JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Fungal Microflora and Ochratoxin A Risk in French Vineyards

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To evaluate the ochratoxin A risk in French vineyards, five winemaking regions were investigated. An exhaustive survey of the fungal microflora of 60 grape samples was carried out at two development stages of the berries: end of veraison and harvest time. Potentially toxinogenic fungi isolated from grapes were assessed in vitro for ochratoxin A production. Ochratoxin A was also quantified in musts by high-performance liquid chromatography after cleanup on immunoaffinity columns. Among the 90 species identified, almost half are listed as mycotoxin producers, but only 2 are potentially ochratoxinogenic: *Aspergillus carbonarius* and *Aspergillus niger*. Among these strains, only *A. carbonarius*, isolated from the Languedoc region at harvest time, was found to produce ochratoxin A. These results were in accordance with the presence of ochratoxin A in French southern region musts (0.01−0.43 μg/L) and confirmed the major implication of *A. carbonarius* in ochratoxin A contamination.

KEYWORDS: **Ochratoxin A; *Aspergillus carbonarius*; French vineyards; fungal microflora; grapes; musts**

INTRODUCTION

Contamination of foodstuffs with mutagenic and carcinogenic mycotoxins such as aflatoxins, ochratoxins, or fumonisins is a major concern for human health. Ochratoxin A (OTA) is a mycotoxin that is receiving much attention for its nephrotoxic effects (*1*). OTA is also known for its teratogenic, immunosuppressive, and carcinogenic properties. The toxin has been considered by the International Agency for Research on Cancer to be possibly carcinogenic (group 2B) for humans (*2*).

Recently, more attention has been focused on ochratoxin A levels in commonly consumed foods, especially fruits and cereals (*3*), and in fermentation products such as beer (*4*) and wine (*5*−*8*). Some species of black aspergilli (*Aspergillus* section *Nigri*) are able to produce OTA (*9*−*12*). These species are commonly present in vineyards and have the ability to cause rot in berries, known as *Aspergillus* rot (*13*). Among the species of this group, *Aspergillus carbonarius* shows the highest ochratoxigenic potential, with most of the isolates having the ability to produce OTA in vitro (*14*).

In Europe, according to the Codex Committee on Food Additives and Contaminants, wine is the second most important source of ochratoxin A in the diet (almost 15% of daily ochratoxin A intake) after cereals (*6*, *15*). Contamination levels in southern Europe as high as 7.6 μg/L were reported in red wines (*16*), which were more contaminated than white wines (*6*). New standards have been discussed within the European Union for products such as wine for which a level of 0.5 μg/L was originally proposed (*17*). The application of this European standard, probably 2 μg/L, was reported to the 2005 harvest in order to allow an extensive risk assessment and to search for adapted prophylactic and curative methods (*18*). The aim of this study was to obtain data on OTA origin in French wines. It included the follow-up of fungal flora on grapes, the assessment of OTA contamination in musts, and in vitro ochratoxinogenic potential of strains (all *Penicillium* and *Aspergillus*) isolated from grapes.

MATERIALS AND METHODS

Study Area. Eight vineyards located in five French winemaking regions were studied (**Figure 1**): Alsace, Beaujolais, Côtes du Rhône, Languedoc, and Bordelais. Eight vine plants were concerned: Chardonnay, Chasselas, and Sauvignon (white wines); and Cabernet Sauvignon, Gamay, Grenache, Merlot, and Syrah (red wines). The vineyards underwent various antifungal (anti-*Botrytis*) treatments and prophylactic methods (stripping off leaves and/or seedling grass) likely to influence OTA contents in musts.

Sample Collection. To determine the period of contamination, berries were collected at two development stages in 2000: 23 samples at the end of veraison (when development of the berries was not too premature) and 37 samples at harvest time. At each sampling stage, 10 plants were chosen along the two diagonals of each vineyard, and a bunch was picked halfway up from each plant. Samples were divided to make bunches of two or three berries. Damaged berries were removed and separately analyzed.

Mycological Analysis of the Grapes. For each sample, 100 g of berries was randomly selected and suspended in 200 mL of sterile water containing SDS (0.05%, w/v). After 1 h of magnetic shaking, 1 mL of each suspension was sprayed in a Petri dish (90 mm diameter) containing malt extract (1.5%)/agar (1.5%) medium (MEA) complemented with chloramphenicol (0.05%, w/v) following the soil plates

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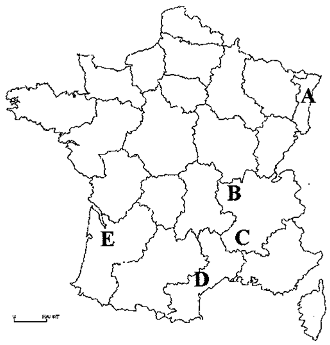
10.1021/jf049497c CCC: $27.50
Published on Web 08/06/2004



Winemaking regions	Vine-plants	Samples *
A : Alsace	Chasselas	4/4
B : Beaujolais	Gamay	4/4
C : Côtes du Rhône	Grenache	3/3
D : Languedoc site 1	Chardonnay	5/5
D : Languedoc site 1	Cabernet Sauvignon	1/1
D : Languedoc site 1	Merlot	1/1
D : Languedoc site 2	Syrah	0/4
D : Languedoc site 3	Syrah	0/5
D : Languedoc site 3	Chardonnay	0/1
E : Bordelais site 1	Merlot	5/5
E : Bordelais site 2	Sauvignon	0/4

Figure 1. Sampling of bunches. *, number of samples: end of veraison/harvest.

method of Warcup (*19*). The plates were incubated at 24 and 37 °C. The identity of each strain, isolated and purified, was achieved through macro- and microscopic examinations (*20*−*27*).

Ochratoxinogenic Ability of the Isolates. All isolates resulting from potentially toxinogenic or not *Penicillium* and *Aspergillus* strains (192 and 75, respectively) were tested in vitro for OTA production. The strains were grown on MEA medium for 1 week before inoculation at three points in 55 mm Petri dishes containing yeast extract sucrose (YES) and Czapek yeast autolysate (CYA) agar (*28*).

For each sample, three agar plugs were removed from the central area of the colony, weighed, and placed into a small vial containing 0.5 mL of methanol. After 1 h, each extract was filtered and analyzed by HPLC.

Detection of Ochratoxin A in Musts. All 37 musts from grapes collected at harvest time were tested for OTA contamination. Each sample (25 mL) was diluted by half with phosphate-buffered saline (PBS) buffer (R-Biopharm), and the pH was adjusted to 7.4. After centrifugation for 30 min at 4000 rpm, a 20 mL aliquot of the diluted sample was applied to an Ochraprep immunoaffinity column (R-Biopharm) preconditioned with PBS buffer (10 mL). After a washing with 20 mL of PBS, the column was eluted with methanol (5 mL) and the eluate was carefully evaporated under nitrogen. The residue was diluted in 1 mL of mobile phase and OTA quantified by reverse-phase HPLC.

Ochratoxin A Detection by HPLC. The extracts were analyzed using a reverse-phase HPLC equipped with a Shimadzu fluorescence detector (333 nm excitation wavelength; 460 nm emission wavelength). Chromatographic separation was performed on a 250 × 4.6 mm i.d., 5 μm, ODS-Hypersil C18 column (SFCC-Shandon) fitted with a precolumn with the same stationary phase. The mobile phase acetonitrile/water/acetic acid [57:41:2 (v/v), pH 3.5] was injected at 1 mL/min. The injection volume was 100 μL.

The OTA standard was supplied by Sigma (St. Louis, MO). Extracts were considered to be positive when the peak gave a retention time similar to the OTA standard peak (5.56 ± 0.03 min) with a height 5 times higher than the baseline noise. Ochratoxin A quantification was done by comparing peak areas with a calibration curve. The detection limit was 0.01 μg/L.

RESULTS AND DISCUSSION

Mycological Analysis of the Grapes. Table 1 presents the survey of fungal microflora isolated from grapes at two development stages: end of veraison and harvest.

Although grapes were visually relatively little contaminated, 91 fungal strains were identified: 77 at the end of veraison (267 isolates) and only 53 at harvest time (262 isolates). All of the identified species were found as spores on the surface of healthy grapes (*29*). Their diversity depended on not only grape variety, maturity, cultural practices but also climatic and geographic conditions. Mycoflora was generally more diversified at the end of veraison, but the 267 isolates belonging to *Penicillium* and *Aspergillus* genera constituted 50% of the whole strains isolated. At the end of veraison mycoflora was diversified in various ways (23−43 strains), depending on winemaking regions, but 5 species were dominant in approximately half of the samples: *Alternaria alternata*, *Botrytis cinerea*, *Cladosporium cladosporioides*, *Penicillium brevicompactum*, and *Penicillium simplicissimum*.

At harvest time 3−39 species were identified according to winemaking regions, among which were *Aspergillus* (0−4 species) and *Penicillium* (2−14 species). The grapes were colonized by only 5 dominant fungal strains: *A. alternata*, *Aspergillus niger*, *B. cinerea*, *P. brevicompactum*, and *Penicillium expansum*. These results agreed with dominant genera usually mentioned on grapes (*30*).

Approximately 10% of isolates were potentially toxinogenic (aflatoxin, patulin, and ochratoxin A). Only 4 species were regularly observed: *A. niger* (ochratoxin A), *A. carbonarius* (ochratoxin A), *Aspergillus parasiticus* (aflatoxin), and *P. expansum* (patulin and citrinin). *A. parasiticus* was present only at the end of veraison, whereas other strains such as *A. carbonarius* or *A. niger* were more frequently identified on ripe grapes.

We observed the predominance of grape contamination by *Penicillium* spp. in northern vineyards (13/34 strains in Alsace, 18/28 in Beaujolais, and 15/37 in Côtes du Rhône), whereas southern vineyards were generally more contaminated by *Aspergillus* spp. In Languedoc-Roussillon, 8 and 4 species were identified at the end of veraison (20% of total isolates) and at harvest time (22% of total isolates), respectively. Among these 4 species isolated on harvested grapes, 3 belonged to black aspergilli (*A. carbonarius*, *A. niger* aggregate, and *A. aculeatus*). This confirmed the presence of *A. niger* and *A. carbonarius* in vineyards with Mediterranean or tropical climates, as described in other studies (*31*−*33*).

The dominant *Penicillium* species, *P. brevicompactum*, *P. expansum*, *P. glabrum*, *P. purpurogenum*, and P. *simplicissimum*, might produce a very wide range of mycotoxins. However, *Penicillium verrucosum*, the major species responsible for ochratoxin A production in temperate countries, was not isolated from grape samples from France.

Among isolated *Aspergillus*, only *A. carbonarius* and *A. niger* were potentially ochratoxinogenic. *A. niger* was observed in the five vineyards from the end of veraison, predominantly in Languedoc-Roussillon (17 isolates/29 total isolates), the only area in which *A. carbonarius* was identified.

Although there was no evidence of contamination of grapes, either by the *A. ochraceus* group or *P. verrucosum*, black aspergilli were by far the most common fungi responsible for OTA production.

Ochratoxinogenic Ability of the Isolates. In our study, no *A. niger* aggregate or *Penicillium* spp. strains were ochratoxinogenic. On the other hand, all of the *A. carbonarius* strains

Table 1. Mycological Analysis of Grapes

fungal strain	winemaking region[a] A	B	C	D	E	fungal strain	winemaking region A	B	C	D	E
total of analyzed samples	4/4	4/4	3/3	7/17	5/9	*Penicillium citreonigrum*	0/0	0/0	0/0	0/0	0/1
Acremonium egyptiacum	2/0[b]	0/0	0/0	1/0	0/0	*Penicillium citrinum*	0/0	1/0	0/0	0/0	0/0
Acremonium strictum	1/0	0/0	1/1	1/0	0/1	*Penicillium corylophilum*	0/0	2/0	1/0	0/1	2/1
Alternaria alternata	3/0	0/0	2/3	6/8	5/6	*Penicillium crustosum*	2/2	0/1	0/0	0/0	0/0
Alternaria tenuissima	0/0	0/0	0/0	1/0	1/1	*Penicillium digitatum*	1/0	1/0	0/0	0/0	0/0
Arthrinium sp.	0/0	0/0	0/1	1/0	0/2	*Penicillium expansum*	2/4	0/2	2/0	0/3	0/4
Aspergillus aculeatus	0/0	0/0	0/0	1/3	1/0	*Penicillium funiculosum*	0/0	0/0	0/0	1/0	0/0
Aspergillus candidus	0/0	0/0	0/0	1/0	0/0	*Penicillium glabrum*	2/0	0/0	2/1	2/7	0/3
Aspergillus carbonarius[c]	0/0	0/0	0/0	**0/10**	0/0	*Penicillium griseofulvum*	0/0	1/0	0/1	1/0	0/0
Aspergillus flavipes	0/0	0/0	0/0	1/0	0/0	*Penicillium herquei*	0/0	0/0	0/1	0/0	0/1
Aspergillus fumigatus	3/0	0/0	1/1	4/2	1/2	*Penicillium implicatum*	0/0	0/0	0/0	1/3	0/0
Aspergillus niger[c]	1/0	1/0	1/2	3/14	1/6	*Penicillium islandicum*	2/0	1/0	0/0	0/0	0/0
Aspergillus parasiticus	0/0	1/0	1/0	2/0	2/0	*Penicillium janczewskii*	0/0	0/0	0/0	0/1	0/0
Aspergillus terreus	0/0	0/0	0/0	2/0	0/1	*Penicillium janthinellum*	0/0	1/0	0/0	1/0	0/0
Aspergillus ustus	0/0	0/0	1/0	0/0	2/0	*Penicillium lividum*	0/0	0/0	0/0	1/2	0/0
Aspergillus versicolor	0/0	0/0	0/0	3/0	0/0	*Penicillium miczynskii*	0/0	0/0	1/0	0/2	0/0
Aureobasidium pullulans	1/0	0/0	0/0	0/0	0/0	*Penicillium minioluteum*	1/0	0/0	0/0	0/0	0/0
Basidiomycète	2/0	1/0	0/0	2/0	1/0	*Penicillium paxilli*	0/0	1/0	1/0	0/0	0/1
Beauveria bassiana	0/0	0/0	0/1	0/0	0/0	*Penicillium purpurogenum*	3/0	1/0	2/1	1/6	0/1
Botrytis cinerea	2/2	4/4	1/3	4/10	0/1	*Penicillium raistrickii*	1/0	0/0	0/0	0/0	0/0
Chaetomium globosum	0/0	0/0	0/0	0/0	0/1	*Penicillium restrictum*	1/0	1/0	0/0	0/0	0/0
Cladosporium cladosporioides	4/0	1/0	2/3	6/4	1/2	*Penicillium simplicissimum*	3/0	3/3	3/0	3/5	2/2
Cladosporium herbarum	2/0	0/0	1/2	0/0	0/1	*Penicillium spinulosum*	1/0	4/1	0/1	0/0	0/1
Cladosporium sphaerospermum	0/0	0/0	0/0	1/0	0/0	*Penicillum thomii*	0/0	4/3	1/0	2/2	0/2
Coniella diplodiella	0/0	0/0	0/0	1/0	0/0	*Penicillium variabile*	0/0	1/1	0/1	0/1	0/1
Coniochaeta velutina	0/0	0/0	0/0	1/0	0/0	*Pestalotiopsis versicolor*	0/0	1/0	0/0	0/0	1/2
Coniothyrium sporulosum	0/0	0/0	1/0	0/0	0/0	*Phialophora hoffmannii*	2/0	0/0	0/0	1/0	0/0
Drechslera spicifera	0/0	0/0	0/2	0/0	0/0	*Phoma eupyrena*	0/0	0/0	0/0	1/1	0/0
Emericella nidulans	0/0	0/0	1/0	0/0	0/0	*Phoma glomerata*	0/0	0/0	0/0	1/0	0/0
Epicoccum nigrum	2/0	0/0	0/1	0/2	4/7	*Phoma herbarum*	0/0	0/0	0/0	0/0	2/2
Fusarium culmorum	0/0	0/0	0/0	0/0	0/1	*Phoma putaminum*	0/0	0/0	0/0	0/0	0/1
Fusarium lateritium	0/0	0/0	0/0	1/0	0/0	*Pleospora herbarum*	0/0	1/0	0/1	4/0	2/2
Geniculosporium sp.	0/0	0/0	0/0	3/0	0/0	*Rhizopus stolonifer*	0/0	0/0	0/0	0/6	0/1
Humicola grisea	1/0	0/0	0/0	0/0	0/0	*Rhodotorula aurantiaca*	3/0	1/0	2/2	0/1	1/1
Mucor hiemalis	3/0	0/0	0/0	2/1	1/1	*Scytalidium lignicola*	0/0	0/0	0/0	0/1	0/0
Myrothecium verrucaria	0/0	0/0	0/0	0/0	1/0	*Sordaria fimicola*	4/0	0/0	0/1	0/1	0/1
Nectria pityrodes	1/0	0/0	0/0	0/0	0/0	*Sordaria macrospora*	1/0	0/0	0/0	0/0	0/0
Nigrospora oryzae	1/0	0/0	0/0	0/0	0/0	*Trichoderma hamatum*	0/0	0/0	0/0	1/0	0/0
Nigrospora sphaerica	0/0	0/0	1/0	0/1	4/2	*Trichoderma harzianum*	1/0	0/0	0/1	1/2	3/5
Nodulisporium sp.	0/0	0/0	0/0	0/0	0/1	*Trichoderma koningii*	0/0	0/0	0/0	0/0	1/0
Oidiodendron tenuissimum	0/0	1/0	0/0	1/0	0/0	*Trichoderma pseudokoningii*	1/0	0/0	0/0	0/0	0/0
Paecilomyces variotii	0/0	1/0	0/0	0/0	0/0	*Ulocladium atrum*	0/0	0/0	0/0	0/0	1/0
Penicillium aurantiogriseum	0/0	3/1	1/0	0/1	0/1	*Ulocladium botrytis*	0/0	0/0	0/1	0/1	0/1
Penicillium brevicompactum	4/0	4/2	1/1	2/10	0/4	*Ulocladium chartarum*	0/0	0/0	1/0	4/3	3/7
Penicillium canescens	3/0	0/0	0/0	2/4	0/0	white yeast	0/0	0/0	0/0	1/2	0/1
Penicillium chrysogenum	0/0	1/0	1/0	1/0	0/0	*Xylaria* sp.	0/0	0/0	0/0	0/0	0/1
total no. of isolates	**67/8**	**43/18**	**33/33**	**81/83**	**43/83**	**total**					
no. of *Aspergillus* isolates	4/0	2/0	4/3	17/29	7/9	75					
no. of *Penicillium* isolates	26/6	30/14	16/7	18/48	4/23	192					
total no. of species	**34/3**	**26/9**	**25/23**	**43/32**	**23/40**	**total**					
no. of *Aspergillus* spp.	2/0	2/0	4/2	8/4	5/3	9					
no. of *Penicillium* spp.	13/2	16/8	11/7	12/14	2/12	29					

[a] Winemaking regions: A, Alsace; B, Beaujolais; C, Côtes du Rhône; D, Languedoc; E, Bordelais (see **Figure 1**). [b] Number of contaminated samples: end of veraison/harvest. [c] Potentially ochratoxinogenic strain.

Table 2. OTA Contents in Musts and OTA Production in Vitro by *A. carbonarius* Strains

area D, Languedoc	vine plants	treatment	OTA in must (μg/L)	OTA production in vitro (μg/g)
site 1	Cabernet Sauvignon	nontreated	0.05	0.02
	Merlot	nontreated	0.02	no strain
site 2	Syrah	prophylactic methods	0.01	0.12
	Syrah	prophylactic methods	0.07	1.90
	Syrah	prophylactic methods	**0.43**	0.24
	Syrah	prophylactic methods	0.02	0.18
site 3	Syrah	nontreated	0.01	0.19
	Syrah	biologic	0.01	0.01
	Syrah	Scala[a]/Teldor[a]	0.02	0.03
	Syrah	Ronilan[a]/Scala[a]	0.01	0.12
	Syrah	Ronilan[a]/Scala[a]/Teldor[a]	0.02	0.11

[a] Anti-*Botrytis*.

isolated from Languedoc produced OTA after 7 days of incubation at 26 °C on CYA, but at very low levels from 0.01 to 1.90 μg/g. These results agree with our previous research on Aude wines (*7*) (southern France) and a study in Spain (*34*).

Another work in wine-producing countries of the Mediterranean basin (*35*) showed that most of the *A. carbonarius* strains produced OTA.

Detection of Ochratoxin A in Musts. All of the musts resulting from bunches of grapes at harvest time previously studied were tested for OTA contamination. Ochratoxin A (>0.01 μg/L) was detected in the 11 musts of red vines from Languedoc **(Table 2)**, which were relatively not much contaminated. The highest level of contamination, 0.43 μg/L, was lower than OTA levels observed in 23 samples of white and red Italian wines produced during the year 2000 (0.01−2 μg/L) (*36*). However, it was not possible to correlate OTA contamination in grapes with in vitro OTA production.

These very low contents of OTA in the musts was in relation to the very low values of bunch contamination, probably due to the particularly favorable climatic conditions before vintage. In consequence, it was not possible to highlight the influence of anti-*Botrytis* treatments or prophylactic methods on OTA production. Nevertheless, our results confirmed the Languedoc region as a risk area among French vineyards. These results were in agreement with the 2001 cartography of French winemaking regions presented by the ONIVins (*37*), which studied 1000 wines and concluded that 30% of the Languedoc wines contained >0.5 μg/L of OTA. Before the application of a European standard (probably 2 μg/L) in 2005, it was obvious that our next studies should be focused on Languedoc "sensitive" vineyards in order to define the period and the origin of contamination and to evaluate the influence of preventive and/or curative treatments applied to grapes on ochratoxinogenic mycoflora and OTA levels.

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Received for review March 26, 2004. Revised manuscript received June 7, 2004. Accepted June 9, 2004. This study was initiated and supported by a grant of the Office National Interprofessionnel des Vins (Paris, France).

JF049497C