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Fungal Flora and Ochratoxin A Production in Grapes and Musts from France

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Eleven samples of grapes and musts used in red table wines were investigated for the occurrence of potential ochratoxin A (OTA)-producing molds. From these samples, 59 filamentous fungi and 2 yeasts were isolated. Among the 30 genera isolated, Deuteromycetes were the most frequent (70%) followed by Ascomycetes (10%). Six of the eleven grapes samples were contaminated by potentially ochratoxinogenic strains (*Penicillium chrysogenum* and *Aspergillus carbonarius*). When cultivated in vitro on solid complex media, the 14 strains of *A. carbonarius* produced OTA. No other species produced OTA under the same conditions. Among must samples, eight of eleven were found to be contaminated by OTA (concentrations from <10 to 461 ng/L). There is a strong correlation between the presence of ochratoxin-producing strains on grapes and OTA in musts. These findings should be connected with the OTA contamination of human blood in these areas and in France.

KEYWORDS: Ochratoxin A; *Penicillium*; *Aspergillus*; grapes; musts

INTRODUCTION

First isolated by van der Merwe et al. (*1*) in cultures of *Aspergillus ochraceus* Wilhelm, ochratoxin A (OTA) was later found in the culture media of various species of *Aspergillus* and *Penicillium* (*2*−*5*). OTA is a widespread contaminant in human food and animal feed, as reviewed by IARC (*4*), Pittet (*6*), SCOOP (*7*), and Speijers and van Egmond (*8*). It has been shown that besides various toxic effects, OTA was nephrotoxic for several animal species, especially swine (*9*, *10*). A kidney disease (Balkan endemic nephropathy) was found to be associated with the presence of OTA in food and the blood of humans suffering from tubulo interstitial nephropathy associated with urinary tract tumors (*11*, *12*).

OTA is also immunosuppressive (*13*, *14*), teratogenic (*15*, *16*), genotoxic (*17*−*20*), and carcinogenic (*21*, *22*) in rodents. Therefore, the International Agency for Research on Cancer has classified it in group 2B as a possible human carcinogen (*4*). OTA had been found in various countries in a large variety of foods: cereals, vegetables, dried fruits, nuts, and meats (pork, poultry, fish, etc.) (*23*−*30*).

Several studies have also found OTA in beverages (wine, grape juice, beer, milk, etc.) (*31*−*34*). First found in various Swiss table wines (*35*), OTA was also found in wines from different regions (*36*, *37*) and in dried vine fruit (*38*). The goal of this study was to obtain further quantitative data on the presence of OTA in grapes and musts used in the Aude department (Southern France) for red table wines and of ochratoxinogenic strains on grapes to estimate the potential contribution of wine in the daily intake of OTA in humans, especially in Southern France.

MATERIALS AND METHODS

Isolation of Fungi from Grapes. We collected 11 samples (1 kg each) of grapes from Southern France used for the making of Aude wines. For each of them, 50 g was collected to ensure a representative sample according to Horwitz et al. (*39*) and suspended in 100 mL of sterile water containing SDS (0.05%, w/v). After 1 h of magnetic shaking, 1 mL of each suspension was deposited in a Petri dish (90 mm diameter) on malt extract (1.5%)/agar (1.5%) medium (MEA) complemented with chloramphenicol (0.05%, w/v), and 1 mL of each of the same suspensions was deposited in a Petri dish and the culture medium poured over it following the soil plates method of Warcup (*40*). The plates were incubated at 24, 30, 37, and 45 °C. *Aspergilli* were identified on Czapek−Dox medium (*41*), *Penicillia* on media proposed by Pitt (*42*), and *Fusaria* on PDA medium (*43*). The purity of each strain and its identity were checked following the morphological criteria of Zycha and Siepmann (*44*), Booth (*45*), Ellis (*46*), Raper and Fennel (*47*), Pitt (*48*), Samson (*49*), Al-Musallam (*50*), and Von Arx (*51*).

OTA Production by Isolated Strains. Strains were grown on MEA medium for 1 week before inoculation at three points in 9 cm Petri dishes containing Yeast Extract Sucrose (YES) agar with 0.5 g/L magnesium sulfate and Czapek Yeast Autolysate (CYA) agar. OTA production was analyzed after 7 and 14 days of growth at 24 °C as

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Table 1. Fungal Strains Isolated from Grape and Their Production of OTA

class/genus (nomenclature)	A	B	C	D	E	F	G	H	J	K	M
Ascomycetes											
Coniochaeta velutina (Fuckel) Cooke		+									
Emericella nidulans (Eidam) Vuillemin		+									
Neosartorya fischeri var. *fischer* (Wehmer) Malloch & Cain							+				
Sordaria conoidea (Ehrenberg) Hughes						+					
Sordaria fimicola (Rob.) Cesati & De Notaris							+				+
Sordaria lappae Potebnia									+		
Basidiomycete						+			+		
Deuteromycetes											
Acremonium alternatum Link	+										
Alternaria alternata (Fries) Keissler	+	+	+		+	+	+	+	+	+	
Aspergillus aculeatus Iizuka	+	+	+		+						
Aspergillus carbonarius (Bainier) Thom[a]	+	+		+	+						
Aspergillus fumigatus Fresenius	+	+									
Aspergillus section *Nigri*	+	+	+	+	+		+			+	+
Aspergillus terreus Thom			+								
Aspergillus ustus (Bainier) Thom & Church											+
Aureobasidium pullulans (de Bary) Arnaud		+					+				
Botrytis cinerea Persoon ex Fries	+			+	+	+	+	+	+		
Cladosporium cladosporioides (Fresenius) de Vries	+	+		+	+		+				
Cladosporium elatum (Harz) Nannfeldt								+			
Curvularia clavata Jain		+									
Curvularia inaequalis (Shaer) Boedijn	+	+									
Curvularia lunata (Wakker) Boedijn								+			
Drechslera spicifera (Bainier) von Arx		+	+								
Epicoccum nigrum Link		+	+				+	+			+
Fusarium moniliforme Sheldon	+										
Fusarium solani (Martius) Saccardo											+
Geotrichum candidum Link ex Persoon							+				
Gliocladium roseum Bainier	+										
*Harzia verrucosa (*Tognini) Holubova Jechova							+				
Microdochium bolleyi (Sprague) de Hoog & Hermanides-Nijhof							+				
Penicillium atramentosum Thom		+				+					
Penicillium brevicompactum Dierckx		+	+			+	+	+	+	+	+
Penicillium canescens Sopp								+			
Penicillium chrysogenum Thom[b]		+					+		+		
Penicillium citreonigrum Dierckx		+									
Penicillium citrinum Thom								+			
Penicillium echinulatum Fassatiova								+			
Penicillium expansum Link					+		+	+	+	+	+
Penicillium glabrum (Wehmer) Westling	+	+			+		+				
Penicillium griseofulvum Dierckx								+			+
Penicillium miczynskii Zaleski		+								+	+
Penicillium minioluteum Dierckx		+	+								
Penicillium oxalicum Currie & Thom		+									
Penicillium paxilli Bainier	+										
Penicillium purpurogenum Stoll	+	+		+							+
Penicillium rugulosum Thom				+							
Penicillium spinulosum Thom		+									
Penicillium thomii Maire	+			+	+		+		+		+
Phoma eupyrena Saccardo	+		+			+		+			
Phoma putaminum Spegazzini	+										
Rhodotorula aurantiaca (Saito) Lodder	+		+					+			
Scopulariopsis brevicaulis (Saccardo) Bainier								+	+		
Stemphylium botryosum Wallroth	+										
Trichoderma harzianum Rifai	+	+						+			+
Ulocladium chartarum (Preuss) Simmons	+	+	+					+			
Verticillium fungicola (Preuss) Hassebrauk		+	+					+			
White Yeast spp.	+					+			+		
Zygomycetes											
Actinomucor elegans (Eidam) Benjamin & Hesseltine			+								
Rhizopus stolonifer (Ehrenberg ex Fries) Lind											+

[a] Strains actually producing OTA in solid YES and/or CYA media. [b] Potentially toxinogenic strains.

previously described (*52*). The YES medium contained (g/L) sucrose (Prolabo) (40.0) and yeast extract (Difco, Detroit, MI) (20.0). The CYA medium contained (g/L) sucrose (Prolabo) (40.0) and yeast extract (Difco) (20.0). Sterilization was carried out by autoclaving for 20 min at 121 °C. After sterilization, the pH of the media was 6.5.

OTA Extraction from Culture. At each sampling, three agar plugs were removed from the central area of the colony. Each plug was weighed and introduced into a small vial, methanol (0.5 mL) was added, and the vial was shaken for 1 h (orbital shaker, 200 rpm). The extracts were filtered (Millipore Corp., Bedford, MA) and injected into an HPLC instrument.

OTA Extraction from Musts. The method described by Visconti et al. (*53*) was used. Each sample (100 mL) was diluted with a solution containing 1% poly(ethylene glycol) and 5% sodium hydrogenocarbonate, filtered, and applied to an OchraTest immunoaffinity column (Vicam Inc., Watertown, MA). The column was washed with a solution

Table 2. Levels of OTA Found in Must Samples and Its Production by Toxinogenic Strains Isolated from the Same Samples

must sample	A	B	C	D	E	F	G	H	J	M	K
[OTA] (ng/L)	a	58		318	461	<10	150	51	20		

		[OTA] (μg/g of medium)			
		YES[a]		CYA	
isolated strain	must sample	7 days	14 days	7 days	14 days
A. carbonarius	A	62 ± 2.1	81.1 ± 0.6	66.4 ± 1.0	87.5 ± 1.1
A. carbonarius	B	traces	0.1 ± 0.0		
A. carbonarius	B	traces	0.2 ± 0.0	2.0 ± 1.1	1.2 ± 0.1
A. carbonarius	D		1.5 ± 0.3	19.9 ± 0.6	18.5 ± 0.6
A. carbonarius	D	0.6 ± 0.1	0.6 ± 0.2	12.0 ± 1.4	11.7 ± 0.9
A. carbonarius	D	0.1 ± 0.0	0.4 ± 0.1	2.5 ± 0.1	3.4 ± 0.2
A. carbonarius	D		0.1 ± 0.0	5.5 ± 0.0	6.9 ± 1.0
A. carbonarius	E	0.2 ± 0.0	1.5 ± 0.1		0.3 ± 0.1
A. carbonarius	E	0.3 ± 0.0	0.9 ± 0.1	7.8 ± 0.0	9.0 ± 0.4
A. carbonarius	E	0.1 ± 0.0	0.2 ± 0.0	0.5 ± 0.2	0.3 ± 0.1
A. carbonarius	E	1.9 ± 0.5	0.7 ± 0.0	13.1 ± 2.0	8.8 ± 0.1
A. carbonarius	E	24.6 ± 2.5	54.6 ± 2.0	65.4 ± 2.2	74.7 ± 1.8
A. carbonarius	E	1.2 ± 0.4	0.9 ± 0.4	9.5 ± 0.3	7.3 ± 0.4
A. carbonarius	E	0.6 ± 0.1	0.9 ± 0.1	15.1 ± 2.4	9.2 ± 0.6
A. section *Nigri*	A–E, G, K, M				
P. chrysogenum	B, H, J				
controls					
A. alliaceus				239.5 ± 6.5	
A. ochraceus				15.4 ± 0.3	

[a] OTA not quantified.

containing sodium chloride (2.5%) and sodium hydrogenocarbonate (0.5%) followed by water. OTA was eluted with methanol and quantified by reversed-phase HPLC. OTA-free samples were spiked with OTA and treated in the same way. About 90% of the OTA was extracted.

Quantification of OTA by HPLC. HPLC was performed with a liquid chromatograph (Shimadzu) equipped with an LC 6A, an SIL-9A automatic injector, a diode array UV detector (SPD-M10A VP), and an RF-530 fluorescence detector with a column (Ø = 4.6 mm, *L* = 250 mm) filled with C_{18} ODS-Hypersil, 5 μm. The mobile phase was acetonitrile/water/acetic acid (99:99:2; pH 3.5). Flow rate: 1 mL/min. Detection: (excitation) 333 nm, (emission) 460 nm. Quantification was achieved with a computing integrator (RC-6A). Following Soleas et al. (*34*), a five-point calibration prepared by adding different volumes of OTA stock standard solution to an OTA-free sample was used with concentrations ranging from 0.5 to 10 μg/L. For the confirmation of OTA production, diode array detection was used. OTA was identified by comparison with pure toxin (Sigma Chemical, St. Louis, MO) used as both external and internal standards.

RESULTS AND DISCUSSION

Fungal Strain Isolation and Production of OTA Detection by Strains. Out of the 11 samples of grapes, 59 filamentous fungi and 2 yeasts were isolated (**Table 1**). The 30 identified genera belong to Deuteromycetes (70%) and Ascomycetes (10%). *Penicillium* (31%) and *Aspergillus* (10%) were the most often encountered genera. *Alternaria alternata* (9/11 samples), *Aspergillus* gr. *niger* (8/11), *Penicillium brevicompactum* (8/11), and *Botrytis cinerea* (7/11) were the natural microfungi from the grape samples most often identified. Though grape sample B was weakly contaminated by fungi, 25 different strains were identified in the cultures. On the contrary, heavily moldy sample E gave only 9 different fungal strains, especially *A.* gr. *niger* and *Penicillium thomii.*

The toxinogenic potential of the isolated fungal strains was compared to *Aspergillus alliaceus* and *A. ochraceus*. Among the strains isolated from grapes, potentially OTA producers were *Aspergillus carbonarius* (14 strains out of 15) and *Penicillium chrysogenum* (3 strains) (**Tables 1** and **2**). Six of the eleven grape samples (A, B, D, E, H, and J) were contaminated by these ochratoxinogenic fungi (**Table 2**).

A. carbonarius was the only species that produced OTA media after 7 or 14 days when cultivated on YES or CYA. According to Bragulat et al. (*52*), the CYA medium is better adapted for OTA production than the YES medium except for two weakly ochratoxinogenic strains. However, concentrations of OTA in CYA medium were much lower than those given by the *A. alliaceus* reference strain (239.5 μg/g) adapted to the culture on a solid medium: the production ranged from 0.5 to 87.5 μg/g for *A. carbonarius* strains and 15.4 μg/g for *A. ochraceus*. As the production of OTA by *A. niger* var. *niger* has recently been reported (*54*), 73 *Aspergillus* section *Nigri* strains isolated from A−E, G, K, and M grape samples were also evaluated for OTA production. After 7 and 14 days of cultivation on YES or CYA medium, none produced any OTA.

The three strains of *P. chrysogenum* isolated from grapes were not toxinogenic, which is consistent with the results of Frisvad (*55*). He performed a survey for the production of mycotoxins by *Penicillium* strains supposed to be toxinogenic selected from the main mycotheques and found that, for several reasons, many *Penicillia* had been misidentified. Finally, they could not detect OTA production by any of the 230 *P. chrysogenum* strains examined. Several other surveys on the fungal contamination of foods have also revealed the low percentage of OTA-producing *Penicillia* (*3, 56*−*58*).

The high frequency of the genus *Aspergillus* in the strains isolated from grapes is consistent with the results of Creppy et al. (*59*), who outline that, in France, ochratoxicosis seems to be connected with the presence of *Aspergilli* while, in Germany and in Scandinavia, it would be connected with the presence of *Penicillia*, owing to their different temperature needs (*60*): ochratoxinogenic *Penicillia* grow well over a wide range of temperature (4−31 °C), whereas *Aspergilli* that produce OTA require higher temperatures (12−39 °C).

OTA Detection in Musts. In this study, 8 out of 11 must samples were found to be contaminated by OTA (**Table 2**).

The concentrations were estimated from 461 to less than 10 ng/L, the higher concentration being found in sample E. There is a strong correlation between the presence of ochratoxin-producing strains on grapes and the finding of OTA in musts. OTA has been found in various agricultural commodities (almost all cereals, animal feeds, other food commodities), as reviewed by Pittet (*6*). Recently, there have been several reports of the presence of OTA in wine and grape juice (*35, 37, 61*−*63*). Surprisingly high concentrations of this mycotoxin were also detected in raisins from Greece and Turkey (*36*). Otteneder and Majerus (*37*) have realized a wide review to describe extensively the situation of OTA contamination of wine. They analyzed 400 samples themselves and collected more than 450 results from the literature. According to these data, OTA is much more commonly detected in red wines (54%) than in rosé (40%) and white (25%) wines. The OTA concentration is remarkably higher in red wines (22−1153 ng/L) than in rosé (119 ng/L) and white (12−108 ng/L) wines, depending on the regions. This situation can be explained by the different processing methods: whereas, for white wines, the grapes are immediately pressed, red grapes are left mashed for several days, which obviously permits fungal growth and production of the toxin. Provisional estimates of the Codex Alimentarius Commission, based on limited European data, suggest that red wine is the second major source of human exposure to OTA following cereals and preceding coffee and beer (*64*).

Risk Assessment. Health concerns regarding mycotoxins depend on the toxicity and the amount of mycotoxin consumed, body weight, the general health conditions of the individual, the presence of other mycotoxins, and other dietary factors. In the present work, except for OTA production, no potentially mycotoxinogenic strains were found in grape samples. It is important to note that others mycotoxins have already been found in wines: trichothecenes from the fungus *Trichothecium roseum* (*65*) and patulin (*66*).

OTA is genotoxic (*17*−*19*) and carcinogenic (*21*, *22*) in rodents. It can be considered as one of the most potent renal carcinogens. Owing to the high level of potential occurrence of OTA in foods and beverages and its relatively long half-life in humans, 35 days in serum, following Studer-Rohr (*67*), the high incidence of this mycotoxin in human sera and milk is not surprising (*7, 68*−*70*). All these data show that OTA is among the most frequent mycotoxins found in human blood in Europe, the U.S., Canada, and elsewhere where it has been investigated (*71*).

At the beginning of the 1990s, the occurrence of OTA was not systematically searched for, and therefore it is almost impossible to establish any conclusion on the possible health hazard for the population resulting from OTA intake. In 1994, the European Commission set up a Scientific Co-Operation on Questions Relating to Food (*7*) task force to provide the Scientific Committee for Food with information on European dietary exposure to OTA. As a result, the mean dietary intake of humans, based on occurrence and consumption data, was established as 1.8 ng/kg bw per day, and based on human blood data, as 0.9 ng/kg bw per day. Because of the increase of scientific reports on OTA contamination in beverages and many kinds of food, the WHO/FAO Joint Expert Committee on Food Additives (JECFA) proposed 100 ng/kg bw per day as a provisional tolerable weekly intake (PTWI) for OTA (*72*). Ueno et al. (*33*) stated that this exposure estimate is not deemed to represent a health hazard. In 1998, The Scientific Committee on Food of the European Union considered it would be advisable to reduce the tolerable daily OTA intake from food as low as possible, i.e., between 1.2 and 14 ng/kg bw per day or lower than 5 ng/kg bw per day (*73*).

CONCLUSION

The aim of this research was to record the fungal population in grapes, particularly ochratoxinogenic strains, and to see the possible connection between the presence of OTA in wine. In the present work, 73% of the must samples contained OTA, produced by strains of *A. carbonarius*. The rate of contamination ranged from 461 to less than 10 ng/L.

These OTA contamination levels were in accordance with previous studies (*35, 37, 61*−*63*). They could explain the human blood content contamination in France of 22−25% of the general population with levels from 0.1 to 130 ng/mL (*68*). Although the number of samples was limited to 11, this study shows a real hazard for the population in the southwestern part of France. Wine was pointed out as one of the possible sources of OTA in human plasma (*33, 37*).

The most efficient way to protect consumers against OTA health hazards is therefore to implement good agricultural practice to lower the presence of fungal strains on the grape and therefore reduce the possibility of OTA production during the wine-making process. An appropriate antifungal treatment of vines and a tight control of the wine-making process would be the main factors.

A new study on grapes (end of veraison and harvest) and musts is in progress in various regions in France. It should allow inventory in each region of ochratoxinogenic strains on grapes at various stages of ripeness, to establish the connection with antifungal treatments applied on the vine and the presence of OTA in musts.

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