STUDIES ON MYCOFLORA AND AFLATOXIN IN REGULAR AND DECAFFEINATED BLACK TEA

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SUMMARY : The mycofloral analysis of twenty different kinds of black tea powder (commonly used in Egypt) indicated that, all these samples have wide range of mould species and counts. A thirty two species and 2 varieties belonging to 9 genera were collected on 1% glucose- (7 genera and 20 species) and 40% sucrose- (7 and 23+2 varieties) Czapek's agar at 28°C. The most prevalent mould on one or two substrates were members of Aspergillus, Penicillum, Cladosporium and Eurotium. The most polluted tea, in total count, were Tayseer, Blue Tea Pot and El-Balabel. The lowest contaminated tea were Khan El-Khallily, Yaquot and El-Arosa.

The total count of tea-borne fungi was more flourished by increasing moisture content. Aspergillus flavus and A. tamarii represent the most prevalent moulds after 20 days of tea storage at 45% m.c. Aflatoxin production by A. flavus IMI 89717 was also increased by the increase of moisture content of both regular and decaffeinated tea. Aflatoxin biosynthesis on decaffeinated tea was more hazardous than the regular tea specially at 45% m.c.

Aflatoxin biosynthesis of A. flavus IMI 89717 was recorded in all kinds of tea after 20 day of storage at 45% m.c. and 28°C. The highest aflatoxin quantity produced in Al-Fares, Ezi-Nasser, Lipton and Tayseer tea, and by their isolates of A. flavus in PD broth. The lowest quantity produced on Khan El-Khallily and Massgeed tea, and by their isolates in liquid medium.

Key words : Mycoflora, aflatoxin, regular tea, decaffeinated tea.

INTRODUCTION

Storage fungi are important factors responsible for tea deterioration. There were several contaminations during initial tea preparation. This was possible because the isolated fungi are common aerial contaminants which are able to proliferate very rapidly especially when remmants of the leaves can provide a rich food source for growth. Spoilage of tea is often accompanied by the formation of mycotoxins which are toxic secondary metabolites produced by specific tea-borne fungi. Aflatoxins are a group of mycotoxins produced by *Aspergillus flavus* and *A. parasiticus* in the field on the developing plant (4) and also during storage of commodities. Aflatoxins are acutely toxic, carcinogenic teratogenic and mutagenic (4, 9)

Several investigations have been carried out on fungi and mycotoxins contaminating seeds, animal feed and human foodstuffs. Some of these studies have been carried on the mycoflora associated with coffee fruits (2) cocoa seeds (6, 7), rooibos tea (13,14) and cocoa, roasted coffee and tea powders (1).

The object of this study was to determine the contamination of twenty kind of black tea powders with fungi. Also, to study the role of moisture content and period of storage on changes in mycoflora and aflatoxin production by *A.*

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flavus IMI 89717 in regular and decaffeinated tea. Finally, to determine aflatoxin production on different kinds of tea inoculated with *A. flavus*.

MATERIALS AND METHODS

Collection of samples

Twenty different kinds of tea powders, commonly used in Egypt, were collected from retail markets (Table 1). The samples were transferred to the Mycological laboratory and kept at room temperature till fungi and toxins analysis.

Estimation of moisture content

A known weight of each sample was dried in an oven for 24h at 105°C, then cooled in a desiccator and re-weighed. The moisture content is expressed as percentage of the dry weight.

Determination of water-soluble extractives

5 g of dried tea was refluxed in a 250 ml round-bottom flask containing 100 ml distilled water for one hour. It was then filtered, added a further 100 ml of water and refluxed for another hour. It was filtered again and the residue was dried in the oven, and after cooling in a desiccator, it was re-weighed (5).

Estimation of fungi

For the mycofloral analysis of the tea samples the dilution-plate method as described (8) was used. Czapek - Dox agar basal medium (g/L : potassium nitrate 3.0, magnesium sulphate 0.5, potassium chloride 0.5, di-potassium hydrogen phosphate 1.0, agar 15.0) in which the 3% sucrose were substituted separately with either 1% glucose or 40% sucrose, was used for isolation of glucophilic and osmophilic (or osmotolerant) fungi, respectively. Chloramphenicol (0.05 g/ml) and rose-bengal (30 ppm) were added to the medium as bacteriostatic agents. Six plates (3 plates for each medium) were used per sample. The plates were incubated at 28°C for 1-2 weeks and the growing fungi were counted, identified and calculated per mg dry tea.

Storage of a tea sample at various levels of moisture content

Portions of 25 g of tea were adjusted to the desired moisture contents (15, 25, 35 and 45%) by adding the required amount of sterile distilled water in sterile polyethylene bags and were sealed by rubber bands. The samples were shaken thoroughly, placed in a refrigerator at 5°C for 24 h. Thereafter the bags were incubated at 100% relative humidity in a desiccator at 28°C. At intervals of 10, 20 and 30 days the samples were assayed for their fungal content as mentioned above.

ARTIFICIAL INFECTION OF TEA

a. Infection of a tea sample at various levels of moisture content

75 g of variously moistened autoclaved tea in 250 ml Erlenmeyer flasks were inoculated with spore suspension of *Aspergillus flavus* IMI 89717. The cultures were incubated at 28°C and at intervals of 10, 20 and 30 days the aflatoxin production was assayed as mentioned earlier.

b. Infection of the different kinds of tea

25 g of each of moistened (45% H_2O), autoclaved tea in 250 ml Erlenmeyer flask were inoculated with spore suspensions of *A. flavus* IMI 89717. The cultures were incubated for 20 days at 28°C for evaluating aflatoxin production.

Cultivation of *Aspergillus flavus* isolates for aflatoxin screening

Twenty isolates of *A. flavus*, isolated during the current study, were inoculated separately on potato-dextrose broth. The cultivation was made in 250 ml Erlenmeyer flasks, each containing 50 ml of the medium. The flasks were sterilized at 1.5 atmosphere for 30 min and inoculated after cooling with 1 ml of spore suspension of 1 week-old cultures of the pure organism. The cultures were incubated at 28°C for 7 days as stationary cultivation.

AFLATOXIN ANALYSIS

a. Extraction procedure

The cultures were extracted with chloroform, and the extract was concentrated in vacuum. The dry material was transferred to 1-dram vials with small amounts of chloroform. The solution was evaporated to dryness under a stream of nitrogen. The crude extract was cleaned up by silica gel column (3). Aflatoxins were dissolved in chloroform and separated by thin layer chromatography on silica gel 60 plates using chloroform-methanol (97:3 v/v) as the developing solvent.

b. Determination of aflatoxin

The spots of aflatoxin B_1, B_2, G_1, G_2 were removed from the plates, eluted with methanol and estimated spectrophotometrically (11).

RESULTS AND DISCUSSION

The moisture content of the tea samples was fluctuated from 5.2-6.8% but in the great majority of the samples the values were more than 6.1% (Table 1).

Water-soluble extractive of different kinds of tea ranging from 31.6-42.1%. El-Arosa, Arcom, El-Balabel and Massgeed tea have the high levels of water extractives. While, Ezi-Nasser and El-Jawhara represent the lowest extractives (Table 1). A low water soluble extractive indicate the presence of spent leaves (5).

The tea samples tested were already heavily infected by storage fungi and the total counts widely varied from 4.1-10.8 and 4.0-11.6 colonies/mg tea on 1% glucose-and 40% sucrose-Czapek's agar at 28°C, respectively (Table 1). Abdel-Hafez and El-Maghraby (1) reported that the total filamentous glucophilic fungi in some drinks widely ranged between 0.12-1.10 colonies/mg in cocoa, 0.11-0.98 in roasted coffee and 0.16-1.28 in tea powders. The broadest spectra of genera and species were recorded in El-Balabel (6 genera and 11 species), El-Keef (5 and 10) and Lipton (5 and 9) on 1% glucose; and Arcom (6 and 12+1 variety), Brooke Bond (5+11+1) and El-Moftah tea (5+11+1) on 40% sucrose-Czapek's agar. The richest samples in total count were estimated in Tayseer, Blue Tea Pot, and El-Balabel. This means that there is no correlation between the total count and number of genera and species. The lowest polluted tea were Khan El-Khallily, Yaquot and El-Arosa which have low total count of fungi.

Thirty-two species and 2 varieties belonging to 9 genera were isolated from the tea samples tested on 1% glucose (7 genera and 20 species) and 40% sucrose (7 and 23+2 varieties) Czapek's agar at 28°C. The most prevalent fungi were members of *Aspergillus, Penicillium, Cladosporium* and *Eurotium*. These results are in agreement with findings of Hor (7) who noticed that *Penicillium* and *Aspergillus* were the most damaging fungi on cocoa seeds.

Aspergillus was the most predominant genus and encountered in all samples comprising 92.3 and 88.9% of total fungi on the two isolation media, respectively (Table 2). From the genus, 11 species and 1 variety were identified of which *A. flavus, A. fumigatus, A. niger* and *A. terreus* were the most common. They occurred in 90-100% of the samples constituting 9.3-45.8 and 6.8-37.5% of total *Aspergillus* and 8.6-42.2 and 6.0-33.4% of total fungi, on media used, respectively. Abdel Kader and Al-Hubaishi (2) isolated 12 species of *Aspergillus* from coffee fruits and the most common species were *A. flavus, A. niger, A. ustus* and *A. terreus*. Also, Abdel -Hafez and El-Maghraby (1) indicated that *A. flavus, A. fumigatus* and *A. niger* were the most prevalent on some drinks including tea.

Penicillium (6 species) ranked second in the number of cases of isolation and total count emerged in 90 and 95% of the samples comprising 4.5 and 5.7% of total isolates on the two media, respectively (Table 2). *P. chrysogenum* (80 and 45% of the samples and 3.8 and 1.5% of total fungi) was predominant on the two media *P. corylophilum* (40 and 1.5%) and *P. oxalicum* (70 and 2.6%) were prevalent on 40% sucrose and not encountered on 1% glucose-Czapek's agar. Most of the above *Penicillium* species were isolated from some drinks or their seeds and leaves (1, 2, 7).

Cladosporium came behind the previous two genera and found in 60 and 65% of the samples contributing 1.5 and 2.2% of total fungi on glucose-and sucrose-Czapek's agar, respectively. It was represented by 3 species of which *C. herbarum* was the most prevalent one. *C. cladosporioides* and *C. sphaerospermum* were less encountered (Table 2). The first two species were common on some drinks including tea (1).

Eurotium was isolated in high occurrence on 40% sucrose-Czapek's agar and completely absent on 1% glucose. It was found in 55% of the samples matching 2.1% of total fungi. Of 5 species isolated from the genus *E. amstelodami* was the most common, represented in 35% of the samples having 55.2% of total *Eurotium* and 1.1% of total fungal isolates. Hansen reported that A. glaucus (*+Eurotium*) was common in seeds of cocoa.

Emericella, (3 species +1 variety) and *Alternaria* (2 species were also, common on one or two isolation media. They occurred in 45, 25, 35 and 109 of the samples comprising 0.8, 0.5, 0.7 and 0.2% of total fungi, respectively. From the two genera *E. rugulosa, E. nidulans var. Lata* and *A. alternata* were common. The remaining genera and species were isolated in rare frequency of occurrence (Table 2). The above species were also isolated from human food and various types of drinks, their seeds, or leaves all over the world as reported by many researchers.

The selective effect of moisture contents on tea mycoflora and aflatoxin biosynthesis was showed in (Table

3). The total count of fungi was regularly increased with the rise of moisture content and storage periods. The highest count was obtained after 20 days at all levels of moisture content. *Aspergillus flavus* and *A. tamarii* were significantly flourished after 20 days of incubation at 45% m.c.

Aflatoxin production on regular tea was regularly increased with the rise of moisture content. The best water content for aflatoxin production was 45% after 20 days of inoculation at 28°C (Table 3).

In decaffeinated tea, aflatoxin production increased by approximately 3 folds than the regular tea. These results agree with these of Nartowicz *et al.* (17) who found that high levels of aflatoxin were produced on decaffeinated coffee beans inoculated with *A. parasiticus*. Lenovich (10) have suggested the presence of caffeine in cocoa beans an having anti-aflatoxigenic properties.

The results in (Table 4) were directed to establish whether a potential hazard might exist due to contamination of tea with aflatoxin-producing members of *Aspergillus flavus*. Twenty isolates of *A. flavus* isolated during this study were examined for the presence of aflatoxin. Fifteen isolates produced B_1, B_2, G_1 and G_2, while five isolates produced B_1 and B_2 only. The highest amount of aflatoxin was produced by species isolated from Blue Tea Pot, El-Fares, Shimto and Tayseer tea. The lowest quantity was produced by fungi isolated from El-Nakhil, Khan El-Khallily and Massgeed tea (Table 4).

Aflatoxin production on different tea kinds was listed in (Table 4). *Aspergillus flavus* had the ability to produce aflatoxin in all kinds of tea after 20 days of incubation at 45% m.c.

The amount of aflatoxin varied from 26-81 μg/kg dry tea. These results agree with the finding of Abdel-Hafez and El-Maghraby (1). They proved that tea powder was contaminated by 72 μg aflatoxin per kg dry tea. The highest quantity was produced on Al-Fares, Ezi-Nasser, Al-Nakhil and Blue Tea Pot. The lowest one was produced on Shimto, Yaquot, Brooke Bond and Khan El-Khallily. This may be attributed to the caffeine content of these commodities.

Contamination of tea by mycoflora and mycotoxin is favored by high humidity and high water activity. To control aflatoxin formation and tea-borne fungi, on the basis of moisture, the moisture content must be maintained below a certain critical level for each kind of tea.

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Table 1: Moisture content (%MC), water - soluble extractives (%WSE) total count (TC, calculated per mg tea), number of genera (NG) and number species (NS) of different kinds tea on 1% glucose - and 40% sucrose-Czapek's agar at 28°C.

No	Kind of tea	Producing Company	% MC	% WSE	1% GLUCOSE			40 % SUCROSE		
					TC	NG	NS	TC	NG	NS
1	Al-Fares (Cylan-Kenya)	Awllad Padawy Co.	6.3	35.5	10.2	3	8	6.7	4	9
2	Al-Nakhil (Kenya)	A.F. Cairo Co.	6.2	39.5	10.4	5	8	5.4	4	6+1
3	Al-Saied (Cylan- Kenya and Indonesia)	El-Sharq Center	6.0	36.7	8.3	4	7	5.4	3	7+1
4	Arcom (Kenya)	El-Nasr Co.	6.1	40.4	6.1	3	6	9.4	6	12+1
5	Blue Tea Pot (India)	Brooke Bond India Limited calcutta (El-Saadawy Co.)	6.2	35.0	10.8	3	8	7.5	3	8+1
6	Brooke Bond (India)	Fine food Co.	6.6	39.3	6.2	3	6	6.6	5	11+1
7	Crown (India-Indonesia)	Crawn manufacture	5.9	39.4	7.4	3	7	6.5	3	5+2
8	El-Arosa (Kenya)	Awllad Padawy Co.	6.0	42.1	6.4	2	5	6.3	1	6
9	El-Balabel (Kenya-Cylan)	El-Tayseer Co.	6.7	40.0	10.3	6	11	7.9	3	9
10	El-Jawhara (India)	El Jawhara manu. (Hamdy Korietum and Co.)	6.2	33.2	8.9	3	8	8.5	4	9+1
11	El-Keef (Endonesia)	Magdy Rehouma and Co.	6.8	35.9	8.9	5	10	6.0	5	9+1
12	El-Moftah (Kenya)	Mahmoud Fawzy and Co	6.0	36.0	9.2	3	10	4.3	5	11+1
13	Ezi-Nasser (Endonesia)	Awllad Nasser manufacture	6.5	31.6	8.4	4	7	8.0	3	9
14	Khan El-Khallily (Cylan-India and Kenya)	Shimto Co.	6.1	34.6	6.3	4	8	5.4	3	7+1
15	Lipton (Ceylonta-Ceylon)	Lipton Ceylon Limited-Colombo	6.6	35.7	4.1	5	9	8.6	3	7+1
16	Massgeed (Kenya)	Egyptian Nationally Co.	6.1	40.0	8.1	3	6	10.0	5	9
17	Rehouma (Kenya-Cylan and Endonesia)	Magdy Rehouma and Co	6.5	36.5	10.0	4	8	7.0	2	7+1
18	Shimto- (Cylan-India-Kenya)	Shimto Co.	5.8	38.2	9.7	3	7	7.1	4	8
19	Tayseer (Cylan)	El-Tayseer Co.	6.5	35.2	10.3	3	7	11.6	3	6+1
20	Yaquot (Cylan-India-Kenya)	Shimto Co.	5.2	34.8	8.4	2	6	4.0	2	4

Table 2: Total count (TC, calculated per mg tea), number of cases of isolation (NCl, out of 20 samples) and occurrence remark (OR) of fungal genera and species recovered from 20 kinds of tea on 1% glucose-and 40% sucrose-Czapek's agar at 28°C.

Genera and species	1 % glucose - 40 % sucrose			
	TC	NCl and OR	TC	NCl and OR
Alternaria	1.2	7M	0.3	2R
A. Alternata (Fries) Keissler	0.8	5L	-	-
A. chlamydospora Mouchacca	0.4	3R	0.3	2R
Aspergillus	155.4	20H	126.5	20H
A. flavus Link	49.8	20H	41.7	20H
A. flavus var. columnaris Raper and Fennell	-	-	2.8	12H
A. fumigatus Fresenius	71.1	20H	47.5	20H
A. japonicus Saito	-	-	0.1	1R
A. niger Van Tieghem	14.4	19H	19.1	18H
A. ochraceus Wilhelm	0.6	4L	0.2	2R
A. tamarii Kita	0.6	3R	-	-
A. terreus Thom	17.9	18H	8.6	18H
A. terricola Marchal	-	-	0.3	2R
A. ustus (Bain.) Thom and Church	1.0	4L	3.8	4L
A. versicolor (Vuill.) Tiraboschi	-	-	0.2	1R
A. wentii Wehmer	-	-	2.2	6L
Cladosporium	2.5	12H	3.1	13H
C. cladosprorioides (Fres.) de Vries	0.8	5L	0.7	4L
C. herbarum (Pers.) Link ex Gray	1.6	8M	2.4	10H
C. sphaerospermum Penzing	0.1	1R	-	-
Emericella	1.3	9M	0.7	5L
E. nidulans (Eidam) Vuillemin	0.3	3R	-	-
E. nidulans var. Lata (Thom and Raper) Subram.	-	-	0.6	4L
E. quadrilineata (Thom and Raper) Benjamin	-	-	0.1	1R
E. rugulosa (Thom and Raper) Benjamin	1.0	8M	-	-
Eurotium	-	-	2.9	11H
E. amstelodami Mangin	-	-	1.6	7M
E. chevalieri Mangin	-	-	0.7	3R
E. repens De Bary	-	-	0.1	1R
E. rubrum Kong. Spieckermann and Bremer	-	-	0.4	3R
E. tonophilum Ohtsuki	-	-	0.1	1R
Fusarium poae (Peck) Wollenw.	-	-	0.6	1R
Gibberella fujikuroi (Sawada) Ito	0.3	2R	-	-
Nectria haematococca (Berk and Brown)	0.2	2R	-	-
Penicillium	7.5	18H	8.1	19H
P. aurantiogriseum Dierckx	0.6	6L	-	-
P. chrysogenum Thom	6.4	16H	2.2	9M
P. citrinum Thom	0.4	3R	-	-
P. corylophilum Dierckx	-	-	2.2	8M
P. duclauxii Delacroix	0.1	1R	-	-
P. oxallicum Currie and Thom	-	-	3.7	14H
Total count	168.4		142.2	
Number of genera = 9	7		7	
Number of species = 32 + 2 var.	20		23+2 var	

Occurrence remarks (OR); H=high occurrence from 11-20 (out of 20); M=moderate occurrence from 7-10; L=low occurrence from 4-6; R=rare occurrence from 1-3 samples.

Table 3: Effect of different moisture contents on common mycoflora and aflatoxin production by *A. flavus* IMI 89717 on a tea sample.

				Common tea-borne fungi (count/mg dry tea)			Total aflatoxin (μg/kg dry tea)	
Storage periods (days)	Moisture contents	Total count	Aspergillus	A. niger	A. tamarii	Penicillium sp.	Regular tea	Decaffeinated tea
10	15	18	9	3	0	1	6(0.4)	8(0.5)
	25	41	2	38	0	1	10(0.6)	31(1.4)
	35	24	12	9	0	1	25(1.3)	88(5.1)
	45	23	11	9	1	1	38(2.1)	123(7.1)
20	15	59	20	7	30	2	8(0.5)	12(1.0)
	25	90	35	0	20	35	12(0.7)	39(3.2)
	35	100	50	0	50	0	32(2.3)	120(6.0)
	45	130	60	0	70	0	47(3.1)	144(9.0)
30	15	46	15	0	10	4	6(0.3)	10(0.8)
	25	70	20	0	15	35	12(1.0)	50(3.3)
	35	70	35	0	35	0	28(1.4)	96(4.2)
	45	90	40	0	50	0	36(1.7)	114(5.7)

Values in parentheses are S.D. of three replicates.

Table 4: Aflatoxin production by *A. flavus IMI* 89717 on different kinds of tea and aflatoxin - producing potential of 20 isolates of *A. flavus* in PD broth.

Aflatoxin produced by A. flavus species in PD broth (ν g/50 ml medium)				Total aflatoxin produced on different kinds of tea
Tea kinds	Aflatoxin B_1+ B_2	Aflatoxin G_1 + G_2	Total	(νg/kg dry tea)
1	375(19.1)	96(4.3)	471(31.2)	83(4.1)
2	104(5.0)	32(1.1)	136(11.1)	70(6.2)
3	130(7.2)	44(2.2)	174(12.2)	40(2.2)
4	260(13.3)	52(3.3)	312(14.3)	35(3.2)
5	384(20.2)	104(5.6)	488(28.2)	67(4.3)
6	312(19.2	0(0)	312(21.3)	32(1.1)
7	135(6.3)	74(0.4)	209(10.2)	34(2.2)
8	270(14.1)	78(0.5)	348(17.2)	45(3.2)
9	156(7.7)	39(0.3)	195(5.1)	39(1.4)
10	316(16.8)	0(0)	316(17.3)	35(1.5)
11	322(12.3)	0(0)	322(14.2)	45(1.2)
12	240(10.4)	37(0.2)	277(8.2)	38(1.3)
13	318(18.6)	0(0)	318(17.3)	72(3.1)
14	114(5.2)	38(0.2)	152(14.1)	32(1.2)
15	364(17.4)	0(0)	364(20.1)	55(1.7)
16	108(4.8)	45(0.3)	153(12.2)	38(3.4)
17	160(8.9)	76(0.7	236(11.1)	56(5.5)
18	338(14.2)	94(0.5)	432(22.0)	26(4.1)
19	332(13.7)	91(0.5)	423(41.0)	54(3.4)
20	165(7.8)	110(0.7)	275(20.7)	32(5.2)

Values in parentheses are S.D. of three replicates.