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Mycotoxin-producing and other fungi isolated from grapes for wine production, with particular emphasis on ochratoxin A

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Abstract

Mycotoxins are toxic secondary metabolites produced by filamentous fungi that have been detected in food commodities, including grapes and wine. A survey was conducted to assess mycotoxin-producing fungi in grapes destined for wine production. The mycotoxigenic capacity of the isolates was tested in culture media. Grapes were analyzed by plating methods from 4 Portuguese wine-growing regions at 3 maturation stages (pea berry, early veraison and ripe berry) between 2001 and 2003. From the 10 602 strains detected and identified, the most frequent genera were *Cladosporium* (25%), *Alternaria* (24%), *Botrytis* (15%), *Penicillium* (9%) and *Aspergillus* (8%). Most (92.0%) were non-mycotoxigenic or produced mycotoxins of unknown relevance to health. Potential producers of ochratoxin A (OTA) (*Aspergillus niger* aggregate, 5.4%, *Aspergillus carbonarius*, 0.6%) and trichothecenes (*Fusarium* spp., 0.4%; *Trichothecium roseum*, 0.8%) were the most frequent mycotoxigenic species isolated from grapes. OTA was detected in all cultures of *A. carbonarius* and 4% of *A. niger* aggregate strains. There was potential for OTA and trichothecene production in grapes by *A. carbonarius* and *T. roseum*, respectively, prior to harvest time. Data presented herein indicate that *A. carbonarius* is most likely to occur in vineyards with Mediterranean climates, while *T. roseum* is more likely to occur in more temperate climates, and is associated with gray rot. The present work emphasizes the need to use grapes under good condition so as to reduce the risk of contamination with mycotoxigenic fungi and subsequent mycotoxin occurrence in wine.

Keywords: Mycotoxins; Ochratoxin A; Trichothecenes; Grapes; *Aspergillus carbonarius*; *Trichothecium roseum*; *Aspergillus niger*

1. Introduction

The concern about filamentous fungi in the vineyard has been traditionally linked to spoilage of grapes due to fungal growth. However, the discussion in the European Union concerning the establishment of a maximum limit for the presence of the mycotoxin ochratoxin A (OTA) in wines has increased concern about mycotoxin production.

Mycotoxins are toxic metabolites produced by filamentous fungi that have been detected in several food commodities. Levels that cause risk to populations are unacceptable, and several countries have set regulations for mycotoxins in various food commodities [13,14,17,22,29,30].

There exist hundreds of mycotoxins [5], but relatively few are frequently detected in foods and are considered relevant to human health. The significance of mycotoxins for human health is not easily assessed, as the effects are often subtle. Toxicity is associated with continued ingestion of low doses, hence the designation “insidious poisons”. Mycotoxins considered most relevant for human health by the Council of Agricultural Science and Technology (CAST) are aflatoxins, trichothecenes, fumonisins, zearalenone, OTA and ergot alkaloids [8], although this list is continuously revised. Other mycotoxins are considered of less importance, due to limited occurrence or lack of evidence of their toxicity in humans. In some cases they are an important indicator of the use of poor quality raw materials, such as patulin in apple products [21].

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Table 1
Mycotoxins considered to be most relevant for human health by CAST [8] or for which official regulation limits in food commodities exist, and the main organisms responsible for their production in foods

Mycotoxin	Fungal species
Aflatoxins	*Aspergillus flavus* and *A. parasiticus*
Ochratoxins	*A. ochraceus, A. alliaceus, A. niger* aggregate, *A. carbonarius*; *Penicillium verrucosum, Penicillium nordicum*
Trichothecenes	*Fusarium* species; *Trichothecium roseum*
Zearalenone	F. culmorum
Fumonisins	*F. verticillioides, F. proliferatum*
Patulin	*P. expansum*

Different mycotoxins are likely to occur depending upon the food commodity under consideration. Mycotoxins are produced by various fungal species (Table 1) with distinct ecological requirements affecting their worldwide distribution and incidence in foods. By elucidating the mycoflora of foods, mycotoxin hazards can be predicted and the appropriate control measures undertaken, such as implementation of HACCP programs.

Mycotoxin production can occur in the field and/or in postharvest situations. It has been found that the synthesis of mycotoxins can occur in grapes before harvest, and thus they may be present in wine [27]. Therefore, it is relevant to determine the mycoflora of grapes and the potential for mycotoxins to be present in wine.

The mycoflora of Portuguese grapes for wine production was evaluated together with the maturation process, in distinct wine-growing regions. The ability of the fungi to produce mycotoxins was tested to assess the potential for mycotoxin synthesis in grapes.

2. Materials and methods

2.1. Study area

Eleven vineyards were studied during a 3-year period (2001–2003) in 4 Portuguese wine-growing regions: Vinhos Verdes, Douro, Ribatejo and Alentejo (Table 2).

According to European Community regulation no. 822/87 revoked by EC regulation no. 1493/1999 of May 17th 1999, Portugal has two distinct wine-growing zones, CIa and CIIb. Wine-growing zones are defined as geographic regions with distinct climatic conditions for grape cultivation. The wine-growing regions of Alentejo, Douro and Ribatejo have Mediterranean climates and belong to the wine-growing area CIIIb. The Vinhos Verdes region has a sub-Mediterranean climate. This particular type of climate is a variant of the temperate climate with Mediterranean influence, as it is more humid than typical Mediterranean climates. This region belongs to the wine-growing zone CIa. The maximum temperatures observed in high summer were between 38 and 42 °C.

Table 2
Number of vineyards and geographical coordinates of the wine-growing regions studied in the Portuguese mainland

Region	No. vineyards	Geographical coordinates	
		Latitude	Longitude
Alentejo	2	38° N	7° W
Douro	3	41° N	7° W
Ribatejo	3	39° N	8° W
Vinhos Verdes	3	41–42° N	8° W

2.2. Sampling time and maturation stages

Samples were collected from June 2001 to September 2003, in the maturation stages corresponding to pea berry (June/early July), early veraison (late July/August) and harvest (late August/September).

2.3. Mycological analysis of grapes

The mycoflora of grapes was determined as described elsewhere [26]: a total of 50 berries (5 berries per bunch) from each sample were plated in Dichloran Rose Bengal Chloramphenicol agar medium (DRBC) and incubated at 25 °C in the dark for one week. The spore-producing filamentous fungi detected were identified to genus level based on morphological characters according to the manuals of Ellis [11] for dematiaceous fungi, and according to the manuals of Barron [2], Domsch et al. [10], Pitt and Hocking [21] and Von Arx [31] for the other fungi. *Penicillium* and *Aspergillus* strains were isolated and identified to species level based on morphological characters according to the manuals of Pitt [19,20] for *Penicillium* and the manual of Klich and Pitt [12] for *Aspergillus* and their teleomorphs. Additionally, grapes were surface-disinfected according Pitt and Hocking [21] and plated in the same medium.

2.4. Fungal strains

Representative strains of the filamentous fungi detected were preserved in 10% glycerol at −80 °C and deposited in the MUM (Micoteca da Universidade do Minho) culture collection.

2.5. Mycotoxigenic capacity of the isolates

OTA-producing ability of the *Aspergillus* isolates was determined according to the method described in Serra et al. [26] by HPLC with fluorescent detection. In addition, the mycotoxigenic ability was tested in a medium with 50% grape juice extract (GJ50). The grape juice extract was obtained by homogenizing wine grapes harvested for winemaking. It was centrifuged at 8500 rpm for 1 min and filtered through a glass microfiber filter (1.5 μm). The grape extract obtained was autoclaved at 90 °C for 30 min and added to autoclaved water with agar 2% (121 °C, 15 min). The mixture was autoclaved at 102 °C for 5 min.

2.6. Statistic analysis

All statistic analyses and graphics were performed with the Statistic Package for Social Sciences (SPSS) for Windows version 11.0. Non-parametric tests were used since most of the variables did not follow a normal distribution. To evaluate whether significant differences existed between the fungal incidence in grapes and variable factors year, region and maturation stage, the Kruskal–Wallis test, with approximation to the chi-square test, as a hypothesis test was used. When statistically significant, the differences were explored between values using the same test. The statistical analyses performed were considered significant when $P < 0.05$.

3. Results

3.1. Mycoflora of grapes during the maturation stage

The filamentous fungi identified from June 2001 to September 2003 by the direct plating method are indicated in Table 3. Without surface disinfection, a total of 10602 strains belonging to 39 genera were identified. The 5 most abundant genera found by descending order were *Cladosporium*, *Alternaria*, *Botrytis*, *Penicillium* and *Aspergillus*. *Epicoccum nigrum*, *Aureobasidium pullulans*, *Rhizopus*, *Stemphylium*, *Ulocladium*, *Trichoderma* and *Trichothecium roseum* were detected in more than 1% of the berries analyzed. The remaining 27 genera were detected in less than or equal to 1% of the berries.

The mycoflora changed significantly with maturation stage. The most frequent genera by descending order at pea berry were *Alternaria*, *Cladosporium*, *Botrytis*, *Epicoccum*, *Penicillium*, *Aureobasidium*, *Stemphylium* and *Aspergillus*. These constitute 92% of the fungi identified. The highest incidence of *Arthrinium*, *Cunninghamella*, *Drechslera*, *Fusarium*, *Gliocladium* and *Pestalotiopsis* in grapes was observed at this stage.

Significant changes occurred in the incidence of *Botrytis* ($P < 0.01$), *Penicillium* ($P < 0.001$) and *Stemphylium* ($P < 0.01$) at early veraison. *Botrytis* and *Penicillium* increased, while the *Stemphylium* incidence decreased. The most frequent genera by descending order were *Cladosporium*, *Alternaria*, *Botrytis*, *Penicillium*, *Epiccoccum*, *Aspergillus*, *Aureobasidium* and *Ulocladium*, representing 93% of the fungi identified at this stage.

Significant changes occurred in the incidence of *Aspergillus* ($P < 0.01$), *Penicillium* ($P < 0.01$) and *Rhizopus* ($P < 0.01$), which increased significantly from early veraison to ripe berry. In addition, a significant decrease was observed in *Alternaria* ($P < 0.001$), *Arthrinium* ($P < 0.05$), *Chaetomium* ($P < 0.05$), *Drechslera* ($P < 0.01$), *Epicoccum* ($P < 0.001$), *Fusarium* ($P < 0.05$), *Phoma* ($P < 0.01$), *Stemphylium* ($P < 0.001$) and *Ulocladium* ($P < 0.01$). The most frequent genera by descending order were *Cladosporium*, *Botrytis*, *Alternaria*, *Aspergillus*, *Penicillium*, *Aureobasidium*, *Rhizopus* and *Epicoccum*, representing 96% of the fungi identified at this stage.

From the most frequent fungi found in grapes, *Cladosporium* and *Aureobasidium* were the only ones that did not vary significantly with maturation.

The *Aspergillus* and *Penicillium* strains were isolated and identified to species level. The isolation rates for *Aspergillus* and *Penicillium* from the berries were 94 and 89%, respectively. From the 770 *Aspergillus* strains identified, the most frequent were from section *Nigri* (84%), namely the bisseriate species *A. carbonarius* and *A. niger* aggregate (Table 4).

Table 3
Fungi identified in Portuguese wine grapes from June 2001 to September 2003 by the direct plating method

Fungi	Number of colonized berries			
	Pea berry	Early veraison	Harvest	Total
Acremoniella Sacc.	5	7	1	13
Acremonium Link	1	8	1	10
Alternaria Nees: Fr.	931	1009	572	2512
Arthrinium Kunze	11	8	0	19
Aspergillus Fr.: Fr.	80	178	548	806
Aureobasidium Viala & G. Boyer	118	168	170	456
Beauveria bassiana Vuill.	1	0	0	1
Botrytis P. Micheli: Fr.	342	706	584	1632
Chaetomium Kunze	2	10	0	12
Chrysonilia Arx	0	1	4	5
Cladosporium Link	735	1052	858	2645
Cunninghamella Matr.	5	2	2	9
Curvularia Boedijn	2	5	2	9
Dendryphiella	1	0	0	1
Drechslera S. Ito	20	12	3	35
Emericella Berk.	1	6	5	12
Epicoccum nigrum Link	241	244	111	596
Eurotium Link: Fr.	1	1	3	5
Fusarium Link	24	16	3	43
Geotrichum Link: Fr.	2	0	0	2
Gliocladium Corda	6	1	1	8
Histoplasma Darling	2	0	0	2
Mucor P. Micheli: Fr.	4	3	11	18
Neurospora tetrasperma Shear & Dodge	0	2	2	4
Nigrospora Zimm.	0	5	1	6
Paecilomyces Bainier	1	2	0	3
Penicillium Link	137	340	520	997
Periconia Tode ex Fr.	4	0	0	4
Pestalotiopsis Steyeart	7	1	0	8
Phoma Sacc.	2	12	1	15
Pithomyces chartarum Ellis	9	11	4	24
Rhizopus Ehrenb.	29	39	130	198
Scytalidium Pesante	1	2	0	3
Stemphylium Wallr.	114	55	2	171
Syncephalastrum racemosum J. Schröt.	1	0	0	1
Trichoderma Pers.	32	44	25	101
Trichotecium roseum Link	16	23	40	79
Truncatella Steyeart	1	0	0	1
Ulocladium Preuss	39	78	19	136
Total identified fungi	2928	4051	3623	10602
Total berries analyzed	1450	1645	1600	4695

Table 4
Aspergillus species identified in Portuguese wine grapes from June 2001 to September 2003 by the direct plating method

Aspergillus species	Number of colonized berries			
	Pea berry	Early veraison	Harvest	Total
A. aculeatus Iizuka	0	1	0	1
A. alliaceus Thom & Church	0	1	0	1
A. auricomus Saito	2	1	0	3
A. candidus Link	1	0	0	1
A. carbonarius Bainier	0	2	66	68
"A. ibericus"	0	1	7	8
A. carneus Blochwitz	0	1	0	1
A. clavatus Desm.	2	1	0	3
A. flavipes Thom & Church	0	0	1	1
A. flavus Link	5	8	14	27
A. fumigatus Fresen.	9	5	3	17
A. japonicus Saito	0	0	2	2
A. niger aggregate	45	113	413	571
A. ochraceus K. Wilh.	1	0	0	1
A. ostianus Wehmer	0	0	1	1
A. terreus Thom	2	2	3	7
A. terreus var. africanus Raper & Fennell	0	1	0	1
A. ustus Thom & Church	5	4	1	10
A. versicolor Tirab.	3	9	1	13
A. wentii Wehmer	2	10	4	16
Emericella Berk.	1	6	5	12
Eurotium amstelodami L. Mangin	0	1	3	4
Eurotium chevalieri L. Mangin	1	0	0	1
Total strains identified	79	167	524	770
Total berries analyzed	1450	1645	1600	4695

A possible new *Aspergillus* species was found in section *Nigri*, designated here as *A. ibericus*, for which formal characterization is in progress [7]. From the 885 *Penicillium* strains identified, the most frequent were *P. brevicompactum*, *P. glabrum*/*spinulosum* and *P. thomii* (Table 5), which represented 71% of the isolates.

The berries from the vineyards sampled were generally in good condition. Fungal rot in the vineyards studied was observed only on one occasion. The gray rot caused minor damage from grapes of the Douro vineyard in the 2002 harvest. *Trichothecium roseum* and *A. carbonarius* were observed together with *Botrytis cinerea* gray rot in some grape bunches (Figs. 1 and 2).

3.2. Potential mycotoxin-producing fungi found in grapes

Species described as producers of mycotoxins represented 8.0% of the grape mycoflora (Tables 1, 2, 3 and 4), distributed as follows: potential producers of aflatoxins (0.3%), OTA (6.0%), patulin (0.5%) and trichothecenes (1.2%). The remaining 92.0% were described as non-mycotoxigenic or as producers of mycotoxins of unknown importance to health.

A. flavus was the only isolated species to be described as an aflatoxin producer. Strains were isolated from berries of all maturation stages without surface disinfection. Its presence was detected in the 4 wine-growing regions colonizing 2–4% of the sampled berries.

Table 5
Penicillium species identified in Portuguese wine grapes from June 2001 to September 2003 by the direct plating method

Penicillium species	Number of colonized berries			
	Pea berry	Early veraison	Harvest	Total
P. aurantiogriseum Dierckx	8	4	3	15
P. bilaiae Chalabuda	0	0	2	2
P. brevicompactum Dierckx	19	92	142	253
P. canescens Sopp	0	1	0	1
P. chrysogenum Thom	2	1	0	3
P. citrinum Thom	3	10	24	37
P. corylophilum Dierckx	3	1	3	7
P. crustosum Thom	3	6	12	21
P. echinulatum Fassatiova	0	2	1	3
P. expansum Thom	3	2	9	14
P. fellutanum Biourge	1	0	0	1
P. funiculosum Thom	7	3	2	12
P. glabrum/*spinulosum*	17	41	63	121
P. griseofulvum Dierckx	1	1	0	2
P. implicatum Biourge	0	0	4	4
P. janczewskii K.M. Zalessky	1	0	1	2
P. miczynskii Zaleski	3	0	1	4
P. minioluteum Dierckx	2	5	1	8
P. novae-zeelandiae J.F.M. Beyma	3	4	2	9
P. olsonii Bainier & Sartory	0	4	0	4
P. oxalicum Currie & Thom	3	11	10	24
P. pinophilum Hedgcock	1	3	0	4
P. purpurogenum Stoll	2	4	6	12
P. raistrickii G. Sm.	0	0	1	1
P. restrictum J.C. Gilman & E.V. Abbott	1	0	1	2
P. roqueforti Thom	2	5	8	15
P. rugulosum Thom	1	0	0	1
P. sclerotiorum van Beyma	0	0	4	4
P. simplicissimum Thom	5	10	12	27
P. solitum Westling	1	0	0	1
P. thomii Maire	38	84	131	253
P. variabile Sopp	1	4	2	7
P. verruculosum Peyronel	0	2	0	2
P. waksmanii Zaleski	2	6	1	9
Total fungi identified	133	306	446	885
Total berries analyzed	1450	1645	1850	4945

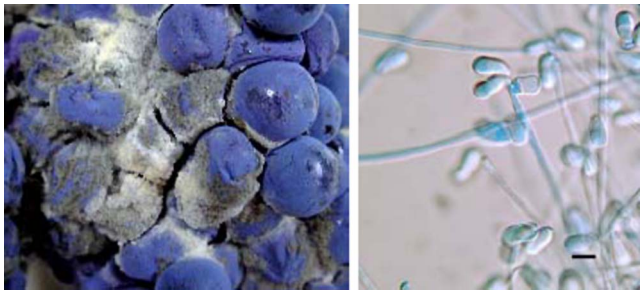

Fig. 1. Aspect of *T. roseum* growing over a rotten bunch of grapes with *B. cinerea* gray rot (left); *T. roseum* conidiophores (right): scale bar = 10 μm.

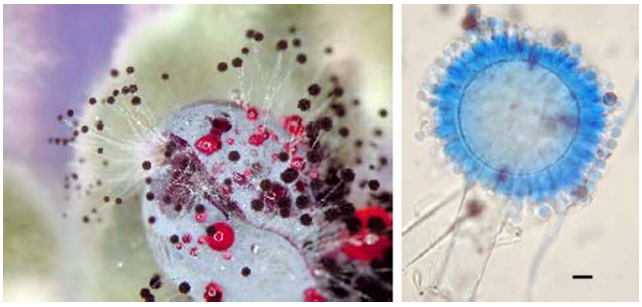

Fig. 2. *A. carbonarius* growing on a grape in an isolation plate (left); *A. carbonarius* conidiophore (right): scale bar = 10 µm.

Table 6
Fungi detected in Portuguese grape berries after surface disinfection

Fungi	Pea berry	Early veraison	Ripe berry
Alternaria	+	+	+
Arthrinium	+	−	−
Aspergillus			
A. carbonarius	−	−	+
A. fumigatus	+	−	−
A. niger aggregate	−	+	+
A. versicolor	−	+	+
Aureobasidium	+	+	+
Beauveria bassiana	+	−	−
Botrytis cinerea	+	+	+
Chaetomium	+	−	−
Cladosporium	+	+	+
Dendryphiella	+	−	−
Drechslera	+	−	−
Emericella	−	−	+
Epicoccum nigrum	+	+	+
Fusarium	+	+	−
Penicillium			
P. brevicompactum	−	−	+
P. citrinum	−	−	+
P. expansum	+	−	+
P. glabrum/spinulosum	+	+	+
P. novae-zeelandiae	+	−	−
P. olsonii	−	−	+
P. thomii	+	+	+
P. variabile	−	−	+
Phoma	+	−	−
Rhizopus	−	−	+
Stemphylium	+	+	+
Ulocladium	+	−	+
Total berries analyzed	450	500	1200

Four *Aspergillus* species described previously as OTA producers were isolated from the grapes: *A. ochraceus*, *A. alliaceus*, *A. niger* aggregate and *A. carbonarius*. From these, *A. niger* aggregate and *A. carbonarius* strains were the most frequent. They were mainly detected at harvest time, with and without surface disinfection (Tables 4 and 6). *A. niger* aggregate and *A. carbonarius* were detected in the 4 regions, and their distribution is given in Fig. 3. *A. niger* aggregate had a significantly higher incidence in the vineyards with a Mediterranean climate (Alentejo, Douro and Ribatejo) when compared to Vinhos Verdes vineyards, of temperate climate with Mediterranean influences ($P < 0.01$). *A. carbonarius*

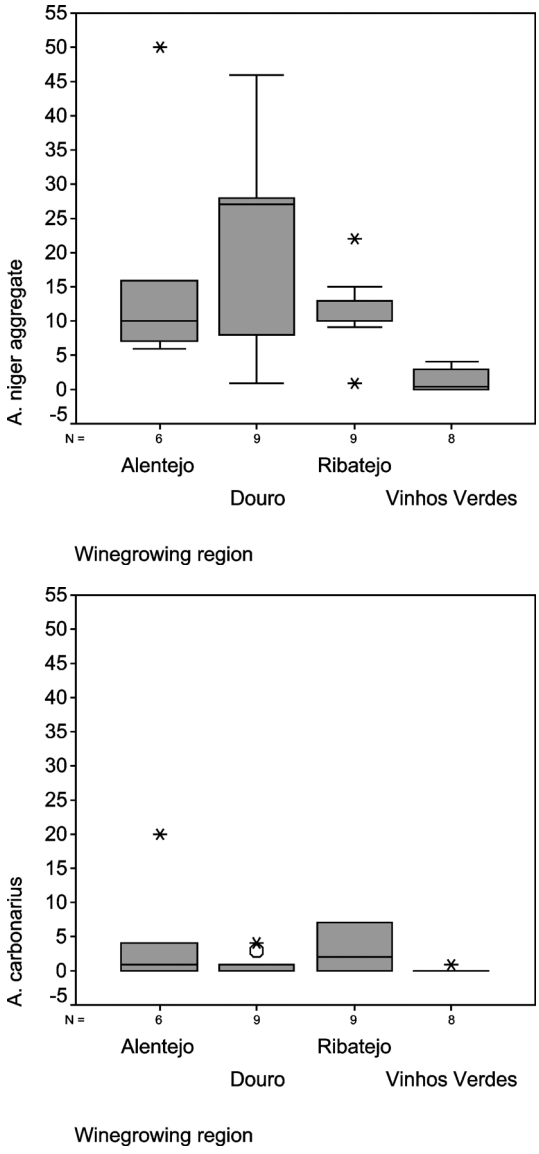

Fig. 3. Boxplots with the distribution of the number of colonized berries with *A. niger* aggregate (left) and *A. carbonarius* (right) in vineyards from the 4 Portuguese wine-growing regions at harvest time (N = number of vineyards; * = extreme values; o = outliers).

was also more frequent in vineyards with Mediterranean climate. The presence of *A. carbonarius* in grape samples was occasional, and the differences observed between regions were only significant between Ribatejo and Vinhos Verdes ($P < 0.05$).

Aspergillus and *Penicillium* species described previously as patulin producers were isolated from grapes: *A. clavatus*, *P. expansum*, *P. funiculosum*, *P. griseofulvum*, *P. novae-zeelandiae* and *P. roqueforti* (Table 4). From these, only *P. expansum* was found after surface disinfection at harvest time (Table 6).

Two genera described as containing trichothecene-producing species were found in grapes: *Fusarium* and *Trichothecium*, the last with a single species in the genus *T. roseum*. *Fusarium* strains were detected at a lower frequency than

Table 7
Mycotoxin-producing ability of potential OTA-producing fungi isolated

Species	No. of isolates tested	OTA-producing strains (%)	Mean production (μg/kg)	
			CYA/YES	GJ50
A. ochraceus	1	0	ND	NT
A. alliaceus	1	100	NQ	NT
A. niger aggregate	571	4	137[a]	26[a]
A. carbonarius	68	100	1129[b]	327[b]

ND—not detected (limit of detection: 0.1 μg/kg); NQ—not quantified; NT—not tested.
[a] Calculations based on the production of OTA by 4 strains in CYA and GJ50.
[b] Calculations based on the production of OTA by 12 strains in CYA and GJ50.

T. roseum from grapes. *Fusarium* strains were primarily detected at the early maturation stages of grapes, with and without surface disinfection (Tables 3 and 6). *T. roseum* was detected primarily at harvest time with a mean percentage of 0.4% berries in the samples from Douro and Ribatejo and 9% from Vinhos Verdes. Nevertheless, the differences found between the two regions were not statistically significant. *T. roseum* was not found after surface disinfection, but was frequently detected from grapes that were infected with *Botrytis*. *T. roseum* growth was observed in some rotten bunches collected in 2002 from a Douro vineyard, and in practically all bunches with gray rot harvested for wine-making delivered to wineries of the Vinhos Verdes region. However, rotten grapes were not observed in the Vinhos Verdes vineyards sampled.

3.3. Mycotoxigenic capacity of fungal isolates

The ability of *Aspergillus* isolates to produce OTA was evaluated. OTA was detected in cultures of *A. alliaceus*, *A. carbonarius* and *A. niger* aggregates (Table 7). *A. carbonarius* was the predominant OTA producer, with all isolates producing the mycotoxin. In the *A. niger* aggregate, 4% of the strains produced OTA in detectable amounts. The OTA-producing isolates also produced the mycotoxin in GJ50 medium. Strains of the possible new species *A. ibericus* of section *Nigri* did not produce OTA in detectable amounts in any of the media tested.

4. Discussion

In a general way, as maturation advances, the incidence of field fungi (e.g., *Alternaria*, *Epicoccum*, *Fusarium*, *Stemphylium* and *Ulocladium*) decreases, while active spoilage agents such as *Aspergillus*, *Penicillium* and *Rhizopus* increase in grapes. Conditions are more favorable for fungal invasion at harvest time, when more damage to the berries is likely to occur.

The mycoflora of the grapes was composed mainly of species that did not produce the main mycotoxins. Nevertheless, low frequencies of aflatoxin, OTA, patulin and trichothecene producers were isolated from apparently healthy berries.

A method for detecting aflatoxins in wine using spiked samples has been described [28], but the natural occurrence of aflatoxins in wines has not been reported. Although *A. flavus* is present in the vineyards, it was not found in grapes after surface disinfection, and rot with this fungus was never observed. *A. flavus* competes with *A. niger* in the field, and its incidence in grapes was low when compared to this species aggregate. Therefore, aflatoxin is unlikely to occur in grapes and wine.

Patulin is a mycotoxin reported in several fruit juices, particularly apple juice. *P. expansum* is considered to be mainly responsible for patulin production in fruits. It is a broad-spectrum plant pathogen that produces the mycotoxin as it rots. *P. expansum* can cause rot in grapes, but does not usually attack grapes before harvest. However, it was isolated from rotten grapes in overmaturation stages and the strains produced patulin in grape juice media [1]. Patulin may be present in grapes with *P. expansum* rot, and consequently in grape juice; however, it is degraded to some extent during the fermentation process [25] and therefore is not likely to occur in wine at high concentrations.

OTA is considered at present to be the most relevant mycotoxin in wine with respect to human health. It is more prevalent in wines originating from the Mediterranean basin [16] and it has been shown to have immunosuppressive effects in doses currently found in the blood of human populations [18]. The International Organization of Wine (OIV) has set a provisional maximum limit of 2 μg/l of OTA. In grapes, the mycotoxin is produced by species of the section *Nigri*, namely *A. carbonarius* and occasionally, *A. niger* [3,4,6,9,15,26]. OTA was detected in apparent healthy Portuguese grapes before harvest, at levels up to 0.061 μg/l [27]. Nevertheless, in a rotten grape bunch where *A. carbonarius* growth was observed, the OTA content determined according to the method described by Serra et al. [27] was 7.5 μg/l (unpublished results). This indicates that OTA may be a problem in wines originating from Mediterranean climates, where OTA-producing fungi are present especially if grapes of poor quality are used.

According to the present results, *T. roseum* mycotoxins may occur in grapes. *T. roseum* metabolites (trichothecin, trichothecolone, rosenonolactone) were previously detected in wines [24]. The relevance of *T. roseum* mycotoxins to human health is neglected due to the rare spoilage of foods by this fungus [21]. Nevertheless, the presence of even low quantities of trichothecin in wine can inhibit alcoholic fermentation, and *T. roseum* rot in grapes has been reported to be increasing [23]. *T. roseum* is described in the literature as a mycoparasite of *Botrytis* [10], and was observed in large numbers of rotten grapes delivered to Vinhos Verdes wineries in 2002 for wine-making. *T. roseum* was not detected after surface disinfection in apparent healthy berries, suggesting that *T. roseum* mycotoxins are likely to occur only if

gray rot bunches with *T. roseum* are used in wine-making. In this way, the presence of these toxins may be indicators of the poor quality of grapes. The contamination of grapes with *T. roseum* seems more likely in regions where rain during harvest time is frequent, such as the Vinhos Verdes region, which has climatic conditions that favor gray rot.

From our study we conclude that the potential for mycotoxin production exists in Portuguese grapes before harvest. The mycotoxins considered relevant for human health and most likely to occur in wines are OTA in regions with a Mediterranean climate and *T. roseum* mycotoxins in more temperate regions.

It should be pointed out that the presence of these mycotoxins appears to be especially relevant when grapes in poor condition are used in winemaking. It is worth emphasizing that the use of good quality raw materials is essential for mycotoxin control in food products.

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References

[1] L. Abrunhosa, R.R. Paterson, Z. Kozakiewicz, N. Lima, A. Venâncio, Mycotoxin production from fungi isolated from grapes, Lett. Appl. Microbiol. 32 (2001) 240–242.
[2] G.L. Barron, The Genera of Hyphomycetes from Soil, Krieger, New York, 1971.
[3] P. Battilani, A. Pietri, T. Bertuzzi, L. Languasco, P. Giorni, Z. Kozakiewicz, Occurrence of ochratoxin A-producing fungi in grapes grown in Italy, J. Food Protect. 66 (2003) 633–636.
[4] M. Bau, M.R. Bragulat, M.L. Abarca, S. Minguez, F.J. Cabañes, Ochratoxigenic species from Spanish wine grapes, Int. J. Food Microbiol., in press.
[5] J.W. Bennett, M.A. Klich, Mycotoxins, Clin. Microbiol. Rev. 16 (2003) 497–516.
[6] F.J. Cabañes, F. Accensi, M.R. Bragulat, M.L. Abarca, G. Castellá, S. Minguez, A. Pons, What is the source of ochratoxin A in wine?, Int. J. Food Microbiol. 79 (2002) 213–215.
[7] F.J. Cabañes, A. Venâncio, G. Mulè, G. Castellá, R. Serra, G. Perrone, Z. Kozakiewicz, DNA characterization of a new species in the *Aspergillus* section Nigri, in: Proc. of Academy Colloquium Fungal Phylogenomics, Amsterdam, The Netherlands, 11–12 May, 2004.
[8] CAST—Council for agricultural science and technology, Mycotoxins, risks in plants, animal and human systems, Report No. 139, CAST, Ames, 2003.
[9] C.A. Da Rocha Rosa, V. Palacios, M. Combina, M.E. Fraga, A. De Oliveira Rekson, C.E. Magnoli, A.M. Dalcero, Potential ochratoxin A producers from wine grapes in Argentina and Brazil, Food Addit. Contam. 19 (2002) 408–414.
[10] K.H. Domsch, W. Gams, T. Anderson (Eds.), Compendium of Soil Fungi, vol. I, Academic Press, London, 1980.
[11] M.B. Ellis, Dematiaceous Hyphomycetes, Commonwealth Mycological Institute, Kew, 1971.
[12] M.A. Klich, J.I. Pitt, A Laboratory Guide to Common *Aspergillus* Species and Their Teleomorphs, Commonwealth Scientific and Industrial Research Organization, Division of Food Processing, North Ryde, 1988.
[13] W.J. de Koe, Regulations of the European Union for mycotoxins in foods, Arh. Hig. Rada. Toksikol. 50 (1999) 37–46.
[14] F.M. Ellner, Mycotoxins—Quantification, Control and Regulation in Agricultural Produce, Human Exposure to Pesticide Residues, Natural Toxins and GMOs: Real and Perceived Risks, 2000, pp. 43–53.
[15] C. Magnoli, M. Violante, M. Combina, G. Palacio, A. Dalcero, Mycoflora and ochratoxin-producing strains of *Aspergillus* section Nigri in wine grapes in Argentina, Lett. Appl. Microbiol. 37 (2003) 179–184.
[16] H. Otteneder, P. Majerus, Occurrence of ochratoxin A (OTA) in wines: Influence of the type of wine and its geographical origin, Food Add. Contam. 17 (2000) 793–798.
[17] D.L. Park, T.C. Troxell, U.S. perspective on mycotoxin regulatory issues, Adv. Exp. Med. Biol. 504 (2002) 277–285.
[18] E. Petzinger, A. Weidenbach, Mycotoxins in the food chain: The role of ochratoxins, Livest. Prod. Sci. 76 (2002) 245–250.
[19] J.I. Pitt, The Genus *Penicillium* and its Teleomorphic States *Eupenicillium* and *Talaromyces*, Academic Press, London, 1979.
[20] J.I. Pitt, A Laboratory Guide to Common *Penicillium* Species, Commonwealth Scientific and Industrial Research Organization, Division of Food Processing, North Ryde, 1988.
[21] J.I. Pitt, A.D. Hocking (Eds.), Fungi and Food Spoilage, Blackie, London, 1997.
[22] H. Rosner, Mycotoxins: Limits in the European Union and Effects on Trade, Mycotoxins and Phycotoxins—Developments in Chemistry, Toxicology and Food Safety, 1998, pp. 203–212.
[23] S. Schwenk, B. Altmayer, K.W. Eichhorn, Simultaneous detection of trichothecenes and rosenonolactone in grape juice and wine by capillary gas chromatography, J. Chromatogr. A 448 (1988) 417–423.
[24] S. Schwenk, B. Altmayer, K.W. Eichhorn, Significance of toxic metabolites of the fungus *Trichothecium roseum* Link ex Fr. for viticulture, Z. Lebensm. Unters. Forsch. 188 (1989) 527–530.
[25] P.M. Scott, T. Fuleki, J. Harwig, Patulin content of juice and wine produced from mouldy grapes, Agric. Food Chem. 25 (1977) 434–437.
[26] R. Serra, L. Abrunhosa, Z. Kozakiewicz, A. Venâncio, Black *Aspergillus* species as ochratoxin A producers in Portuguese wine grapes, Int. J. Food Microbiol. 88 (2003) 63–68.
[27] R. Serra, C. Mendonca, L. Abrunhosa, A. Pietri, A. Venâncio, Determination of ochratoxin A in wine grapes: Comparison of extraction procedures and method validation, Anal. Chim. Acta 513 (2004) 41–47.
[28] D.M. Takahashi, Reversed-phase high-performance liquid chromatographic analytical system for aflatoxins in wines with fluorescence detection, J. Chromatogr. 131 (1977) 147–156.
[29] H.P. van Egmond, Mycotoxins: Regulations, quality assurance and reference materials, Food Addit. Contam. 12 (1995) 321–330.
[30] H.P. van Egmond, Worldwide regulations for ochratoxin A, IARC Sci. Publ. (1991) 331–336.
[31] J.A. Von Arx, The Genera of Fungi Sporulating in Pure Culture, J. Cramer, Vaduz, 1981.