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ORIGINAL ARTICLE

Fungi and ochratoxin A detected in healthy grapes for wine production

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Abstract

Aims: The mycoflora of healthy grapes (i.e. without visible symptoms of rot) for wine production in Portuguese winemaking regions was assessed and its potential for ochratoxin A (OTA) production evaluated. The OTA content of grapes was also determined.

Methods and Results: A total of 386 fungal strains were isolated by plating methods. The most frequent genera found in grapes were non-ochratoxigenic species: *Cladosporium* (28%), *Penicillium* (24%), *Botrytis* (13%) and *Aspergillus* (9%). Two OTA-producing strains were isolated, belonging to the species *Aspergillus carbonarius* and *Aspergillus ochraceus*. OTA was detected in three of four grape samples, up to 116 ng l^{-1}.

Conclusions: OTA is being produced in healthy berries by *Aspergillus* species, namely *A. carbonarius*, at levels below the maximum recommended limit of 2000 ng l^{-1} in wine.

Significance and Impact of the Study: The OTA concentration detected in healthy Portuguese grapes does not represent a risk to wine regarding the legal limit established.

Introduction

Filamentous fungi can cause spoilage of grapes and/or contaminate them with toxic secondary metabolites named mycotoxins (Bennett and Klich 2003). Several mycotoxins have been detected in wine, namely ochratoxin A (OTA), *Tricothecium roseum* metabolites, and alternariol (Serra 2005). Additionally, the mycotoxin patulin was detected in grapes and grape juice (Scott *et al.* 1977); however, it is degraded to some extent during the fermentation process and it has not been detected in wine.

Ochratoxin A is nowadays the most relevant mycotoxin found in wine. It is considered one of the main mycotoxins hazardous for human health (CAST 2003). Exposure of the EU population to this contaminant was confirmed over the last few years. In a European assessment of the contribution of each food commodity to the mean total dietary intake of OTA, wine was considered the second major source of OTA intake, contributing with 10% (Miraglia and Brera 2002). In this report, cereals were the main source of OTA intake (44%). Other beverages such as beer and coffee represent an estimated OTA intake of 7% and 9% respectively. EU legislation regarding maximum admissible limits for OTA presence in food exists for cereals and its by-products and for dried vine fruits (Commission of the European Communities 2002). Recently, EU legislation was extended to foods for infants and children (Commission of the European Communities 2004) to coffee, grape juice and wine. The maximum limit proposed for OTA in grape juice and wine is 2000 ng l^{-1} (Commission of the European Communities 2005).

A recent survey of 340 Portuguese wines revealed that 69 samples had detectable levels of OTA. Of these three exceeded 500 ng l^{-1} OTA, the maximum concentration detected being 2100 ng l^{-1} (Ratola *et al.* 2004).

The contamination of wines with OTA varies with the type of wine (red, white, rosé and sweet wines) and with the region of origin. Some sweet wines, followed by red wines, from the Mediterranean basin are the ones most frequently contaminated with the mycotoxin and at highest concentrations. The OTA content of the distinct types

of wine is related to differences in the winemaking process. However, the variation between the OTA content of wines and the geographical origin is related to the climatic conditions of the winemaking regions and to the mycoflora of grapes. The grapes are exposed to OTA-producing fungi in the vineyard. Only two fungal genera have species capable of producing OTA: *Aspergillus* and *Penicillium*. Previous studies on the contamination of grapes with OTA indicate that the species responsible for OTA production in this commodity are *Aspergillus* species. The most frequent OTA-producing strains found in grapes belong to the species *Aspergillus carbonarius*. Strains of other OTA-producing species have been isolated from grapes less frequently, such as *Aspergillus niger* and *Aspergillus ochraceus* (Serra 2005).

The contamination of grapes with OTA can occur in the field, while grapes are still in the vineyard (Serra *et al.* 2004). It is known that the contamination of agricultural commodities with mycotoxins can occur without visible fungal contamination; therefore, it is important to assess the OTA content of healthy grapes (i.e. without visible symptoms) in order to define critical control points for preventive measures for contamination of wine with OTA.

In the present work, the mycoflora of Portuguese grapes of two winemaking regions was investigated, in order to assess (i) the OTA-producing strains present and (ii) the OTA contamination of healthy grapes for wine production.

Materials and methods

Sampling

Grapes for wine production were collected from two winemaking areas: Dão and Madeira Island. The geographical coordinates and annual temperature and precipitation values are shown in Table 1. Dão is located in the Portuguese mainland. Red and white wines are produced in this region. Madeira Island is located in the North Atlantic Ocean. In this region is produced the sweet wine 'Madeira'. Both regions have Mediterranean climate, but Dão is more humid than Madeira.

Table 1 Geographical coordinates, mean annual temperature and precipitation values, and type of climate of the two winemaking regions studied

	Region	
	Madeira Island	Dão
Latitude	32°38′N	40°39′N
Longitude	16°56′W	7°54′W
Mean annual temperature* (°C)	18·7	13·0
Annual precipitation* (mm)	513·7	1229·3
Type of climate	Mediterranean	Mediterranean

*Values based on the climatologic data of the Portuguese Meteorological Institute over a 30-year period between 1931 and 1960 for Madeira Island, and 1951 and 1980 for Dão.

The samples were collected in Dão and Madeira at harvest, which took place in the first week of October and September respectively. Two vineyards were studied in each winemaking region at the 2003 harvest with distinct grape varieties. In Madeira, the grape varieties studied were Boal (white variety) and Tinta Negra Mole (red variety). In Dão, the red varieties Cabernet Sauvignon and Touriga Nacional were selected. From each vineyard, 10 grape bunches apparently healthy, without visible fungal rot, were collected across two diagonal transects. Grapes were transported to the laboratory in closed paper bags in refrigerated boxes. The grapes collected in Madeira Island were transported by airplane in rigid boxes. The time between sample collection and laboratory analysis was <6 h.

Mycoflora survey of grapes

The mycoflora of grapes was determined as described elsewhere (Serra *et al.* 2003): a total of 50 berries (five berries per bunch) of each sample were plated in Dichloran Rose Bengal Chloramphenicol medium (Oxoid CM727) and incubated at 25°C in the dark for 1 week. The spore-producing filamentous fungi detected were identified to genus level and *Aspergillus* and *Penicillium* strains were isolated and identified to species level.

Preservation of fungal strains

Representative strains of the filamentous fungi genera detected, as well all the isolated *Aspergillus* strains were preserved in 10% glycerol at −80°C and are deposited in MUM (Micoteca da Universidade do Minho) culture collection.

OTA production by fungal strains

Detection of OTA in the cultures consisted of extracting, from cultures grown 7–9 days in Czapek Yeast extract Agar (CYA) medium, agar plugs with methanol, filtering and injecting the samples into the HPLC system (Bragulat *et al.* 2001). Although the OTA production was assessment in CYA rather than a natural media, it was previously found that all strains that are positive for OTA production in CYA are also positive in grape media and vice versa (Serra *et al.* 2005b). Strains were considered positive for OTA production if the extract yielded a peak at a retention time similar to the OTA standard (approx. 11 min). The identity of OTA was confirmed by derivati-

zation of OTA in its methyl ester with boron trifluoride in methanol (BF_3) as described by Hunt *et al.* (1980). The detection limit of the method was 0·1 μg OTA per kg of agar plugs.

OTA analysis of grapes

The grapes (10 bunches per sample) were analysed according to a previously described method by Serra *et al.* (2004). The berries were detached from the rachis, visually inspected for eventual fungal rots and homogenized in a blender. An aliquot of 50 g was extracted with a 5% sodium bicarbonate and 1% polyethylene glycol solution. The mixture was centrifuged and the supernatant was applied to an immunoaffinity column (OchraTest; Vicam, Boston, MA, USA). The columns were used according to the manufacturer instructions. OTA was eluted with HPLC grade methanol and quantified by reverse-phase HPLC using a fluorescent detector. The average recovery rate of this method was 76% and the relative standard deviations in repeatability and intermediate precision conditions were of 8% and 12% respectively (Serra *et al.* 2004). The equipment used was a Jasco FP-920 system (Jasco, Tokyo, Japan) set at 330 nm excitation and 460 nm emission wavelengths. Chromatographic separations were performed on a Waters Spherisorb ODS2 (4·6 × 250 mm; 5 μm) column (Waters, Milford, MA, USA), fitted with a precolumn with the same stationary phase operated at 30°C. The mobile phase was pumped at 1·0 ml min^{-1} and consisted of an isocratic program as follows: acetonitrile : water : acetic acid (99 : 99 : 2, v/v). The injection volume was 100 μl. The limit of detection in grapes was 4 ng kg^{-1}.

Results

The fungal species isolated from grapes are indicated in Table 2. The genera most frequently detected were *Cladosporium* (28%), *Penicillium* (24%), *Botrytis* (13%) and *Aspergillus* (9%). The fungal genera more frequently detected in the grapes grown in Dão were by descending order *Cladosporium*, *Aureobasidium*, *Aspergillus*, *Alternaria*, *Rhizopus* and *Botrytis*. Altogether, these genera represented 94% of the strains identified. The fungal genera more frequently detected in the grapes from Madeira were by descending order *Penicillium*, *Cladosporium*, *Botrytis* and *Rhizopus*, which altogether represented 92% of the identified strains.

Twelve *Penicillium* species and three *Aspergillus* species were isolated from grapes. None of the 12 *Penicillium* species identified is recognized as OTA producer. On the contrary, the *Aspergillus* species *A. carbonarius*, *A. niger* aggregate and *A. ochraceus* are OTA-producing species. The 35 *Aspergillus* strains isolated were analysed for its OTA-producing ability. OTA was detected in the cultures of *A. carbonarius* and *A. ochraceus*, in the vineyards shown in Table 3. None of the *A. niger* isolates could produce the mycotoxin in detectable levels. OTA was detected in three of four grape samples (Table 3), in concentrations ranging from 9·5 to 115·6 ng kg^{-1}.

Table 2 Fungi detected in grape samples of Dão, Madeira, and in total berries and number of colonized berries in the samples

	No. of colonized berries		
Fungi	Dão	Madeira	Total
Alternaria spp.	25	4	29
Aspergillus			
A. carbonarius	0	1	1
A. niger (aggregate)	28	5	33
A. ochraceus	0	1	1
Aureobasidium pullulans	31	1	32
Botrytis cinerea	16	35	51
Chrysonilia sp.	0	2	2
Cladosporium spp.	71	36	107
Epicoccum nigrum	1	0	1
Fusarium sp.	1	0	1
Neurospora sp.	0	1	1
Penicilium			
P. aurantiogriseum	1	0	1
P. bilaie	0	1	1
P. brevicompactum	1	56	57
P. chermesinum	0	8	8
P. citrinum	0	1	1
P. glabrum	1	6	7
P. oxalicum	1	0	1
P. raistrickii	0	3	3
P. sclerotiorum	0	2	2
P. spinulosum	1	0	1
P. thomii	1	4	5
P. variabile	0	5	5
Rhizopus spp.	17	13	30
Trichoderma spp.	5	0	5
Total of fungi identified	201	185	386
Total of analysed berries	100	100	200

Table 3 OTA-producing (OTA^+) fungi and OTA content of the grape samples analysed

Region	Grape variety	OTA^+ fungi	OTA (ng ml^{-1})
Dão	Cabernet Sauvignon	ND	115·6
	Touriga Nacional	ND	9·5
Madeira	Boal	*A. carbonarius*	94·1
	Tinta Negra Mole	*A. ochraceus*	<LOD

ND, not detected; LOD, limit of detection; OTA, ochratoxin A.

Discussion

The mycoflora of healthy wine grapes is mostly dominated by non-mycotoxigenic species and species capable of

producing mycotoxins of unknown relevance for human health. This is in agreement with previously published data on the mycoflora of Portuguese wine grapes (Serra *et al.* 2005a). The filamentous fungi isolated in this study have been previously reported in Portuguese grapes (Abrunhosa *et al.* 2001; Serra *et al.* 2003, 2005a), with the exception of *Penicillium chermesinum*, a rare species not previously isolated from this commodity. However, for the first time, *Penicillium* genus was found as the most frequent in Madeira. In other Portuguese regions, such as Dão, *Cladosporium* genus has been reported as the most frequent one. The mycoflora of grapes is composed of common field fungi, such as *Alternaria*, *Cladosporium* and *Aureobasidium*, as well by pathogenic agents of grapes, such as *Aspergillus*, *Botrytis*, *Penicillium* and *Rhizopus*. Although all the mentioned genera were isolated from the grapes of Dão and Madeira, the grapes grown in Madeira were dominated by pathogenic species and had a lower incidence of field fungi. *Aspergillus*, *Botrytis*, *Penicillium* and *Rhizopus* species represented 78% of the total fungi isolated, while *Alternaria*, *Aureobasidium* and *Cladosporium* represented 21%. On the contrary, the mycoflora of Dão samples was dominated by field fungi. *Alternaria*, *Aureobasidium* and *Cladosporium* accounting for 60% of the total fungi isolated, while the pathogenic species *Aspergillus*, *Botrytis*, *Penicillium* and *Rhizopus* represented 37% of the total fungi isolated in the grapes of the last mentioned region.

Regarding the pathogenic species found, the main differences between regions concerned the incidence of *Aspergillus* and *Penicillium* in grapes. *Aspergillus*, namely *A. niger* aggregate, were the most frequent pathogenic species isolated from the grapes from Dão; *Penicillium* species, namely *Penicillium brevicompactum*, were the most frequent pathogenic species in Madeira grapes.

None of the *Penicillium* species isolated is described as OTA producer. The only known *Penicillium* species capable of OTA production are *Penicillium verrucosum* and *Penicillium nordicum*, common in cereals and meat products respectively (Larsen *et al.* 2001). From all the surveys conducted in grapes, these species were never isolated. Nevertheless, other mycotoxin-producing *Penicillium* species were isolated from grapes, namely *Penicillium expansum*, a patulin and citrinin producer (Abrunhosa *et al.* 2001).

From the three *Aspergillus* species isolated, the most frequent was *A. niger* aggregate. *Aspergillus niger* aggregate is the most common *Aspergillus* species in grapes, being dominant in ripe berries at harvest time (Serra *et al.* 2003). Nevertheless, this is not the species responsible for OTA production in this commodity. OTA production by *A. niger* strains is rare. In previous studies, 4% of the *A. niger* strains isolated from grapes were ochratoxigenic (Serra *et al.* 2003, 2005a). In the present study, no OTA-producing *A. niger* strains were found. The higher incidence of the *A. niger* aggregate in the Dão Region, could be explained by its proximity with the Douro Region (41°N, 7°W), where the *A. niger* aggregate is dominant and *A. carbonarius* is rarely isolated (Serra *et al.* 2004).

From the 35 *Aspergillus* strains isolated, only two positive strains were found: one isolate of *A. carbonarius* and one isolate of *A. ochraceus*. The *Aspergillus* species considered responsible for OTA production in grapes is *A. carbonarius*. Unlike *A. niger*, 100% of the *A. carbonarius* strains have the ability to produce the mycotoxin. In Portugal and Spain, the only strains identified as *A. carbonarius* that did not produced the mycotoxin (Abarca *et al.* 2003; Serra *et al.* 2003) were latter proposed as belonging to a new species, *Aspergillus ibericus* sp. nov., which formal description is in progress (Cabañes *et al.* 2004). Other researchers claimed that certain *A. carbonarius* strains do not produce OTA, but this needs further investigation (Bau *et al.* 2005).

The OTA-production ability of *A. carbonarius* strains in Portuguese grape varieties was recently confirmed (Serra *et al.* 2005b). *Aspergillus carbonarius* strains are the most frequent OTA-producing strains isolated from grapes, and its presence was confirmed in the grapes grown in the winemaking regions that produce wines contaminated with the mycotoxin (Cabañes *et al.* 2002; Tjamos *et al.* 2004).

On the contrary, *A. ochraceus* is rarely isolated from grapes, and therefore, is not considered a relevant species concerning OTA production in this commodity. Nevertheless, Pardo *et al.* (2005) recently reported the ability of *A. ochraceus* strains to produce the mycotoxin in grapes. It may play a secondary role in OTA contamination in this commodity, but is probably negligible compared with *A. carbonarius*.

Ochratoxin A was detected in three of four samples analysed. The fact that OTA was detected in healthy berries at harvest confirms that despite OTA-producing strains rarely isolated from grapes, the infection of grapes by OTA-producing fungi occurs in the vineyard, without visible signs of rot. However, OTA was also detected in samples from where no OTA-producing fungi were isolated (Dão samples). This is most probably because of sampling issues, as the OTA-producing species are rare compared with the remaining mycoflora of grapes. Nevertheless, the most frequent *Aspergillus* species in Dão was *A. niger* aggregate. In previous studies we found a significant positive correlation between the presence of *A. niger* and *A. carbonarius* in other winemaking regions (R. Serra and A. Venâncio, unpublished data), suggesting that the more common and less ochratoxigenic species *A. niger* aggregate is an indicator of the rarer but more ochratoxigenic species *A. carbonarius* in grapes in Portugal.

Our results demonstrate that although OTA production can occur in grapes with no visible fungal growth, its levels do not seem to present concern considering the maximum limit proposed for OTA in wine. This is in agreement with our previous studies concerning OTA concentration of the grapes of other Portuguese winemaking regions (Serra *et al.* 2004). However, OTA detection in healthy berries confirms the presence of OTA-producing fungi and its ability to infect berries in the vineyard. This is of importance for defining a potential risk for a severe OTA contamination resulting from a heavy fungal infection. As *Aspergillus* species are not considered primary pathogens, the damage of grapes due to different causes, such as attack by other fungi or mechanical injuries, increase dramatically the risk of fungal infection by these species and OTA contamination. It was found that the OTA content of a rotten bunch with *Botrytis cinerea*, *A. carbonarius* and *T. roseum* had 7500 ng kg^{-1} of OTA (Serra *et al.* 2005a). In the winemaking process, some OTA is removed with the removal of the solid parts of grapes (Fernandes *et al.* 2003, 2005; Ratola *et al.* 2005), but inevitably high levels of OTA in grapes present a hazard for wine.

Based on the data found in this work and corroborated by data from the literature (Ratola *et al.* 2004; Serra *et al.* 2004, 2005b), the main conclusion is that the OTA contamination of healthy berries is not significant, but vinification of rotten berries in areas where OTA-producing fungi and OTA are detected must be avoided to minimize the risk of mycotoxin presence in wine.

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