and Taniwaki *et al*. (2003) stated that 2-3% of *A. niger* separated from coffee beans was able to produce ochratoxin whereas 13% of *A. niger* taken from Thailand coffee was able to produce OTA and OTB. But these contaminations are few compared with *A. carbonarius* which is considered as the most contaminating source for the Thailand coffee.

The existence of ochratoxin A in the coffee is not desirable because it could hamper the coffee trade and affect the economics of coffee-producing countries. The European Commission did not specify minimum levels of ochratoxin A in coffee. There is only one recommendation, in which there is a reference to the level of 3 mg/kg, which was a proposal of a member in the European Union (Romani *et al*., 2000). Another study, Silva *et al*. (2000) reported that contamination of Arabic coffee beans (*Coffea arabica* L.) with ochratoxin A as a result of fungal contamination with *Aspergillus* species including: *A. suphureus, A. ochraceus, A. melleus, A. dimorphicus, A. Sclerotiorum, A. auricomus, A. ochraceus, A. sulphureus, A. sclerotiorum* were producing ochratoxin, as well as *A. niger. A. foetidus* taken from 128 samples, (44.29%) of them is capable of producing ochratoxin A while (30.80%) is not able to produce this toxin.

In a previous study carried out by Joosten *et al*. (2001) on more than 14 samples of green coffee from Southern Thailand, it was found to be contaminated by black molds, and it was observed that half of them were related to *A. carbonarius*. Based on this, we assume that the black fungus, especially fungus *A. carbonarius* plays an important role in the contamination of coffee beans with OTA in Southern Thailand.

Through the studies carried out by Taniwaki *et al*. (2003) on the arabic coffee, the results indicate that *A. niger* is the major fungus exists in the coffee, where it produced ochratoxin with the rate of 63% of the total ochratoxin produced, while the *A. ochraceus* was 31% of the total isolates and produced 3% of the total ochratoxin, and he also stated that 75% of fungi isolated were able to produce ochratoxin. Also *A. carbonarius* was isolated from 6% of the samples taken from the warmer areas during storage, and 77% of the isolates of fungus was capable of producing the toxins.

The increase in the production of ochratoxin in coffee stored at different moisture levels for a period of 20-30 days of storage was observed. There was also a notable difference in the variation and physiological characters of fungi, and usually the poor storage and high humidity caused the production of ochratoxin within ten days (Ahmed and Magan 2006), while Betancourt and Frank (1983) stated that it is necessary that the humidity content in the coffee beans should be less than 14.5% to prevent the mold during storage.

Premila and Sanchez (2006) studied the effects of temperature and various farm environments on the growth of *A. flavus* and the production of Aflatoxins contaminated peanuts in Georgia. The results have shown that the fungus could grow at a temperature of 10^{o}C and produced aflatoxin, as well as the temperature of 37^{o}C. They got the highest production of fungal growth and toxin at a temperature of 27, 30^{o}C in three different farm environments which are potato Dextrose agar (PDA) and nutrient agar (NA) and corn maltose agar (CMA).

Bokhari (2007) reported that the beans of coffee were highly polluted with toxins fungi, especially those producing ochratoxin A. The incidence of this pollution and production of mycotoxins starts from the harvest, where production is affected by the moisture content of the beans and grow during transport and during storage and marketing.

In a study performed by Ilic *et al*. (2007) on Vietnamese robusta coffee beans, it was found that *A. nige*r is the only type which produces OTA. Leong *et al*. (2007) tested the ability of 13 isolates of the fungus *A. carbonarius* isolated from Arabic coffee samples and robusta coffee arbica on toxin OTA production and found that 11 of them were able to produce these toxins.

Moslem *et al*. (2010) were able to isolate a group of fungi associated with the beans of coffee where *A. flavus* achieved highest frequency rate of 50%, followed by the *A. carbonarius*. They found that 80% of the isolates of the *A. flavus* were able to produce OTA toxins. Alborch *et al*. (2011) studied the ability of *A. niger and A. carbonarius* isolated from corn beans to produce OTA toxins where they noted that the optimum temperature to produce the highest rate of toxins of the two fungi was 15^{o}C.

Iqbal *et al*. (2011) found that contamination of samples of chilli with Aflatoxin increased to 61% of samples stored at a temperature of 25, 30^{O}C from those stored at 20^{O}C and number of fungi increased directly proportional to increasing the storage period.

Aflatoxin contaminated coffee is considered a global problem. Taniwaki (2006) mentioned that a lot of green coffee beans samples were contaminated with these toxins in varying degrees ranging from 2.0 - 360 µg/kg. This study aimed at studying the effect of storage period, temperature, humidity and degree of roasting on fungal

pollution and producing aflatoxins and ochratoxin in ground coffee.

MATERIALS AND METHODS

Storage studies on ground coffee

The Fungal invasion and ability of *Aspergillus* and *Penicillium* to produce aflatoxins and ochratoxins were studied, where plastic boxes were equipped with 100 grams of ground coffee for tested varieties and then placed in the center of each packet Cup small glass by concentration calculated from sulfuric acid so that each can has the required concentration (10, 25, 45%) of the relative humidity (Solomen, 1951). Then all boxes were stored at room temperature (25 ± 2) and for different periods of time (30, 20, 10 days). The results were counted of numbers of fungal colonies per 1gram of ground coffee (Alvnindia and Acda, 2010).

Mycotoxins analysis

Apparatus of High Performance Liquid Chromatography (HPLC) system

The High Performance Liquid Chromatography (HPLC) system consisted of Waters Binary pump Model 1525, a Model Waters 1500 Rheodyne manual injector, a Waters 2475 Multi-Wavelength Fluorescence Detector, and a data workstation with software Breeze 2. A phenomenex C_{18} (250x 4.6mm i.d), 5um from Waters corporation (USA) for aflatoxins**.** A HyperClone 5µ ODS column (C_{18}) 120A° , DIM: 250 x 4.60mm (Phenomenex).

Extraction of Aflatoxins by VICAM method (2000)

Sample Extraction

Weigh 25g sample with 5g salt sodium chloride and place in blender jar. Add to jar 125ml methanol: water (70:30). Cover blender jar and blend at high speed for 1 minute. Remove cover from the jar and pour the extract into fluted filter paper. Collect filtrate in a clean vessel.

Extract Dilution

Pippet or pour 15ml filtered extract into a clean vessel. Dilute extract with 30mL of purified water, mix well. Filter diluted extract through the glass microfiber filter into a glass syringe barrel using markings on barrel to measure 4ml.

Immunoaffinity Chromatography

Pass 15 ml filtered diluted extract (15ml = 1g sample equivalent) completely through AflaTest ®-P affinity column at a rate of about 1-2 drops/second until the air comes through column. Pass 5ml of purified water through the column at a rate of about 2 drops/second. Elute affinity column by passing 1.0ml HPLC grade methanol through the column at a rate of 1-2 drops/second and collecting all of the sample eluate (1ml) in a glass vial. Evaporated to dryness under a stream of nitrogen and was determined of HPLC.

Detection and determination of Aflatoxins by HPLC

Derivatization

The derivatives of samples and standard were done as follow:100µl of trifluoracetic acid (TFA) were added to samples and mixed well for 30 s and the mixture stand for 15min. 900µl of water: acetonitrile (9:1 v/v) were added and mixed well by vortex for 30 s and the mixture was used for HPLC analysis.

HPLC conditions

The mobile phase consists of Acetonitile/Water/ methanol (1:6:3). The separation was performed at ambient temperature at a flow rate of 1.0 ml/min. The injection volume was 20µl for both standard solutions and sample extracts. The fluorescence detector was operated at an excitation wavelength of 365nm and an emission wavelength of 450nm. AFB_1 concentration in the samples was determined from the standard curve, using peak area for quantification.

Ochratoxin analysis

HPLC Equipment

The HPLC system consisted of Waters Binary pump Model 1525, a Model Waters 1500 Rheodyne manual injector, a Watres 2475 Multi-Wavelength Fluorescence Detector, and a data workstation with software Breeze 2.

Chemicals and Reagents

OTA standard, Chartist, microfiber filter 1.5µm, and filter papers were purchased from VICAM. Milford, MA USA. Acetonitrile, glacial acetic acid HPLC grade were obtained from BDH, England. Sodium chlorid, sodium biocarbonate, sodium hydrogen phosphate, potassium dihydrogen phosphate and potassium chloride were purchased from (BDH, Merck chemicals). And tween -20 obtained from Sigma (St. Louis, MO, USA).

HPLC condition

A Symmetry C_{18} (5 µm particle size, 150 mm X 4.6 mm i.d.) from the Waters Corporation (USA), were used along with a mobile phase of Acetonitile/Water/ acetic acid (55:43:2). The separation was performed at ambient temperature at a flow rate of 1.0 ml/min. The injection volume was 50µL for both standard solutions and sample extracts. The fluorescence detector was operated at an excitation wavelength of 330nm and an emission wavelength of 470nm. OTA concentrations in coffee extracts were determined from the standard curve, using peak area for quantitation.

Instant coffee

The instant coffee extraction carried out according to the method of Pittet *et al*. (1996). Briefly: Five gram of soluble coffee was weighed accurately into plastic centrifuge bottles and mixed with respectively 100ml of methanol-3% aqueous sodium hydrogen carbonate

(50:50). The suspension was blended for 3min at medium speed using a blander. Then homogenized sample was filtered through 11cm Whatman GF/B glass microfiber filter under reduced pressure. Then 4 ml of filtrate was transferred to a graduated cylinder and diluted to 100 ml with PBS pH 7.4. The whole diluted extract was applied to an immunoaffinity column, at a slow, steady flow rate of 2-3 m/min. After washing the column with 10 ml of distilled water, OTA was eluted with 1.5ml of methanol. To ensure complete removal of the bound toxin, the methanol was left in contact with the column for at least 3 min. This was achieved by reversing the flow of methanol (back flushing) two or three times. The elute was then evaporated to dryness under a stream of nitrogen at 40°C, and the residue was redissolved in 3 ml of HPLC mobile phase.

Statistical Analysis

The results obtained in this research were analyzed statistically using the sixteenth version of SPSS16 program where transaction averages were compared at the abstract level (0.05) using the least significant difference test (LSD) designed by Norusis (1999).

RESULTS

1. Effect of storage period and the percentage of moisture and the degree of roasting on the fungi presence in ground coffee for Arabic coffee types

Results in table 1, show the storage of ground coffee for 10days on the humidity rates of 10, 25, 45% and at room temperature indicate the presence of many fungi in ground coffee as varied in their distribution to the different classes and gave the following percentages 61, 41, 25.19, 9.44, 1.57, 0.78, 0.78% for each of *A. alliaceus, A. niger, A. flavus, A. melleus, A. fumigates, F. solani, Penicillium* sp., respectively.

Table 2 related to the storage of ground coffee according to the previous conditions for a period of 20days indicates the occurrence of fluctuation in the fungi existence, and the isolated fungi were in descending order as follows: *A. niger* (76,03%), followed by *A. tubingensis* (13,22%), *A. flavus* (7,43%), *A. melleus* (1,65%), *A. alliaceus* (0,82%) and *Aspergillus* sp. (0.82%).

The results of table 3 related to the storage of the ground coffee for the period of 30days at different humidity ratios a diversity of fungal isolates in terms of the number and types and in descending order were as follows:

70.87, 12.62, 5.82, 4.85, 2.91, 1.94, 0.97% for the *A. niger, A. alliaceus, A. flavus, A. melleus, Aspergillus* sp., *Penicillium* sp., *F. solani* respectively.

Table 1. Effect of storage (10 days), relative humidity, and the degree of roasting on the fungal diversity of coffee samples of Arabica ground coffee for harari and barri varieties.

Isolates		Harari dark			Harari mild			Harari white			Barri dark			Barri mild			Barri white			Total isolates	% Frequency
		Humidity %																			
		10	25	45	10	25	45	10	25	45	10	25	45	10	25	45	10	25	45		
A. niger		5	3	8	4	2	2	3	4	-	-	-	-	1	-	-	-	-	-	32	25.19
A. flavus		-	-	-	1	1	-	1	1	1	-	2	-	2	-	1	1	-	1	12	9.44
A.fumigatus		-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	0.78
A. melleus		-	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2	1.57
A. alliaceus		-	2	6	1	6	10	-	1	3	9	11	5	5	4	5	4	4	2	78	61.41
F. solani		-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	1	0.78
Penicillium sp.		-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	1	0.78
Total isolates	Total	5	7	14	7	9	12	6	6	4	9	13	5	8	4	6	5	4	3		
	Each of roasted degree	26			28			16			27			18			12			127	
	Each variety	70									57										

Table 2. Effect of storage (20 days), relative humidity, and the degree of roasting on the fungal diversity of coffee samples of Arabica ground coffee for harari and barri varieties.

Isolates		Harari dark			Harari mild			Harari white			Barri dark			Barri mild			Barri white			Total isolates	% Frequency
		Humidity %																			
		10	25	45	10	25	45	10	25	45	10	25	45	10	25	45	10	25	45		
A. niger		7	8	5	4	4	7	3	2	-	7	7	12	2	3	14	1	3	3	92	76.03
A. flavus		-	1	1	1	1	1	1	-	-	-	-	-	-	2	-	1	-	-	9	7.43
A. melleus		-	-	-	1	-	-	1	-	-	-	-	-	-	-	-	-	-	-	2	1.65
A. alliaceus		-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	1	0.82
A. tubingensis		-	-	4	7	-	1	2	-	-	-	1	-	1	-	-	-	-	-	16	13.22
Aspergillus sp.		-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	1	0.82
Total isolates	Total	7	9	10	31	5	9	8	2	-	7	8	12	3	6	14	2	3	3		
	Each of roasted degree	26			27			10			27			23			8			121	
	Each variety	63									58										

Table 3. Effect of storage (30 days), relative humidity, and the degree of roasting on the fungal diversity of coffee samples of Arabica ground coffee for harari and barri varieties.

Isolates		Harari dark			Harari mild			Harari white			Barri dark			Barri mild			Barri white			Total isolates	% Frequency
		Humidity %																			
		10	25	45	10	25	45	10	25	45	10	25	45	10	25	45	10	25	45		
A. niger		3	6	10	3	3	6	3	2	2	10	8	2	-	4	1	6	2	2	73	70.87
A. flavus		-	-	1	-	-	-	-	-	-	2	-	1	-	1	-	-	-	1	6	5.82
A. melleus		1	-	-	1	-	1	-	-	-	-	-	-	1	1	-	-	-	-	5	4.85
A. alliaceus		-	-	-	-	-	-	-	-	1	-	-	9	1	2	-	-	-	-	13	12.62
F. solani		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	1	0.97
Penicillium sp.		-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	1	2	1.94
Aspergillus sp		1	1	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	3	2.91
Total isolates	Total	5	7	11	4	3	7	4	2	3	13	8	12	1	8	2	6	3	4		
	Each of roasted degree	23			14			9			33			11			13			103	
	Each variety	46									57										

Table 4 related to averages general activity of fungi at tested humidity levels and periods of storage and the degree of roasting varieties of ground Arabic coffee indicates that the storage period has an effect, where there was a decrease in the rate of fungal pollution with increased storage up to 30 days, where the rate was 36.18, 34.47, 29.34% after storage for 10, 20, 30 days.

With regard to the items, the results show that was more polluted with fungi where the rate of fungi was 50.99% compared Barri varieties in which the percentage of fungal contamination is up to 49.01%.

2. Effect of the storage period and the percentage of moisture on the fungal presence in the types of ground coffee of Robusta Coffee

Results shown in table 5 related to robusta ground coffee stored for 10days indicate the existence of fungi isolated from tested samples and a group of fungi were isolated in descending order by repetition of isolation as follows: 23.40, 27.21, 21.27, 12.76, 10.63, 4.25, 2.12, 2.12, 2.12% for each of *A. melleus, A. flavus, A. alliaceus, A. niger, Alternaria* sp., *Paecilomyces variotii, A. fumigates, F. solani, Penicillium* sp., respectively.

The results shown in table 6 related to the storage of coffee for 20days indicate a diversity of fungi isolated from tested samples and were, in descending order as follows: 22.44, 20.40, 18.36, 16,32, 14.28, 8, 16%) for each *of E. nidulans, A. flavus, A. tubingensis, A. melleus, A. niger, F. solani*, respectively.

Whereas results in table 7 related to the storage for the period of 30days indicate noted decrease in fungi, and ordered dissentingly as follow: *A.niger* (40,74%), followed by *A. melleus* (33,33%), *A. flavus* (7,40%), *A. alliaceus* (7,40%), *E. nidulans* (7.40%) and finally *Aspergillus* sp. (3.70%).

Also the storage period may affect the incidence averages as the general averages show a rise in the number of fungi with the increase in the storage period up to 20days and then decreased again after 30days of storage where it was 38.21, 39.84, 21.95% after 10, 20, 30days, respectively.

With regard to the percentage of humidity, the results in table 8 indicated that there is a difference in the numbers of fungi where a decrease generally observed in the number of fungi with the increased humidity ratio to 35.77, 33.33, 30.09%, when humidity rates are 10, 25, 45%, respectively.

As for the different varieties of coffee it has been observed that there is a remarkable variation in the degree of fungal contamination where pollution averages show the pollution of Turkish coffee at 83.26%, followed by cappuccino with the rate of 58.23% and french coffee with the rate of 33.20% and finally Nescafe without caffeine in 38, 11%.

Table 4. Average general activity of fungi at tested humidity levels and periods of storage and the degree of roasting varieties for ground arabic coffee.

Impact factor			Harari dark	Harari mild	Harari white	Barri dark	Barri mild	Barri white	Total isolates
Total isolates	Each humidity levels	10%	17	24	18	29	12	13	113 (32.19%)
		25%	23	17	10	29	18	10	107 (30.48%)
		45%	35	28	7	29	22	10	131 (37.32%)
	Each periods of storage	10 days	26	28	16	27	18	12	127 (36.18%)
		20 days	26	27	10	27	23	8	121 (34.47%)
		30 days	23	14	9	33	11	13	103 (29.34%)
	Each periods of storage		75 (21.37%)	69 (19.66%)	35 (9.97%)	87 (24.78%)	52 (14.81%)	33 (9.40%)	
	Each variety		179(50.99%)			172(49.01%)			

Table 5. Effect of storage for 10 days and relative humidity on the fungal diversity of coffee samples of Robusta ground coffee.

Isolates		Nescaffee			Cappuccino			Turkish coffee			French coffee			Nescaffee with out			Total isolates	% Frequency
		Humidity %																
		10	25	45	10	25	45	10	25	45	10	25	45	10	25	45		
A. niger		-	1	-	-	-	-	-	2	-	1	-	1	-	-	1	6	12.76
A. flavus		-	-	-	-	5	-	-	1	1	-	2	-	1	-	-	10	21.27
A. fumigatus		1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	2.12
A. melleus		1	-	-	2	4	-	-	4	-	-	-	-	-	-	-	11	23.40
A. alliaceus		-	-	-	-	-	-	6	-	-	-	-	3	-	-	1	10	21.27
F. solani		1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	2.12
Alternaria		-	-	-	5	-	-	-	-	-	-	-	-	-	-	-	5	10.63
P. variotii		-	-	-	-	-	-	-	-	-	-	-	2	-	-	-	2	4.25
Penicillium		-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	1	2.12
Total isolates	Total	2	1	1	7	9	-	7	7	-	1	3	6	1	-	2	47	
	Each variety	4			16			14			10			3				

Table 6. Effect of storage for 10 days and relative humidity on the fungal diversity of coffee samples of Robusta ground coffee.

Isolates		Nescaffee			Cappuccino			Turkish coffee			French coffee			Nescaffee with out			Total isolates	% Frequency
		Humidity %																
		10	25	45	10	25	45	10	25	45	10	25	45	10	25	45		
A. niger		-	-	-	-	-	-	-	-	1	1	-	3	-	1	1	7	14.28
A. flavus		-	-	-	1	1	1	-	1	-	1	3	-	-	-	2	10	20.40
A. melleus		-	-	-	-	-	2	3	-	-	1	1	1	-	-	-	8	16.32
A. tubingensis		1	-	-	-	-	-	5	-	-	1	-	-	1	-	1	9	18.36
E. nidulans		11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	11	22.44
F. solani		2	-	-	-	-	-	-	-	-	-	-	-	-	-	2	4	8.16
Total isolates	Total	14	-	-	1	1	3	8	1	1	4	4	4	1	1	6	49	
	Each variaty	14			5			10			12			8				

Table 7. Effect of storage for 10 days and relative humidity on the fungal diversity of coffee samples of Robusta ground coffe.

Isolates		Nescaffee			Cappuccino			Turkish coffee			French coffee			Nescaffee with out			Total isolates	% Frequency
		Humidity %																
		10	25	45	10	25	45	10	25	45	10	25	45	10	25	45		
A. niger		-	1	-	-	2	2	-	3	-	1	1	-	-	-	1	11	40.74
A. flavus		-	-	-	-	1	-	3	-	1	-	-	-	-	-	-	2	7.40
A. melleus		-	-	1	1	-	1	1	3	-	-	-	1	-	1	-	9	33.33
A. alliaceus		-	1	-	-	-	1	-	-	-	-	-	-	-	-	-	2	7.40
E. nidulans		1	-	-	-	-	-	-	-	-	-	-	-	1	-	-	2	7.40
Aspergillus sp.		-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	1	3.70
Total isolates	Total	1	2	1	1	3	4	1	7	1	1	1	1	1	1	1	27	
	Each variaty	4			8			9			3			3				

Table 8. Averages general activity of fungi at tested humidity levels and periods of storage in ground Robusta coffee

Impact factor			Nescaffee	Cappuccino	Turkish coffee	French coffee	Nescaffee with out	Total
Total isolates	Humidity %	10%	17	9	9	6	3	44(35.77%)
		25%	3	13	15	8	2	41(33.33%)
		45%	2	7	9	11	9	38(30.09%)
	Each periods of storage	10 days	4	16	14	10	3	47(38.21%)
		20 days	14	5	10	12	8	49(39.84%)
		30 days	4	8	9	3	3	27(21.95%)
	Each variaty		22 (17.89%)	29 (23.58%)	33 (26.83%)	25 (20.33%)	14 (11.38%)	123

3. Qualitative and quantitative assessment of aflatoxins and ochratoxin in ground coffee of arabica and robusta coffee

Results shown in table 9 regarding the qualitative and quantitative assessment of mycotoxins of aflatoxin and ochratoxin in crushed of harari and barri coffee, and also the types of robust stored at room temperature for 30days at the different humidity ratios indicated that these toxins were produced in all samples with varying degrees and it wasn't observed that there is a correlation between the number of fungi and the concentrations and types of these toxins. It was also found that the concentration of toxins was not associated with a fixed relationship with humidity, and in general it has been observed that in the samples stored at 10% samples of Barri coffee samples which is black roasted, the Aflatoxin toxins were 4.382µg/g while cappuccino samples were not having any concentration of Aflatoxins.

Regarding the samples with a relative humidity of 25%, the most polluted samples were French coffee, where Aflatoxin concentration reached 463.10µg/g, while the coffee samples of Barri type which are mild roasted are the least polluted with toxins where total aflatoxins were 547.0µg/g of the coffee.

Table 9. Qualitative and quantitative assessment of aflatoxins and ochratoxin in ground coffee of arabica and robusta coffee after stored for 30 days at 25 ± 2°C and different degree of humidity.

Varieties	Humidity 45 %						Humidity 25 %						Humidity 10 %					
	B1	B2	G1	G2	Total	OTA	B1	B2	G1	G2	Total	OTA	B1	B2	G1	G1	Total	OTA
Harari dark	1.184	0.5	0.586	0.806	3.076	ND	1.422	0.5	1.006	0.604	3.532	ND	2.322	0.468	0.008	0.756	3.554	ND
Harari mild	1.138	0.028	ND	ND	1.166	0.61	1.184	0.032	ND	ND	1.216	0.31	2.748	ND	ND	1.362	4.11	ND
Harari white	0.616	0.188	0.05	0.04	0.894	0.39	0.710	0.182	ND	0.202	1.094	0.62	0.616	ND	ND	0.202	0.818	0.39
Barri dark	1.422	0.344	1.002	0.554	3.322	0.23	1.468	0.406	0.920	0.604	3.398	0.20	2.086	0.468	1.172	0.656	4.382	0.30
Barri mild	1.896	0.656	1.172	0.604	4.328	0.15	0.355	0.091	ND	0.101	0.547	0.35	1.848	0.406	1.006	0.6.4	3.864	25
Barri white	2.086	0.624	1.088	0.806	4.604	0.30	1.80	0.648	0.920	0.656	4.024	0.76	1.848	0.656	1.170	0.604	4.278	0.30
Nescaffee	ND	ND	ND	ND	-	0.6	6.634	0.156	ND	ND	6.79	ND	1.326	0.188	0.334	0.302	2.152	0.17
Cappu-ccino	0.094	0.124	ND	ND	0.218	0.6	2.086	0.592	1.006	0.908	4.592	0.18	ND	ND	ND	ND	-	1.40
Turkish coffee	0.468	0.188	0.334	0.352	2.342	ND	1.942	0.250	1.256	0.454	3.902	ND	1.322	ND	ND	ND	1.322	ND
French coffee	0.355	ND	ND	ND	0.355	ND	5.687	ND	3.264	1.512	10.463	0.18	1.421	ND	ND	ND	1.421	0.22
Nescaffee with out	1.468	0.406	1.172	0.554	3.6	ND	0.758	ND	0.026	ND	0.784	ND	0.710	ND	0.05	ND	0.760	ND
Average	1.066	0.278	0.491	0.338	2.173	0.262	2.186	0.259	0.764	0.458	3.668	0.236	1.477	0.199	0.340	0.408	2.424	2.526
L.S.D. at 0.05%	5.01	3.79	3.11	3.31	4.21	3.39	3.54	3.60	2.59	3.23	4.11	3.03	6.06	2.62	2.21	3.11	4.89	1.12

The samples with relative humidity of 45% and stored also for 30days at room temperature were sampled product of Barri variety of light roasting, which were more samples in terms of Aflatoxins totaling 604.4µg/g while there is no producing of aflatoxins in samples of Nescafe.

With regard to the production of fungi to ochratoxin in ground coffee, it was observed that twelve samples of the total of thirty-three samples, the fungus were unable to produce of ochratoxin, that is 64.63% of the samples produced fungi with this toxin, where cappuccino samples stored for thirty days with 10% humidity were contaminated with Ochratoxin by 40.1µg/g, while the highest production of this poison in moisture of 25% were in the samples of light barri where it was 76.0µg/g. Also, the pollution of harari variety was noted in the relative humidity of 45% with the highest percentage of ochratoxin which was 61.0µg/g.

DISCUSSION

The results of qualitative and quantitative assessment of aflatoxin and ochratoxin in ground coffee of arabica and robusta stored at room temperature for 30days at different humidity ratios indicated that these toxins were produced in all samples with varying degrees and there wasn't any correlation between the number of fungi and the concentrations and types of these toxins. And it was also found that the concentration of toxins was not associated with a stable relationship with the humidity.

With regard to the production of ochratoxin in ground coffee, it was observed that in twelve of the total samples of thirty-three fungi cannot produce of Ochratoxin, that is in 64.63% of the samples the fungus produced this toxin. This high rate corresponds with the results of many researchers such as (Nehad *et al.*, 2007; Moslem *et al*., 2010; Iqbal *et al.*, 2011). Another study, Taniwakin *et al*. (2003) and Logrieco *et al*. (2003) stated that *A. ochraceus* can be the primarily responsible for the pollution of coffee with ochratoxin. While, Taniwaki *et al*. (2003) considered that *A. carbonarius, A. niger, A. ochraceus* are the main producing for OTA toxins in Brazilian coffee beans, which found that 77% of the isolates fungus were able to produce toxins of OTA.

RECOMMENDATIONS

The current research proved that there is serious damage happens to the coffee during storage and this damage is in contamination with fungi of store which is harmful, especially *Aspergillus, Penicillium, Fusarium* which are resulted in the produce of several mycotoxins. The matter increases this damage is the high temperature of storage and humidity. Therefore, this study recommends the following as an attempt to avoid the fungal attack and minimize the damage as much as possible, where toxins of ochratoxin and aflatoxin were found in tested coffee as well as the ability of all fungal isolates to produce mycotoxins which requires us not to stand idly by, so we recommend the need for periodic inspection of samples of coffee in stores and eliminate what may be contaminated with these mycotoxins. We also advise more stringent laws to prevent the entry of any shipments of coffee products contaminated with either fungi or its toxins.

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