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Roasting coffee beans (*Coffea arabica*) artificially contaminated with ochratoxin A strongly reduces the analytical ochratoxin A content but not the genotoxic effects

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ABSTRACT

The effect of roasting coffee beans artificially contaminated with ochratoxin A (OTA) on its genotoxic properties was studied in *Allium cepa*. Green coffee beans, artificially contaminated with OTA, had an OTA concentration of 12 ng/g, and after roasting at 230 °C for 5, 8 and 12 minutes showed OTA concentrations of 9, 5 and 2.2 ng/g, respectively. OTA-non-contaminated coffee beans were used to compare the results. Based on the results from the *Allium cepa* test, ochratoxin proved to be toxic and genotoxic at different concentrations for meristematic cells. OTA-non-contaminated coffee beans also showed genotoxic effects. *A. cepa* species is an efficient test organism to assess the toxic effects induced by mycotoxins such as ochratoxin A. Genotoxicity analyses on meristematic cells of *A. cepa* indicated that the roasting process was not efficient enough for OTA degradation because the clastogenic and mutagenic effects were not reduced. This shows that OTA-degraded compounds could have possibly combined with *Coffea arabica* compounds forming compounds toxic to vegetable cells. In the present study, this assumption is based on the transformation of OTA-non-contaminated coffee cell compounds during the roasting process, which is proved by their genotoxic effects on *Allium cepa* meristematic cells.

KEYWORDS: coffee beans, ochratoxin A, roasting, genotoxicity

INTRODUCTION

Ochratoxin A (OTA) is a mycotoxin that contaminates a wide variety of foods such as cereals, beer, wine, coffee, cocoa, grapes, raisins and spices. OTA has been proved to have hepatotoxic, carcinogenic, immunosuppressant and teratogenic properties. It has been classified as possibly carcinogenic for humans (Group 2B) [1, 2]. In tropical areas OTA is produced in coffee beans mainly by *A. ochraceus* and *A. westerdijkiae*. The natural appearance of OTA in green coffee beans has been reported since 1974 [3]. Gopinandhan and co-workers [4] found OTA rates between 0.2 and 13.5 $ng.g^{-1}$ in green coffee samples. The presence of OTA in roasted and prepared coffee was reported by Tsubouchi and co-workers [5]. Before these reports, it was generally accepted that OTA was eliminated during roasting; nevertheless, concentrations above 20 $ng.g^{-1}$ have been reported in commercialized roasted coffee [6]. Several reports concerning the impact of roasting on the OTA content in coffee have shown OTA reduction ranging from 0-12%

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to 90-100% [7]. Such variations may be related to the different analytical conditions, on the roasting process or to the heterogeneity in the toxin distribution [8]. The use of *Allium cepa* as a test system was introduced by Levan [9], who proved that there were mitotic spindle alterations due to colchicine use. Since then, technical modifications have been made in *Allium cepa* test in order to obtain a more complete evaluation of chemical substances such as complex mixtures, which cover most environmental samples and pure substances [10].

The aim of the present study was employ the current knowledge on OTA degradation during coffee roasting and then to apply the *Allium cepa* genotoxicity test on the roasting-products, using contaminated and non-contaminated coffee beans.

MATERIALS AND METHODS

Coffee sample

Green *Coffea arabica* beans resulting from drying and de-husking of coffee fruits, were harvests from Sao Paulo State, Brazil, acquired directly from Terra Forte exporter, Sao Joao Da Boa Vista, SP, Brazil.

Artificial OTA contamination of coffee beans

Ten 2 L Polyethylene Terephthalate (PET) flasks were surface decontaminated with 70% alcohol and dried under UV light for 15 min. Following this, 1 kg of green coffee beans was placed in each flask and 120 mL of the fungal inoculum was added. Additionally 120 mL of peptone water (0.1%) without fungal inoculum was added to one flask containing the same amount of green coffee beans to achieve similar water activity conditions and used as a control. Flask tops were loosened and the flasks were incubated at 25 °C for 30 days. Flasks were shaken daily and opened for aeration inside an aseptic cabinet flow. After 30 days, all the *A. westerdikiae* contaminated coffee and the non-inoculated control were homogenized and analyzed for OTA.

Coffee roasting

Coffee samples (300 g) were roasted in duplicate in a vertical roaster herthware at 230 °C for 5 min, 8 min and 12 min. The roaster was pre-heated with hot air until the desired temperature was reached. After roasting, the samples were immediately cooled to room temperature. Colorimetric values were obtained using an Agtron Model E10-CP spectrophotometer (Agtron, Reno, NV-USA) and compared with the color disks from the "Roasting Color Classification System" (Agtron) for determination of roasting degrees.

OTA analysis

In green coffee: coffee samples were frozen at -80 °C, then ground to pass through a 0.5 mm sieve and analyzed for OTA content [11]. The samples (10 g) were extracted for 30 min with 100 mL of methanol/ 3% sodium bicarbonate (50:50). The extracts (10 mL) were filtered and diluted with 30 mL of phosphate-buffered saline (PBS) and passed through to an immunoaffinity column (Neogen, USA). OTA was eluted with 3 ml HPLC grade methanol. The eluate was evaporated to dryness under a stream of nitrogen gas at 70 °C and the residue was re-dissolved in 1 mL of HPLC mobile phase and then quantified by HPLC with fluorescence detector. The mobile phase consisted of distilled water/acetonitrile/glacial acetic acid (51:48:1). The injection volume was 100 µL and the flow rate was 1 $mL.min^{-1}$. OTA was detected by absorption at 333 nm excitation and 460 nm emission at a retention time of 13.3-13.5 min. A standard curve of OTA was established from an ochratoxin A standard (Sigma, USA) and the limit of detection was 0.05 ng mL^{-1}.

In roasted coffee: 10 g of roasted, ground coffee was extracted for 30 min with 100 mL of 3% methanol/bicarbonate solution (20/80) at 60 °C; 5 mL of the filtered extract was diluted with 40 mL of PBS buffer and cleaned through an immunoaffinity column (Neogen-USA). The OTA analysis of the extract was then performed as described previously for green coffee. Green coffee beans without OTA was used as control.

Allium cepa assay

Coffee solutions were prepared from green and roasted coffee. The coffee beans were ground in a home coffee mill (Philips) for 2 min. Twenty-five grams of ground coffee were extracted with 250 mL of boiling tap water (filter sterilized) in a home coffee maker (Moulinex) with coffee filter paper. The extract was lyophilized and the dry material was dissolved in distilled water (concentration of 8 mg/mL) for the *Allium* assay.

Onion bulbs, Perla variety, of weight 20 g were used. Adventitious roots were obtained by placing the bulb bases in tubes with natural mineral water and equipped with a constant bubbling system (10-20 mL min^{-1}) in an incubator at 25 +/- 0.5 °C in darkness. Pure, crystalline OTA solutions (Sigma Chemical Co, St Louis, MO, USA) were prepared at concentrations of 0.25, 0.50, 1.0, 2.0 and 5.0 $\mu g.mL^{-1}$ in Dimethylsulphoxide (DMSO) and diluted with distilled water. Water was used as a negative control. In order to determine the concentrations necessary for the genotoxicity test, the root growth was monitored over 72 h. Between 20 to 30 roots were used for each concentration of OTA, and the average of root lengths were measured and used for calculation of percentage growth of exposed root (positive control) compared to the negative control (water). These values were then used to prepare a dose-response curve. Samples of green and roasted coffee obtained as above were used for *Allium cepa* assay. All preparations were administered when the roots were 2-3 cm long.

Proliferative activity

Proliferative activity was assessed by determination of the frequency of mitotic cells at the root tip. The roots obtained at 0, 12, 24, 36 and 48 h after starting the assay were fixed in ethanol-acetic acid (3:1 v/v) at 4 ºC for 24 h. The meristematic area of the roots was squashed and colored in acetic orcein. The frequency of mitotic cells in 1000 squashed cells was determined.

Chromosomal aberrations (CA) and Micronucleus (MN)

On reaching 3-5 cm length, the roots were exposed to OTA content in green and roasted coffee for 48 h. Then, they were exposed to colchicine at 0.1% for 3 h. Afterwards, the roots were cut and fixed in ethanol-acetic acid (3:1 v/v) at 4 ºC for 24 h and then stained with acetic orcein. About 5000 cells were counted for the frequency and type of chromosomal aberrations and nuclear abnormality in the meristematic cells. Ethyl-methyl-sulfoxide (EMS) at 0.2% was used as positive control and natural mineral water and Dimethylsulphoxide (DMSO) at 1% as negative control. The frequency (percentage) of aberrant cells from the total number of counted cells and the number of cells in division was determined. The evaluation of mutagenic effects was carried out by scoring micronucleated cells of meristematic regions.

Statistics

The Fisher test (Z) for exact probability was carried out in order to determine the significant difference between the bulbs of exposed roots and controls in CA.

RESULTS AND DISCUSSION

The conditions and degree of roast as well as ochratoxin A content are shown in Table 1. Color development was directly related to the time and temperature of roasting. Color ranged from moderately clear to very dark, measured with spectrophotometer. Ochratoxin A content in green and roast coffee in samples and controls (absence of OTA) is also shown in this table.

In proliferative activity, the frequency of mitotic cells decreased progressively with the increase in OTA concentration in contaminated samples; however, non-contaminated samples modified the frequency of mitotic cells only in sample 0_{12} (Table 2).

This inhibition was transient, as recovery of the proliferation activity was observed after 48 h of OTA incubation. The non-contaminated sample 0_{12} modified the frequency of mitotic cells (Table 2). These findings suggest that OTA blocks the cell division cycle at a stage before mitosis, supporting the report of Lerda and co-workers [12]. OTA induces clastogenic effects in meristematic roots of *Allium cepa.* Data on the frequency of aberrant cells are shown in Table 3. An increase in the frequency of aberrant cells in 5, 9 and 12 ng/g OTA concentrations was observed. Aberrant cells were observed in non-contaminated sample 0_{12}. The Fisher test (Z) for exact probability was carried out using the total number of aberrant cells where OTA concentrations of 9 and 12 ng/g produced cell rupture, bridge and binucleated cells that differ from the positive control. The Fisher test (Z) was 8.33 (>1.90) and significant to $P < 0.05$. Detected CA included cromatide breaks, bridge and binucleated cells among other cell types. The mutagenic effect, evaluated by the MN test, was recorded by scoring meristematic cells in

Table 1. Condition, degree of roasting and OTA content in green and roasted coffee samples.

Condition of roasting		Ochratoxin A (ng/g)[a]		Degree of roasting (Spectrophotometer)
Temperature (ºC)	Time (min)	Samples	Control[b]	
230	0[c]	12 +/- 7.0	0	
	5	9 +/- 0.2	0_5	Moderate clear
	8	5 +/- 2.1	0_8	Dark
	12	2.2 +/- 1.0	0_{12}	Very dark

[a] : The result is the average of SD +/- of four analysis.
[b] : Control, coffee beans not contaminated with OTA and identified according to roasting time 0, 0_5, 0_8 and 0_{12}.
[c] : Sample of non-roasted green coffee.

Table 2. Effect of OTA on mitotic index in the meristematic zone of the root tip.

Samples	Mitotic index (%)			
	Exposure time			
	12 hr	24 hr	36 hr	48 hr
Negative Control	14.2 +/- 0.9	13.9 +/- 0.8	13.8 +/- 0.6	14 +/- 0.9
12 ng/g[a]	4.1 +/- 1.7	1 +/- 2.6	10.3 +/- 3.5	12 +/- 3.5
9 ng/g[a]	8.2 +/- 2.1	6 +/- 0.8	6.3 +/- 2.4	11 +/- 1.1
5 ng/g[a]	10 +/- 1.1	11.3 +/- 2.3	12.5 +/- 0.9	12 +/- 3.1
2.2 ng/g[a]	11.5 +/- 0.7	12.4 +/- 3.3	12.9 +/- 1.2	12.3 +/- 0.7
0[b]	13.9 +/- 0.8	13.8 +/- 1.1	13.7 +/- 2.2	14 +/- 3.3
0_5[c]	11.2 +/- 1.1	12.1 +/- 0.8	12.1 +/- 3.5	12 +/- 2.2
0_8[c]	11 +/- 0.9	12 +/- 2.3	12 +/-1.2	12 +/- 0.7
0_{12}[c]	7.5 +/-3.4	6.1 +/-1.5	7 +/-0.9	12 +/- 3.4

[a] : Coffee beans contaminated with OTA.
[b] : Sample of non-roasted green coffee.
[c] : Coffee beans not contaminated with OTA and identified according to roasting time 0_5, 0_8 and 0_{12}.

A. cepa (Table 4). In 5, 9 and 12 ng/g OTA concentrations, an increase of MN frequency was observed when compared with the negative control. Non-contaminated coffee sample 0_{12} showed an MN increase compared with the negative control. No increase of MN frequency was found at 2.2 ng/g OTA concentration or in non-contaminated coffee beans 0, 0_5 and 0_8. Different tests have been carried out with this plant, *Allium cepa*, in order to identify the presence of potential genotoxic and mutagenic chemical compounds [10]. Rank and Nielsen [13] showed a correlation of 82% of the *A. cepa* test in relation to the carcinogenicity test in rodents. The same authors also showed that, in this comparison, the *A. cepa* test was more sensitive than the Ames and the Microscreen tests. In the present study, we observed that the roasting process produces a substantial OTA reduction; the remaining compounds can combine with those of coffee and produce some toxicity in plant cells. This is probably due to intrinsic components such as caffeic acid and other chlorogenic acids, as well as hydrogen peroxide. Strong electrophiles

Table 3. Frequency and spectrum of cytological aberrations induced by OTA content in green and roasted coffee and roasted coffee with no contamination in the *Allium cepa* assay.

Ochratoxin A (ng /g)	No. of cells	Cells in div.	Abnormalities			Frequency of aberrant cells % based in		
			Breaks	Bridges	Binucleate cells	Total of aberrant cells	Total of cells	No. of div. cells
0[a]	5100	385	-	-	-	-	-	-
0_5[a]	5400	344	-	-	-	-	-	-
0_8[a]	5200	360	-	-	-	-	-	-
0_{12}[a]	5350	381	1	3	2	6*	0.11	1.57
2.2	5200	390	-	-	-	-	-	-
5	5220	317	1	4	1	6*	0.11	1.89
9	5700	341	0	5	2	7*	0.12	2.05
12	5100	360	1	6	4	11*	0.22	3.05
Negative control	5220	350	-	-	-	-	-	-
Positive[b] control	5410	347	7	13	11	31	0.57	8.90

- : no clastogenic effects are observed.
* : Z = 8.01 significant to P < 0.05 compared to the positive and negative control.
[a] : coffee beans not contaminated.
[b] : Ethyl methyl sulfoxide 0.2%.

Table 4. Micronucleus (MN) frequency in meristematic cells of *A. cepa* induced by OTA content in green and roasted coffee and roasted coffee with no contamination.

Ochratoxin A (ng/g)	Micronucleus frequency (% +/- SD)
0[a]	-
0_5[a]	-
0_8[a]	-
0_{12}[a]	1.80 +/- 1.02
2.2	-
5	1.30 +/- 1.03
9	2.10 +/- 1.10
12	3.40 +/- 1.01
Negative control	0.03 +/- 0.07
Positive control	4.55 +/- 1.40

[a] : coffee beans not contaminated.
- : absence of micronuclei.

generated through Fenton type chemistry then react with DNA and other cell components, and measurable change is surely visible. The most interesting finding in the present study was that coffee beans non-contaminated with OTA and roasted at 230 °C for 12 minutes showed clastogenic and mutagenic effects. This reveals that some coffee cell compounds change during the roasting process producing several compounds that have harmful effects on plant cells. Ames and Gold [14] identified more than 20 rodent carcinogens in coffee, several of them linked to chlorogenic acids, aldehydes and other components. In the present study, it was observed that the highest OTA concentrations in green and roasted coffee showed chromosomal aberrations and the non-contaminated coffee sample 0_{12} showed a concentration-dependent reduction in the mitotic index and most of the common abnormalities were the bridges. Bridges probably occur by the interruption and joining of chromosomes or chromatids [15], or as a result of chromosome stickiness, or it could be attributed to unequal translocation or inversion of chromosome segments [16]. Cytologic changes observed in the *Allium* chromosome aberrations assay showed that OTA was able to induce genotoxicity at the chromosome level. The efficacy of the micronucleus test for mutagenicity screening has been well established. Micronuclei are chromosome fragments or whole chromosomes that were not incorporated in the daughter cell nuclei and appear in the cytoplasm. Micronuclei are a manifestation of the chromosome breakages and the failure of the normal spindle function. It is likely that MN formation is due to clastogenic effects.

CONCLUSIONS

Genotoxicity analyses on meristematic cells of *A. cepa* indicated that the roasting process was not efficient enough for OTA degradation because the clastogenic and mutagenic effects were not reduced. This shows that OTA-degraded compounds could have possibly combined with *coffea arabica* compounds forming compounds toxic to vegetable cells.

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CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest.

REFERENCES

1. IARC (International Agency for Research on Cancer), 1993, Some naturally occuring substances: Food ítems and constituents, heterocyclic aromatic amines and mycotoxins, IARC monographs on the evaluation of carcinogenic risks to humans, IARC Press, Lyon, 56, 489-521.
2. Pfohl-Leszkowicz, A. and Casteganro, M. 1999, L`ochratoxine A., A. Pfohl-Leszkowicz, (Ed.) Les Mycotoxines dans l`alimentation: évolution et gestion des risques (Ed.), Paris, France : Lavoisier, 249-277.
3. Levi, C., Trenk, H. and Mohr, H. 1974, Journal Association of Official Analytical Chemists, 57, 866.
4. Gopinandhan, T. N., Kannan, G. S., Panneerselvam, P., Velmourougane, K., Raghuramulu, Y. and Jayarama, J. 2008, Food Addit. Contam. Part B, 1, 51.
5. Tsubouchi, H., Yamamoto, K., Hisada, K., Sakabe, Y. and Udagawa, S. 1987, Mycopathologia, 97, 111.
6. Mounjouenpou, P., Durand, N., Guyot, B. and Guiraud, J. 2007, Food Additives & Contaminants, 24, 730.
7. Amezqueta, S., Gonzalez-Penas, E., Murillo-Arbizu, M. and Lopez de Cerain, A. 2009, Food Control, 20, 326.
8. Suarez-Quiroz, M., De Louise, B., Gonzalez-Ríos, O., Barel, M., Guyot, B., Schorr-Galindo, S. and Pierre Guiraud, J. 2005, International Journal of Food Science and Technology, 40, 605.
9. Levan, A. 1938, Hereditas, 24, 471.
10. Lerne, D. and Marin-Morales, M. 2009, Mutation Research, 682, 71.
11. Nakajima, A., Horikoshi, T. and Sakaguchi, T. 1977, J. Agric Chem., 51, 507.
12. Lerda, D., Pelliccioni, P., Biagi, M. and Litterio, N. 2010, Plant Biology, 12, 685.
13. Rank, J. and Nielsen, M. 1994, Mutat. Res., 312, 17.
14. Ames, B. N. and Gold, L. S. 1990, Proc. Natl. Acad. Sci., 87, 7772.
15. Turkoglus, S. 2007, Mutation Research, 626, 4.
16. Gömürgen, A. 2005, Cytologia, 70, 119.