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# Detection and Genotoxicity of Ochratoxin A (OTA) in Raisins

***Daniel Lerda, PhD***
Molecular Genetic Laboratory. Medicine School, Cordoba Catholic University, Jacinto Ríos 571. Bº General Paz, 5000 Córdoba, Argentina
***Eduardo Miotti, MD***
***Nicolas Litterio, PhD***
Toxicology Laboratory. School of Agrarian Sciences, Cordoba Catholic University, Arnada Argentina, 5016 Córdoba, Argentina

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**Abstract**

Ochratoxin A (OTA) is a mycotoxin that contaminates a wide variety of foods such as cereals, beer, wine, coffee, cocoa, grapes, raisins and spices. It possibly affects the genetic material of organisms that are in contact with the OTA, causing mutation in their DNA. The aim of this study was to evaluate the presence of the OTA-producing fungi in raisins, its detection and the induction of chromosomal aberrations using the *Allium cepa* test. Samples of dried grapes were studied in which the microflora and the presence and content of OTA were determined. *Allium cepa* roots were exposed to OTA content (12 and 15 ng ml-1) for 48 h. Then they were collected from each assay and analyzed. OTA induces alterations in the genetic material of plant cells which suggests that OTA presents genotoxic effects at the concentration studied.

**Keywords**: Raisins, OTA, *Allium cepa*, genotoxicity

## Introduction

According to FAO more than 25% of the food produced worldwide is contaminated to some extent with mycotoxins (Lawlor and Lynch, 2001); however, the incidence of their contamination and concentration varies, depending on the time of year and the geographic area (Leung, Diaz-Llano & Smith, 2006). 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 demonstrated to have hepatotoxic, carcinogenic, immunosuppressive and teratogenic properties. It has been classified as possibly carcinogenic to humans (Group 2B) (IARC, 1993; Pfohl-Leszkowicz and Casteganro, 1999). Grape belongs to the genus *Vitis* and can have different

destinations: winemaking, fresh consumption or raisins. Raisins are obtained from grape dehydration and from the different varieties not all produce raisins, there are some varieties that are specific for raisins and others are multipurpose. During the drying process raisins lose weight, and although it varies depending on the variety, we can say that to get a kilo of raisins four kilos of fresh grapes are needed. Grapes are exposed to OTA-producing fungi in the vineyard. Only two genera of fungi have species capable of producing OTA: *Aspergillus* and *Penicillium*. Previous studies on the contamination of grapes with OTA indicate that the species responsible for the production of OTA in this product are the *Aspergillus* species. OTA producing strains, which are most frequently found in grapes, belong to the *Aspergillus carbonarius* species. Less frequently, strains of other OTA producing species, such as *Aspergillus niger* and *Aspergillus ochraceus* have been isolated in grapes (Serra, 2005). Contamination of grapes with OTA can occur in the field, while the grapes are still in the vineyard (Serra, Mendonca, Abrumhosa, Pietri & Venancio, 2004)). It is known that the contamination of agricultural products with mycotoxins can occur without visible fungal contamination; therefore, it is important to evaluate the content of OTA in healthy grapes (i.e. with no visible symptoms). Argentina accounts for 3% of the worldwide raisin production, ranking seventh among the major exporting countries. In Argentina, the available information on the presence of micoflora and ochratoxin A (OTA) in grapes, wine, grape juice and dried vine fruit is limited (Chulze et al.,2006)). *Aspergillus niger* is predominant in the Argentine varieties and a survey of wine grape mycobiota showed that while *Alternaria alternata* was predominant, the species *Aspergillus* section *niger* were isolated in 60% of samples. Around 41% of the isolates of *Aspergillus niger* produced OTA with levels between 2 and 24.5 ng ml-1. In another study, around 83% of *Aspergillus carbonarius* isolated from dried vine fruit produced OTA, with levels from 2 to 5200 ng ml-1 (Chulze et al.,2006). The legislation of most importing countries sets, for this mycotoxin, a maximum limit of 10 ug / kg and the Argentine legislation has no restrictions on the amount of mycotoxins permitted in raisins for domestic consumption (SAGPyA, 2006). Among the higher plants, *Allium cepa* is indicated as an efficient organism in genotoxicity assays. This efficiency is based on the characteristics of the kinetics of proliferation of the plant, the rapid growth of its roots, the large number of dividing cells, high tolerance to different growing conditions, availability all year round, ease of use, and the small number (2n = 16) and the large size of its chromosomes (Fiskesjo, 1985; Leme and Marin-Morales, 2009). The *Allium cepa* was originally introduced as a test organism in 1920 (Fiskesjo, 1985; Rank and Nielsen, 1993) and since then it has been used to assess and classify the toxicity of chemical products in the environment (Fiskesjo, 1985). This kind of plant has been used by different researchers as an indicator in the

mutagenicity tests (Brusick, 1987; Lerda, 1992; Rank and Nielsen, 1993; Ma et al, 1995; Khors et al, 1997; Kovalchuck et al, 1998; Lerda, 2005; Matsumoto et al, 2006; Lerda, 2010; Ventura-Camargo et al, 2011; Lerda, 2013; Mazzeo and Marin-Morales, 2015). The aim of this study was to investigate the presence of micoflora of dried vine fruit in order to determine the strains that produce OTA and its genotoxicity in the *Allium cepa* assay.

## Materials and methods

### Samples

Samples of dried vine fruit were analyzed, they were acquired at various retailers in the city of Córdoba, Argentina. All the samples were from different brands and lots which were kept frozen until their analysis.

The mycoflora of raisins was determined as described (Serra, 2003). A total of 10 raisins of each sample were planted in the medium Dichloran Rose-Bengal chloramphenicol (Oxoid CM727) and were incubated at 25 ° C in darkness for 1 week.

Filamentous fungi producers of spores were identified at their genus level and *Aspergillus* and *Penicillium* strains were isolated and identified at species level.

### OTA extraction

OTA detection in crops consisted of the extraction, from 7-9 day growth on Czapek agar (CYA) medium and injecting samples into the HPLC system (Bragulat, Abarca and Cabanes, 2001). The strains were considered positive for OTA production if the extract gave a peak at a similar retention time to the standard OTA (approx. 11 min). OTA identity was confirmed by derivatization of OTA in its methyl ester with boron trifluoride in methanol (BF3) as described by Hunt et.al (1980). The detection limit of the method was 0.1 ug OTA per kg of agar buttons (Serra, Mendonca, & Venancio, 2006).

### Analysis of OTA in dried vine fruit

OTA content was analyzed according to the Senyuva method (Senyuva, Gilbert, Ozcan & Ulken, 2005). Samples were extracted for 30 min with 100 ml of sodium bicarbonate / methanol at 3% (50:50). Extracts (10 ml) were filtered and diluted with 30 ml of phosphate buffered saline (PBS) and passed through an immunoaffinity column (Neogen, USA). OTA was eluted with 3ml methanol HPLC grade. The eluate was evaporated to dryness under a stream of nitrogen gas at 70 ° C and the residue was redissolved in 1 ml of HPLC mobile phase and then quantified by HPLC with fluorescence detection. The mobile phase consisted of acetonitrile / water / glacial acetic acid (51: 48:1). The injection volume was 100 ul, 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 to 13.5 min. A standard OTA curve was established from a standard of ochratoxin A (Sigma, USA) and the detection limit was 0.05 ng ml-1.

**Allium cepa Assay**

**Chromosomal aberrations (CA) and micronucleus (MN)**

Onion bulbs of the pearl variety were used, with an average weight of 20 g. Adventitious roots were obtained by placing the base bulbs in filtered water in glass tubes equipped with a system of constant air bubbling (10 to 20 ml.min-1) in an incubator at 25 ± 0.5 ° C in darkness. When roots reached 3-5 cm in length they were exposed to OTA content (12 and 15 ng/ml-1) for 48 h. Then they exposed to 0.1% colchicine for 3h. Later, the roots were cut and fixed in ethanol-acetic acid (3: 1 v / v) at 4 ° C for 24 h and then they were stained with acetic orcein. Around 5000 cells were counted for the frequency, type of chromosomal aberrations and nuclear abnormalities in the meristematic cells. Ethyl methyl sulfoxide (EMS) at 0.2 % was used as positive control, natural water and dimethylsulfoxide (DMSO) at 1% as negative control. The frequency (percentage) of aberrant cells in the total number of counted cells and the number of dividing cells was determined. The evaluation of the genotoxic effects was carried out to detect meristematic cells with micronuclei. Assays were performed in duplicate and the data obtained were subjected to statistical nonparametric Mann-Whitney test, considering significant values of $p \leq 0.05$.

**Results and discussion**

The most frequently detected genera were *Penicillium* (23%) and *Aspergillus* (12%). OTA was detected in cultures of *Aspergillus carbonarius* of raisins. *Aspergillus niger* did not produce the mycotoxin in detectable levels. OTA was detected in the two samples in concentrations ranging from 10 to 15 ng ml-1. OTA induces clastogenic effects in meristematic roots of *Allium cepa*. An increase in the frequency of aberrant cells in the concentration of 12 and 15 ng ml-1 of OTA is observed. The test results with OTA (Table 1) showed that the two concentrations tested induced changes in the visible chromosomes in the

**Table 1**. Results of chromosomal aberrations and micronuclei in *Allium cepa* cells, after exposure to the OTA.

| **Concentrations (ng/ml-1)** | **CA**<br>**M +/- SD** | **MN**<br>**M +/- SD** |
|---|---|---|
| NC | 0.47 ± 0.81 | 0.31 ± 0.73 |
| 12 | 1.42 ± 1.43* | 13.20 ± 11.45** |

| | | |
|---|---|---|
| 15 | 2.19 ± 1.61* | 12.30 ± 13.55** |
| EMS | 1.20 ± 1.31* | 14.30 ± 11.55** |

**CA**: chromosomal aberrations, **MN** micronuclei observed in meristematic cells, **M**: mean, **SD**: standard deviation**, NC**: negative control, **EMS**: ethyl methyl sulfoxide ( positive control), *p < 0.05, ** p < 0.0001

meristematic cells of *Allium cepa*, as c-metaphase, metaphase with chromosome loss when exposed for 48 h. Regarding the induction of MN, OTA induced significant results. The mutagenic effect, assessed by MN test was recorded by detecting meristematic cells of *Allium cepa* with micronuclei. In the concentration of 12 and 15 ng ml-1 of OTA an increase in the MN frequency compared to the negative control was observed. Different tests have been carried out with this plant, *Allium cepa*, to identify the presence of potential genotoxic mutagenic chemicals (Leme and Marin-Morales, 2009). In the present study, it was observed that the concentrations of OTA 12 and 15 ng ml-1 showed chromosomal aberrations and most of the common abnormalities were bridges. These are probably produced by the disruption and binding of the chromosomes or chromatids (Gömürgen, 2005) or as a result of the rigidity of the chromosome or that could be attributed to translocation or unequal inversion of chromosome segments (Turkoglus, 2007). Cytological changes in *Allium cepa* exposed to OTA show increase of chromosome aberration and MN indicating genotoxicity at concentrations assays. Micronuclei are fragments of chromosomes or whole chromosomes that were not incorporated into the nuclei of the daughter cells and appear in the cytoplasm. Micronuclei are a manifestation of chromosomal breakage and failure of the normal function of the spindle. It is probable that MN formation is due to the clastogenic effects (Mazzeo and Mari-Morales, 2015).

## Conclusion

The results presented indicate that plant cells (*Allium cepa*) have a very sensitive cellular response to the mycotoxin, OTA, in the concentrations used. These concentrations cause clastogenic and mutagenic lesions. On the basis of these findings it is important to evaluate the content of OTA in healthy grapes, without visible symptoms, in order to reduce the impact of this toxin in raisins. It would also be important to study the ecophysiology of ochratoxigenic fungi and their emergence to reduce the impact on the food chain.

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