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Toxigenic fungi isolated from dried vine fruits in Argentina

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Abstract

To evaluate the potential for mycotoxin production by fungi in dried vine fruits, the mycobiota was determined both before and after surface disinfection. Predominant genera were *Aspergillus* (50.2%), *Eurotium* (21.4%) and *Penicillium* (13.5%). *Aspergillus* section *Nigri* ("black aspergilli") were isolated with relatively high frequency. *Aspergillus niger* was the most common species but only 3 of 293 isolates screened were ochratoxin A (OTA) producers. *Aspergillus carbonarius* was less common but 96% of 48 strains screened were ochratoxigenic. OTA was not produced by *A. japonicus*. Other toxigenic fungi detected were *A. ochraceus* (3 strains produced OTA), *Aspergillus flavus* (5 strains produced cyclopiazonic acid but not aflatoxins), *P. citrinum* (19 strains were strong citrinin producers) and *Alternaria alternata* (15 strains were producers of tenuazonic acid, alternariol and alternariol methyl ether). In spite of the high incidence of *A. carbonarius* capable of producing OTA, low levels of this toxin were detected in the samples analysed.

Keywords: Dried vine fruits; Toxigenic fungi; Black aspergilli; Ochratoxin

1. Introduction

Recent studies have demonstrated the synthesis of mycotoxins by fungal species which had not been previously reported as toxigenic (Abarca et al., 2000). For example, the nephrotoxic ochratoxin A (OTA), produced by species of *Aspergillus* sect. *Circumdati* and by *Penicillium verrucosum*, has been reported as produced by some species of *Aspergillus* section *Nigri* which are very common contaminants not only in grains but also in fruits, such as grapes, and coffee beans (Abarca et al., 1994, 2001; Heenan et al., 1998; Taniwaki et al., 2003; Serra et al., 2003). This finding opens new perspectives in the study of potential contamination of different types of foods and beverages that can contribute to increase the risk of human exposure to this toxin.

Black aspergilli are found on the surface of healthy grapes at all stages and are the main fungi responsible

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for post-harvest decay of fresh fruit. OTA production has been observed only in *Aspergillus niger* and *Aspergillus carbonarius* isolates (Varga et al., 2003). In Spain, *A. carbonarius* has been reported as the main source of OTA contamination in wine (Cabañes et al., 2002) and in dried vine fruits (Abarca et al., 2003). High concentrations of OTA were also detected in dried vine fruits from the UK (Anonymous, 1997; MacDonald et al., 1999) and in lower concentrations in those from Greece (Stefanaki et al., 2003). *A. niger* and related species could become predominant during the drying process because the black spores apparently provide protection from sunlight and UV radiation, giving them a competitive advantage in this habitat (Pitt and Hocking, 1997). Dried vine fruits are healthy foods and are also ingredients in muesli, cereal bars, biscuits and cakes, among other foods, and could be an important source of OTA for people who consume large amounts, particularly children. The European Union has recently established maximum OTA limit of 10 μg/kg for these products (currants, raisins and sultanas) (Commission of the European Communities, 2002).

In Argentina, grapes are cultivated in several provinces located at the north-western region and they are destined to production of wine, juice and dried vine fruits. Da Rocha Rosa et al. (2002) reported that 17% of *A. niger* strains ($n=48$) isolated from Argentinean grapes were OTA producers. *A. carbonarius* was not present in fruits cultivated in Argentina but in those from Brazil both species of black aspergilli were detected. Magnoli et al. (2003a) reported that *Aspergillus* section *Nigri* from grapes of the same region (Mendoza province) included *A. niger* var. *niger*, *A. niger* var. *awamori* and *A. foetidus* but not *A. carbonarius*.

The aim of the present study was to determine the incidence of toxigenic fungi in dried vine fruits from some geographical locations of Argentina with special interest in OTA producing black aspergilli.

2. Materials and methods

2.1. Samples

Eleven samples (2200 berries) were selected from the main producer regions of dried vine fruits in Argentina (provinces of Mendoza, San Juan, Salta y Catamarca). Different dried vine fruits varieties were analysed from 2001–2002 harvest. They were supplied both from producers and purchased in retail market.

2.2. Isolation and identification of fungi

Fungi were isolated on Dichloran 18% Glycerol Agar (DG18) (Pitt and Hocking, 1997) with and without surface disinfection. The dried fruits were disinfected externally by immersion in a 2% sodium hypochlorite solution for 1 min, then rinsed with sterile water. One hundred berries (10 per plate) were aseptically placed in Petri dishes for each treatment. All plates were incubated in darkness for 7 days at 25 °C. Strains were subcultured on potato dextrose agar (PDA) slants, allowed to grow at 25 °C for 7 days and stored at 5 °C for final identification to species level. Taxonomic identification was made according to Pitt (1988), Pitt and Hocking (1997), Klich (2002) and Samson et al. (2002).

3. Mycotoxin production

3.1. Ochratoxin A

This toxin was tested by thin layer chromatography (TLC) in all species reported as producers (Abarca et al., 1994, 2001; Heenan et al., 1998). Plates of CYA were inoculated and incubated in darkness for 7 days at 25 °C, and were extracted by shaking with chloroform for one hour and a half at 250 rpm. The chloroform extracts were filtered through Whatman n ° 5 filter paper over a Na_2SO_4 (anh.) layer and were evaporated to dryness at 35 °C, 50 mm Hg. Residues were re-suspended in 500 μl of chloroform:diethyl ether:acetic acid (17:3:1) and spotted (5 μl) on TLC plates (Silica Gel G 60, 20 × 10, Merck). OTA standard was also spotted for Rf comparison with sample spots. Solvents for development of plates were chloroform:diethyl ether:acetic acid (17:3:1) (Filtenborg and Frisvad, 1980; Heenan et al., 1998). OTA was identified under UV light (360 nm) as a bluish-green fluorescent spot. Confirmation assays were carried out in

all cases by developing the respective chromatograms with an alternative solvent, benzene:methanol:acetic acid (18:1:1), and treating the spots with ammonia (OTA bluish-green fluorescence spots change into deep blue). The agar plug method (Filtenborg and Frisvad, 1980) was also used for screening the OTA production.

3.2. Tenuazonic acid (TA), alternariol (AOH) and alternariol methyl ether (AME)

Alternaria alternata strains were cultivated on autoclaved rice at 25 °C for 25 days. The cultures were extracted according to Bottalico and Logrieco (1992).

3.3. Other toxins

Citrinin, aflatoxins and cyclopiazonic acid were determinated by the agar plug method for extracellular toxins (Filtenborg and Frisvad, 1980) and intracellular toxins (Filtenborg et al., 1983). Solvent systems for development of chromatograms were toluene:ethylacetat:90% formic acid (6:3:1) for citrinin and aflatoxins. Cyclopiazonic acid was detected on oxalic acid-treated plates, developed with ethyl acetate:2-propanol:28% NH_3 in water (20:15:10) and revealed with Ehrlich reagent (Filtenborg et al., 1983).

All standards were purchased in Sigma Co. and all solvents were analytical grade.

4. OTA detection in dried vine fruits by HPLC

Samples were prepared and extracted as described by MacDonald et al. (1999). Ochratoxin A immunoaffinity columns were obtained from Rhone Diagnostic Technologies Ltd. OTA was detected and quantified with Shimadzu HPLC (2 pumps LC-10 ADvp, 1 DAD SPD- M10 Avp, 1 System controller SCL-10 Avp) and A BioRad C18 90-5 S (150 × 4.6 mm; 5 μm) column. Each analysis was done with a 20 μl loop, a mobile phase of acetonitrile:water:acetic acid (99:99:2) at a flow rate of 1 ml/min. Detection was made with excitation at 333 nm and emission at 460 nm. Limits of detection was 0.01 μg/kg for ochratoxin A.

All solvents employed were HPLC grade.

5. Results and discussion

A high level of fungal contamination was detected in most of the samples (Table 1). Although surface disinfection generally reduced the number of dried fruits with viable mold, there was considerable internal mold invasion.

The mold genera and species isolated from samples both before and after surface disinfection are shown in Table 2. Predominant genera in the mycobiota were *Aspergillus* (50.2%), *Eurotium* (21.4%) and *Penicillium* (13.5%). Black aspergilli represent almost the 95% of the 665 *Aspergillus* strains. They were found in 82% of the samples, some of which constituted a high proportion of internal infection. The predominance of *Aspergillus* section *Nigri* in the dried fruits was not unexpected because members of this group can survive the drying process due to the relative resistance of black spores to sunlight and UV radiations. In this section, *A. niger* was the most common species (37.3%), *A. carbonarius* and *A. japonicus* were less frequently found (7.3% and 2.9%, respectively). Other relatively frequent contaminants were *A. alternata* and several *Eurotium* species (mainly *Eurotium repens*, *Eurotium rubrum*, *Eurotium amstelodami* and *Eurotium chevalieri*) as well as some *Penicillium* species (*P. citreonigrum* and *P. chrysogenum*) and *Cladosporium* sp.

Due to the strong contribution of black aspergilli to the mycobiota of this substrate, the toxigenic potential of strains belonging to *Aspergillus* section *Nigri* was investigated. From 494 *A. niger* strains isolated, 293

Table 1
Percentage of dried vine fruits contaminated with viable molds

Sample	% of fruits with fungal infection	
	Without surface disinfection	With surface disinfection
1	66	40
2	2	1
3	43	3
4	87	71
5	0	1
6	100	77
7	92	85
8	100	94
9	100	93
10	100	100
11	100	100

Table 2
Strains isolated from dried vine fruit cultivated in Argentina

Species	N° of strains isolated in samples without disinfection	N° of strains isolated in samples with disinfection	Total incidence of species isolated[a]
Alternaria alternata	40	31	5.4%
Aspergillus carbonarius	43	54	7.3%
A. flavus	3	2	0.4%
A. fumigatus	2	0	0.2%
A. japonicus	24	15	2.9%
A. niger	305	189	37.3%
A. ochraceus	2	1	0.2%
A. restrictus	0	1	0.1%
A. sydowi	2	19	1.6%
A. unguis	0	1	0.1%
A. wentii	2	0	0.2%
Aureobasidium pullulans	17	7	1.8%
Chrysonilia sitophila	0	2	0.2%
Cladosporium sp.	16	15	2.3%
Curvularia lunata	1	0	0.1%
Emericella appendiculata	0	1	0.1%
Emericella nidulans	3	0	0.2%
Emericella quadrilineata	0	1	0.1%
Eurotium amstelodami	22	21	3.2%
Eurotium chevalieri	15	16	2.3%
Eurotium repens	50	43	7.0%
Eurotium rubrum	51	37	6.6%
Eurotium spp.	13	16	2.2%
Fusarium sp.	0	1	0.1%
Monascus ruber	7	16	1.7%
Nigrospora sp.	0	1	0.1%
Paecilomyces variotii	0	1	0.1%
Penicillium atramentosum	0	1	0.1%
P. brevicompactum	1	1	0.2%
P. chrysogenum	5	34	2.9%
P. citreonigrum	1	60	4.6%
P. citrinum	10	9	1.4%
P. corylophilum	2	0	0.2%
P. crustosum	11	2	1.0%
P. glabrum	4	5	0.7%
P. implicatum	2	0	0.2%
P. palitans	1	0	0.1%
P. purpurescens	1	1	0.2%
P. raciborskii	1	2	0.2%
P. roquerforti	0	2	0.2%
P. spinulosum	1	0	0.1%
Penicillium spp.	8	14	1.7%
Rhizopus sp.	22	0	1.7%
Syncephalastrum racemosum	8	0	0.6%
Trichoderma sp.	7	0	0.5%
Total	703	622	100%

[a] (n° of strains of the species/ Total of strains isolated). 100.

were screened for their ability to produce OTA. Forty-eight strains of *A. carbonarius* and 20 strains of *A. japonicus* were also screened. Table 3 shows the incidence of ochratoxigenic strains in the three species of black aspergilli that were isolated from the samples. *A. niger* was the most widely spread species, it was the most frequently found and was present in samples from all geographical regions, but only three strains (1%) were OTA producers. The reported percentages of ochratoxigenic isolates of *A. niger* are quite variable depending on the number of isolates studied and geographical origin. In most previous works, percentages range from 0.6 to 18.5% (Abarca et al., 1994, 2003; Tèren et al., 1996; Heenan et al., 1998; Sage et al., 2002; Taniwaki et al., 2003; Battilani et al., 2003; Serra et al., 2003). However, Da Rocha Rosa et al. (2002) reported that 30% of *A. niger* from grapes cultivated in Brazil was OTA producers and Magnoli et al. (2003a) found an unusually high percentage (43.1%) of ochratoxigenic isolates in the species *A. niger* var. *niger* from grapes cultivated in Argentina. *A. carbonarius* was less common and it was present only in samples from Catamarca and San Juan provinces but not from Mendoza, results that confirm those reported in previous studies on mycobiota of wine grapes from this province (Da Rocha Rosa et al., 2002; Magnoli et al., 2003a). A high proportion (96%) of *A. carbonarius* screened in the present work was ochratoxigenic. Similar results were found by Abarca et al. (2003), from 91 *A. carbonarius* isolated, 88 (96.7%) were found to be OTA producers. The consistent ability of this species to produce OTA has also been reported by other authors (Tèren et al., 1996; Heenan et al., 1998; Da Rocha Rosa et al., 2002; Taniwaki et al., 2003; Battilani et al., 2003; Serra et al., 2003) who found percentages of ochra-

Table 3
OTA production by *Aspergillus* sect. *Nigri* isolated from dried vine fruits in Argentina

Sample	Origin	N° of strains toxigenic/screened		
		A. niger	*A. carbonarius*	*A. japonicus*
1	Mendoza (Capital)	0/41	–	–
2	Mendoza (Capital)	–	–	–
3	Mendoza (Capital)	–	–	0/2
4	Mendoza (San Rafael)	1/58	–	–
5	Mendoza (San Rafael)	–	–	–
6	San Juan (Santa Lucía)	0/16	–	–
7	San Juan	0/46	–	–
8	San Juan	0/11	7/7	0/1
9	Salta (Cafayate)	0/25	–	0/5
10	Catamarca (Tinogasta)	1/23	26/27	0/11
11	Catamarca (Fiambalá)	1/73	13/14	0/1
Total		3/293 (1%)	46/48 (96%)	0/20 (0%)

toxigenic *A. carbonarius* strains ranging from 41.7 to 97%.

These results demonstrate that production of OTA by *A. carbonarius* is quite common but it is rare in *A. niger*. Furthermore, *A. carbonarius* strains are stronger OTA producers than *A. niger* strains. Data from several workers demonstrated levels of OTA produced in culture media ranging from 0.5 to 234 μg/g (Bragulat et al., 2001; Sage et al., 2002; Da Rocha Rosa et al., 2002). Most strains of *A. carbonarius* isolated in the present work were able to produce more than 1 μg/g after 7 days in CYA while strains of *A. niger* produced no more than 0.1 μg/g under the same conditions. *A. japonicus* strains were not OTA producers as it was mentioned in the literature (Tèren et al., 1996; Klich, 2002). The three strains of *A. ochraceus* isolated produced OTA. This species is rarely encountered in foods in our country. In the present work, *A. ochraceus* infected only 0.1% of the dried fruits and cannot be considered an important hazard in this foodstuff.

Several other known toxigenic species were also isolated and screened in their ability to produce mycotoxins. None of the 5 strains of *Aspergillus flavus* were aflatoxigenic, but all of them produced cyclopiazonic acid. Some of the *Penicillium* species listed in Table 2 (e.g. *P. chrysogenum*, *P. citreonigrum* and *P. crustosum*) are potential producers of a very wide range of toxic compounds that could be considered as a hazard to human health (Samson et al., 2002). All the isolates of *P. citrinum* (19 strains) were tested and all were strong citrinin producers. As citrinin is a nephrotoxin, it could act synergistically with OTA (Castegnaro and McGregor, 1998). *A. alternata*, isolated in moderate percentage (5.4%) (Table 2), is known as producer of numerous secondary metabolites (Bottalico and Logrieco, 1998). In the present study, 15 strains were analysed for tenuazonic acid (TA), alternariol monomethyl ether (AME) and alternariol (AOH) production. Eight strains (53%) produced TA, 10 (67%) produced AME and 12 (80%) were AOH producers. The presence of moulds capable of producing mycotoxins in this product should be considered as a potential hazard for public health. Industry should work further to reduce fungal contamination and growth of these fungi and mycotoxin production by maintaining good agricultural and manufacturing practices.

Samples analysed in the present study were also tested for OTA contamination (Table 4). Our results

Table 4
Ochratoxin A concentrations in dried vine fruits

Sample	(μg/kg)
1	0.20
2	0.14
3	0.16
4	0.16
5	0.21
6	n.d.
7	0.29
8	n.d.
9	0.39
10	n.d.
11	0.11

n.d.: not detected (<0.01 $\mu g \cdot kg^{-1}$).

indicate a very low level of contamination in comparison with those obtained by MacDonald et al. (1999) who reported a range of 0.2–53.6 μg/kg and by Stefanaki et al. (2003), with an overall mean of 2.6 μg/kg. In spite of the high incidence of ochratoxigenic *A. carbonarius* in the samples, low levels of OTA in dry vine fruits have been found in the present study. The fruit under study was highly prone to infection with this fungus, and the amount of OTA produced by toxigenic isolates was fairly high but only low levels of OTA were found in the natural substrate. This could be due to the local environmental conditions during harvesting and drying of grapes and storage practices that did not allow the development of OTA production. However, another recent study (Magnoli et al., 2003b) showed levels of contamination ranging from 1 to 7.5 μg/kg in dried vine fruits produced in Argentina. Contamination with OTA in dried vine fruits depends on several factors such as the distribution of toxigenic species in the fruits, the traits of the species present, the variety, the geographical origin, the climatic conditions and the drying method. Although further investigations are needed to assess the influence of environmental conditions and geographical differences on the OTA production by black aspergilli, results of the present work indicate that this group of fungi may be an important source of OTA contamination in dried vine fruits in Argentina, as it is in other regions of the world.

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