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Study of Spanish Grape Mycobiota and Ochratoxin A Production by Isolates of *Aspergillus tubingensis* and Other Members of *Aspergillus* Section *Nigri*

Angel Medina,[1] Rufino Mateo,[2] Laura López-Ocaña,[3] Francisco Manuel Valle-Algarra,[2] and Misericordia Jiménez[1]*

Departamento de Microbiología y Ecología, Facultad de Biología, Universidad de Valencia, Dr. Moliner 50, E-46100 Burjasot, Valencia, Spain[1]; Departamento de Química Analítica, Facultad de Química, Universidad de Valencia, Dr. Moliner 50, E-46100 Burjasot, Valencia, Spain[2]; and Colección Española de Cultivos Tipo (CECT), Universidad de Valencia, Dr. Moliner 50, E-46100 Burjasot, Valencia, Spain[3]

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The native mycobiota of five grape varieties grown in Spain has been studied. Four (Bobal, Tempranillo, Garnacha, and Monastrell) were red varieties and one (Moscatel) was white. The main fungal genera isolated were *Alternaria*, *Cladosporium*, and *Aspergillus*. The isolation frequency of *Aspergillus* spp. section *Nigri* in contaminated samples was 82%. Ochratoxin A (OTA) production was assessed using yeast extract-sucrose broth supplemented with 5% bee pollen. Cultures of 205 isolates from this section showed that 74.2% of *Aspergillus carbonarius* and 14.3% of *Aspergillus tubingensis* isolates produced OTA at levels ranging from 1.2 to 3,530 ng/ml and from 46.4 to 111.5 ng/ml, respectively. No *Aspergillus niger* isolate had the ability to produce this toxin under the conditions assayed. Identification of the *A. niger* aggregate isolates was based on PCR amplification of 5.8S rRNA genes and its two intergenic spacers, internal transcribed spacer 1 (ITS1) and ITS2, followed by digestion with restriction endonuclease RsaI of the PCR products. The restriction patterns were compared with those from strains of *A. niger* CECT 2807 and *A. tubingensis* CECT 20393, held at the Spanish Collection of Type Cultures. DNA sequencing of the ITS1-5.8S rRNA gene-ITS2 region of the OTA-producing isolates of *A. tubingensis* matched 99 to 100% with the nucleotide sequence of strain *A. tubingensis* CBS 643.92. OTA determination was accomplished by liquid chromatography with fluorescence detection. OTA confirmation was carried out by liquid chromatography coupled to ion trap mass spectrometry. The results showed that there are significant differences with regard to the isolation frequency of ochratoxinogenic fungi in the different grape varieties. These differences were uncorrelated to berry color. The ability of *A. tubingensis* to produce OTA and the influence of grape variety on the occurrence of OTA-producing fungi in grapes are described in this report for the first time.

Ochratoxin A (OTA) was discovered in 1965 as a secondary metabolite of a strain of *Aspergillus ochraceus* (61). OTA exhibits intestinal fragility, nephrotoxicity, immunosuppresion, teratogenicity, carcinogenicity (11, 19, 24, 27, 34), and cytotoxicity in hepatic cell lines (11) and induces iron deficiency anemia (23). OTA could be responsible for Balkan endemic nephropathy. It has been reported that OTA concentration in the blood serum of Balkan endemic nephropathy patients was 10-fold higher than in the blood serum of people from other regions (4, 57). The International Agency for Research on Cancer classifies OTA in group 2B (possibly carcinogenic to humans) (25).

Fungi from two genera are known to produce ochratoxins. In genus *Penicillium*, OTA is produced by *P. verrucosum* (53) and *P. nordicum* (33) and in genus *Aspergillus* by *A. ochraceus*, *A. melleus*, *A. auricomus*, *A. ostianus*, *A. petrakii*, *A. sclerotiorum*, and *A. sulphureus*, all in section *Circumdati* (formerly the *A. ochraceus* group) (3, 10, 22, 62). *Aspergillus alliaceus* and *Aspergillus albertensis*, formerly placed in section *Circumdati*, but recently shown to be more closely related to section *Flavi*, have also been described as OTA producers (50). In recent years, some members of *Aspergillus* section *Nigri* (formerly the *Aspergillus niger* group) such as *Aspergillus niger* var. *niger* and *Aspergillus carbonarius* have been reported as ochratoxigenic fungi (1, 6, 15, 21, 40). More recently, the ability of the uniseriate species of black aspergilli *Aspergillus japonicus* to produce OTA has been mentioned (9, 17). In the *A. niger* aggregate, it has always been difficult to distinguish one taxon from another by morphological means because the differences are very subtle. The division of this *A. niger* aggregate into two species, namely *A. niger* and *Aspergillus tubingensis*, according to restriction fragment length polymorphism (RFLP) analysis of total DNA was proposed by Kusters van Someren et al. (32). Studies involving a molecular approach followed and substantially confirmed these results (7, 37, 43, 63, 64). Although the ability of *A. niger* to produce OTA has been previously described (1), the species *A. tubingensis* has not been reported to be an OTA producer (2).

Ochratoxin A has been detected in human blood (12, 16, 49, 59) and food and drinks such as cereals (mainly wheat, barley, corn, and oats), seeds, beans, pulses, peanuts, dried fruits, coffee, milk, and beer (30, 54, 56, 58, 60); in recent years, it has been detected in wine (13, 38, 48, 66). Due to the presence of

* Corresponding author. Mailing address: Departamento de Microbiología y Ecología, Facultad de Biología, Universidad de Valencia, Dr. Moliner 50, E-46100 Burjasot, Valencia, Spain. Phone: 34-96-3543144. Fax: 34-96-3543099. E-mail: misericordia.jimenez@uv.es.

TABLE 1. Characteristics of the grape samples used in the study

Color of berries	Grape variety	Town (province) where vineyard is located	No. of samples
Red	Bobal	Requena (Valencia)	3
		Iniesta (Albacete)	2
		Villamalea (Cuenca)	5
	Garnacha	Iniesta (Albacete)	7
	Tempranillo	Iniesta (Albacete)	7
		Haro (Rioja)	11
	Monastrell	Jumilla (Murcia)	3
		El Pinoso (Alicante)	3
		Sax (Alicante)	3
White	Moscatel	Málaga (Málaga)	4
		Titaguas (Valencia)	2
		Villar del Arzobispo (Valencia)	2

OTA in food and drinks typical in the human diet, the study of OTA has become increasingly important. The Joint Food and Agriculture Organization of the United Nations/World Health Organization Expert Committee on Food Additives has discussed the imposition of a maximum tolerable weekly intake of 100 ng of toxin/kg of body weight (28) and a maximum level of 5 to 20 μg OTA/kg in cereals, both processed and nonprocessed (29). The Office International de la Vigne et du Vin fixed 2 μg/liter as a maximum level of OTA in wine (46).

Wine is a product of great economic relevance around the world, especially in wine-producing countries. Recently, it has been shown that OTA is stable in wine for at least 1 year (38).

There are differences between northern and southern European regions regarding OTA levels in wines. Several surveys carried out in different countries have reported OTA levels in grape products and wine ranging from 0.01 to 3.5 μg/liter. These levels were higher in products from southern regions of Europe than in northern regions (9, 40, 41, 47, 48, 51). The fungal mycobiota on ripe grape is very critical in assessing the risk of OTA presence in wine. Therefore, some researchers have recently studied the grape mycobiota in different countries (5, 14, 55), but no attention has been paid to the study of ochratoxigenic fungi cooccurrence in different grape varieties.

The aims of the present study were (i) assessment of the native mycobiota in different grape varieties grown in Spain, (ii) morphological and molecular characterization of potential OTA-producing isolates from each variety by PCR-RFLPs of the rRNA genes internal transcribed spacer (ITS) region, and (iii) identification of ochratoxinogenic isolates among these fungal species.

MATERIALS AND METHODS

Samples. In the present study, a total of 52 grape samples (44 red grapes and 8 white grapes) (*Vitis vinifera*) were analyzed. Table 1 shows the studied grape varieties, the geographical origin of the vineyards, and the number of samples of each grape variety.

The criteria for farm selection within each sampled area were size and the quality of wines derived from their grapes. Samples were harvested in late September during the grape harvest, and plants were chosen along diagonal transects to obtain random sampling. Grape bunches (each about 1 kg) were taken and placed in previously sterilized bags, which were kept at about 4°C until analysis, which was carried out within 24 h of harvest.

Fifty berries were picked from all the parts of bunches and homogenized in a stomacher (IUL Instruments, Barcelona, Spain). From the homogenate, decimal seriate dilutions were made under sterile conditions. These solutions were used to inoculate petri dishes containing malt extract agar (Cultimed; Panreac Química S.A., Barcelona, Spain). Petri dishes were then incubated at 28°C for 5 to 7 days in the dark. After incubation, the number of CFU of filamentous fungi per milliliter of berry homogenate was evaluated.

Identification of fungi. Taxonomic identification of all isolates was achieved through macroscopic and microscopic observation with the aid of guidelines published for each genus or general guidelines (8, 31, 52).

A. carbonarius was identified through microscopic observation, and *Aspergillus niger* aggregate (*A. niger* and *A. tubingensis*) was identified on the basis of the determination of restriction patterns of PCR-amplified rRNA gene products. Fungal DNA was isolated according to the method described by Lee and Taylor (35). The ITS1-5.8S-rRNA gene-ITS2 region was amplified by PCR. Two oligonucleotide fungal primers (ITS1 and ITS2) described by White et al. (67) were used for amplification. Random amplified products were digested overnight at 37°C with restriction endonuclease RsaI (Boehringer Mannheim). PCR products and restriction fragments were separated by electrophoresis in 1% and 2% agarose gels, respectively, with 0.5× Tris-borate-EDTA buffer. After electrophoresis, gels were stained with ethidium bromide (0.5 μg/ml), and the DNA bands were visualized with a UV transilluminator. DNA sizes were estimated by comparison with a DNA length standard (100-bp molecular marker; Gibco BRL Life Technologies, Inc., Rockville, Md.). The restriction patterns obtained for the different isolates from grape samples were compared with those obtained under the same conditions from two type strains (*A. niger* CECT 2807 and *A. tubingensis* CECT 20393) held at the Spanish Collection of Type Cultures (Valencia University, Burjassot, Valencia, Spain). Strain CECT 20393 corresponds to IMI 172296 (International Mycological Institute, Surrey, United Kingdom) and CBS 115.29 (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands).

To perform DNA sequencing, PCR products were cleaned with the Gene Clean II Purification kit (Bio 101, La Jolla, Calif.). Then, PCR products were sequenced using the Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Falmer, Brighton, United Kingdom) and an Applied Biosystems automated DNA sequencer (model 373A) according to the manufacturer's instructions. The primers ITS1 and ITS4 were also used to obtain the sequence of both strands. The National Center for Biotechnology Information (NCBI) Nucleotide Database was used to compare nucleotide sequences.

All isolates assayed for OTA production are held lyophilized at the fungal collection of the Fungi and Mycotoxins in Food Group (Department of Microbiology and Ecology, Valencia University). Due to the originality of the results obtained in the present study on OTA production by *A. tubingensis*, producing isolates Bo56, Bo66, and Mn24 of this species were deposited in the Spanish Collection of Type Cultures under reference numbers CECT 20543, CECT 20544, and CECT 20545, respectively.

Characterization of OTA-producing isolates. Characterization of ochratoxinogenic isolates was carried out by inoculation of Erlenmeyer flasks containing 50 ml of yeast extract-sucrose broth (YES; 2% yeast extract, 15% sucrose) supplemented with 5% bee pollen to increase OTA production (40) with 1 ml of a spore suspension (10^4 spores of each isolate/ml). Bee pollen used as an ingredient came mainly from *Cistus* spp. and, secondarily, from *Echium* spp.; it was a gift of a Valencian company of bee products. Bee pollen was previously assayed to ensure it contained undetectable OTA levels. Before inoculation, culture media was autoclaved for 30 min at 111°C. Spore suspensions used for inoculation were prepared from single-spore cultures made in potato-dextrose agar and grown for 7 days at 25°C. Erlenmeyer flasks containing inoculated media were incubated for 28 days at 25°C in the dark.

OTA extraction from YES–5% bee pollen cultures was accomplished as follows. The content of each flask was filtered through Whatman no. 4 filter paper, acidified to pH 2.8 to 3.0 with 0.1 M phosphoric acid, and extracted in a separatory funnel with chloroform (three times; each extraction, 5 ml). The organic extracts were combined, evaporated to dryness in a rotary evaporator, and suspended in 100 μl of acetonitrile-water-acetic acid (99:99:2 [vol/vol/vol]) for further analysis.

OTA separation and detection were performed by liquid chromatography (LC) according to the method of Visconti et al. (66) with some modifications. The liquid chromatographic system used for OTA analysis consisted of a Waters 600 pump connected to a Waters 474 fluorescence detector. System control and signal treatment were carried out with Millennium 32 software, version 3.01.05 (Waters, Milford, Mass.). Separation was performed with a stainless steel LiChrospher 100 C_{18} reversed-phase column (250 by 4 mm; 5-μm particle size) connected to a guard column (4 by 4 mm; 5-μm particle size) filled with the same phase (Agilent Technologies, Waldbronn, Germany). The mobile phase was acetonitrile-water-acetic acid (99:99:2 [vol/vol/vol]) at a flow rate of 1.0 ml/min. It was filtered through a 0.45-μm nylon membrane filter and degassed by an

TABLE 2. Fungal contamination levels in five grape varieties grown in Spain

Fungus	Grape variety (no. of samples analyzed)												Total (52)	
	Bobal (10)			Tempranillo (18)			Garnacha (7)			Monastrell (9)			Moscatel (8)			
	(*n*)[a]	CFU/ml[b]	(%)[c]	(*n*)[a]	CFU/ml[b]	(%)[c]	(*n*)[a]	CFU/ml[b]	(%)[c]	(*n*)[a]	CFU/ml[b]	(%)[c]	(*n*)[a]	CFU/ml[b]	(%)[c]	CFU/ml[b]	(%)[c]
A. carbonarius	10	1.6×10^2	1.3	14	32	0.2	4	71	0.8	8	4.0×10^2	3.9	7	2.8×10^2	41.7	1.8×10^2	1.2
A. niger aggregate	10	21	0.2	14	5	<0.1	3	2	<0.1	7	27	0.3	7	22	3.3	15	0.1
A. flavus	3	12	0.1													12	<0.1
Alternaria spp.	10	7.0×10^3	58	18	3.8×10^3	24.0	7	6.2×10^2	6.7	9	2.9×10^2	2.8	8	3.2×10^2	47.5	2.8×10^3	20.3
Acremonium spp.										6	30	0.3				30	0.2
Penicillium spp.	6	42	0.4	14	1.4×10^3	8.7	5	46	0.5	3	18	0.2	2	7	1.0	6.7×10^2	4.8
Cladosporium spp.	10	4.3×10^3	35.8	18	6.5×10^3	41.0	7	7.2×10^3	78.2	9	9.3×10^3	92.2	6	34	5.1	5.9×10^3	42.2
Fusarium spp.	7	1.3×10^2	1.1	8	62	0.4				3	26	0.3	3	9	1.4	72	0.5
Rhizopus spp.				13	3.8×10^2	2.4	4	1.3×10^3	13.7							5.9×10^2	4.2
Phoma spp.				7	3.7×10^3	23.2										3.7×10^3	26.4
Total		1.2×10^4			1.6×10^4			9.2×10^3			1.0×10^4			6.7×10^2		1.4×10^4	

[a] Number of samples showing fungal contamination.
[b] CFU/ml of berry homogenate (average of positive samples).
[c] Percentage of CFU of each fungus/ml with respect to the total CFU/ml count in each grape variety.

on-line vacuum device (Waters). Sample extracts (each, 20 μl) were injected by means of an automatic injector (Waters). Excitation and emission wavelengths were 330 and 460 nm, respectively.

LC-ion trap mass spectrometry (MS) was used to unambiguously confirm the presence of OTA in cultures. The analysis was carried out on an Agilent 1100 liquid chromatograph (Agilent Technologies), equipped with a Zorbax SB-C18 column (150 by 4.6 mm; 5-μm particle size) (Agilent Technologies) and coupled to a Bruker Esquire 3000 Plus ion trap mass spectrometer (Bruker Instruments, Billerica, Mass.). The mobile phase was programmed following a linear gradient at a flow rate of 0.5 ml/min. Solvent A was water with 0.05% trifluoroacetic acid, and solvent B was methanol with 0.05% trifluoroacetic acid. The gradient program was as follows: 0 min, 40% B; 1.5 min, 40% B; 15 min, 100% B. The ionization method was electrospray ionization in positive mode by using the following ionization source parameters: N_2 nebulizer gas at 60 lb/in^2, dry gas at 10 liters/min, dry temperature at 220°C, and capillary voltage at 3 kV. Confirmation by MS was based on the protonated molecule $[M + H]^+$ and the most abundant product ion $[(M + H)^+ - HCOOH]$, whose *m*/*z* ratios are 404 and 358, respectively.

Ochratoxin A standard was purchased from Sigma-Aldrich (Alcobendas, Spain). A stock solution of 500 μg/ml was prepared in benzene-acetic acid (99:1 [vol/vol]) and stored at −20°C. Working standards were prepared by evaporation of an aliquot of this stock solution under a stream of nitrogen and redissolution of the residue in acetonitrile-water-acetic acid (99:99:2 [vol/vol/vol]). A calibration curve made with five working standards, which were added to the YES–5% bee pollen medium, was used to determine OTA in cultures during the study.

Chloroform (LC grade) was from Lab-Scan, Ltd. (Dublin, Ireland). Benzene, acetonitrile, and acetic acid (LC grade) were from J. T. Baker (Deventer, Holland). Pure water was obtained from a Milli-Q system (Millipore, Billerica, Mass.).

RESULTS

Fungal contamination of grapes. Eight fungal genera were isolated from the grape samples (*Aspergillus*, *Alternaria*, *Acremonium*, *Penicillium*, *Cladosporium*, *Fusarium*, *Rhizopus*, and *Phoma*). Table 2 shows the contamination levels of the five grape varieties by these fungal genera and the total counts.

Tempranillo was the most contaminated grape variety, with 1.6×10^4 CFU/ml. The genus *Phoma* was isolated quite frequently in this variety while this genus was not found in the remaining varieties. The Moscatel variety showed the lowest level of fungal contamination (6.6×10^2 CFU/ml). The remaining grape varieties (Bobal, Monastrell and Garnacha) showed very similar contamination levels, which were 1.2×10^4, 1.0×10^4, and 9.2×10^3 CFU/ml, respectively.

Generally speaking and considering all the grape varieties included in this study, the most frequently isolated fungi were *Alternaria* spp. and *Cladosporium* spp. In these varieties, the number of CFU/ml ranged from 2.9×10^2 to 7×10^3 for the first genus, and from 34 to 9.3×10^3 for the second genus, depending on the variety. Statistical analysis (analysis of variance) using all data confirmed that these two genera were dominant among the mycobiota of the studied grape samples. The *P* value was 0.0000, which indicates that there are very significant differences between these two genera and the remaining found genera with regard to isolation frequency.

When considering only the *A. niger* aggregate (*A. niger* and *A. tubingensis*) and *A. carbonarius*, the analysis of variance showed that there were significant differences ($P = 0.000$) in contamination levels among the different grape varieties. The most contaminated grape varieties by *Aspergillus* section *Nigri* were Monastrell, Moscatel, and Bobal, where average *A. carbonarius* contamination levels were 4×10^2, 2.8×10^2, and 1.6×10^2 CFU/ml, respectively. The number of CFU of *A. carbonarius*/ml in Garnacha and Tempranillo varieties was signif-

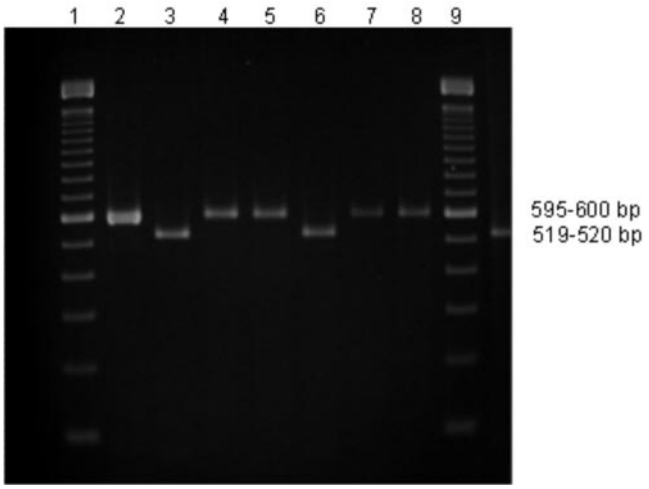

FIG. 1. PCR products digested by enzyme RsaI and separated on 2% agarose gel. Lanes 1 and 9, the 100-bp DNA ladder (Gibco BRL) used as a size marker; lane 2, T pattern (*A. tubingensis* CECT 20393 strain); lane 3, N-pattern (*A. niger* CECT 2807 strain); lanes 4, 5, 7, and 8, isolates Bo44, Bo56, Bo66, and Mn24, respectively; lane 6, isolate Bo62.

icantly lower (71 and 32, respectively). Species belonging to the *A. niger* aggregate were isolated less often.

Aspergillus flavus was isolated only from the Bobal variety at a low level (12 CFU/ml) and in only 3 of the 10 grape samples. Consequently, it cannot be considered a habitual member of the natural mycobiota in the studied grape varieties. *A. ochraceus* was not detected in any sample.

The incidence level of *Penicillium* spp. was usually low (7 to 1.4×10^3 CFU/ml), regardless of the grape variety, and *P. verrucosum* was not detected in any of the studied samples. Occurrence of *Fusarium* spp. was also very low (9 to 1.3×10^2 CFU/ml).

Molecular characterization of isolates within *A. niger* aggregate. Due to morphological similarity within the *A. niger* aggregate, identification of isolates in this group was based on PCR amplification of 5.8S rRNA genes and the two intergenic spacers, ITS1 and ITS2, followed by the subsequent digestion of PCR products with restriction endonuclease RsaI. The ribosomal ITS1-5.8S-ITS2 region was amplified from all 85 isolates of the *A. niger* aggregate. The product size was about 600 bp (596 to 600 bp). The restriction patterns obtained from this amplified region with enzyme RsaI were compared with those from type strains *A. tubingensis* CECT 20393 (T pattern, where enzyme RsaI does not cut the PCR product) and *A. niger* CECT 2807 (N pattern, where two fragments of 519 and 76 bp were obtained). Figure 1 shows the results obtained for these two type strains and five isolates. The 76-bp fragment was too small to remain on the gel. Isolates Bo44, Bo56, Bo66 (Bobal variety), and Mn24 (Monastrell variety) displayed a T pattern and were classified as *A. tubingensis*, while isolate Bo62 (Bobal variety) showed an N pattern and was classified as *A. niger*.

About 55% of the isolates of *A. niger* aggregate contaminating the grape samples belonged to *A. tubingensis*; the remaining 45% belonged to *A. niger*.

Ochratoxin A production in *Aspergillus* section *Nigri*. Of 205 *Aspergillus* section *Nigri* isolates (Table 3) that were tested for OTA production, 92 (44.9%) produced this toxin. The production levels ranged from 1.2 to 3,530 ng/ml of culture medium. Eighty-nine of the ochratoxinogenic isolates were classified as *A. carbonarius* (74.2% of the 120 tested isolates), while the remaining 3 were classified as *A. tubingensis* (14.3% of the 21 tested isolates) on the basis of RFLP. OTA was detected in cultures of isolates Bo56, Bo66, and Mn24 but not in cultures of the remaining isolates of the *A. niger* aggregate. Their OTA production levels varied from 46.4 to 111.5 ng/ml of culture medium. OTA was not detected in cultures of the 64 assayed isolates of *A. niger*. Comparison of the ITS1-5.8S rRNA gene-ITS2 sequences from the amplified regions of isolates Bo56, Bo66, and Mn24 with those available in the NCBI Nucleotide Database showed that the ITS sequences of both isolates Bo56 and Bo66 were identical to the sequences of *A. tubingensis* CBS 643.92 and CBS 127.49 (EMBL accession numbers for the sequenced region are AJ280008 and AJ280007, respectively), except for a single nucleotide (G instead of T) at position 532. Thus, they showed 99% of identity (592 out of 593 bp). Isolate Mn24 showed 100% of identity (593 out of 593 bp) with the same CBS strains.

TABLE 3. OTA production capacity of *Aspergillus* section *Nigri* isolates from grapes grown in Spain when cultured in YES broth supplemented with 5% bee pollen[a]

Fungus	No. of isolates		% Positive isolates	OTA (ng/ml)	
	Assayed	Positive		Avg[b]	Range
A. carbonarius	120	89	74.2	155	1.2–3530
A. niger	64				
A. tubingensis	21	3	14.3	70.7	46.4–111.5
Total	205	92	44.9		

[a] Culture conditions: 28 days at 25°C in the dark.
[b] Average OTA level in cultures of positive isolates

The distribution of the 205 isolates and the grape varieties from which they came was as follows: 37 isolates from Bobal, 53 isolates from Garnacha, 36 isolates from Tempranillo, 31 isolates from Monastrel, and 48 isolates from Moscatel.

Analysis by LC-ion trap MS of the YES–5% bee pollen extracts confirmed the identity of OTA in cultures. The peaks produced by the $[M + H]^+$ ion (*m*/*z* 404) and the $[(M + H)^+ - HCOOH]$ ion (*m*/*z* 358) were observed in the mass spectra of the OTA standard and the cultures where OTA had been detected by LC with fluorescence detection.

Although the occurrence levels of *A. carbonarius* were significantly different with regard to the grape varieties studied in the present report, the percentages of OTA-producing isolates from the different grape varieties were very similar (74%, 85%, 80%, 63%, and 69% for Bobal, Garnacha, Monastrell, Tempranillo, and Moscatel, respectively).

DISCUSSION

After analysis of the occurrence data of fungi in grapes from other varieties and geographic locations reported by other authors (5, 9, 14, 39, 55), it can be observed that the contaminant mycobiota differs from the results found in this work. Abrunhosa et al. (5) did not find *Aspergillus* in samples of grapes grown in Portugal. Battilani et al. (9) found *Aspergillus*

spp. in grapes grown in Italy, with the *Nigri* section largely predominating. The most abundant were molds with biseriate conidial heads (*A. niger* and *A. tubingensis*), followed by *Aspergillus* with uniseriate conidial heads (*Aspergillus aculeatus* and *A. japonicus*) and *A. carbonarius*, in that order. Cabañes et al. (14) did not find molds with uniseriate conidial heads in grapes grown in Spain. Magnoli et al. (39) found a clear dominance of the genus *Alternaria* in grapes grown in Argentina. Their results are similar to those found in the present report. However, the levels of *Cladosporium* were much lower in Argentinean samples. Although Sage et al. (55) did not evaluate the occurrence of the different fungi in French grapes, they found black fungi with uniseriate and biseriate conidial heads, including *A. carbonarius*. No *Aspergillus* section *Nigri* isolates with uniseriate conidial heads were detected in the samples analyzed in the present work, in agreement with Cabañes et al. (14). It should be noted that despite the differences in geographic location, the varieties studied by the different authors were different as well, which could explain the disagreement of the results found among the samples. Our results show that grape variety has a strong influence on the occurrence of *Aspergillus* section *Nigri*. Cabañes et al. (14) found *Penicillium purpurogenum* in all samples of the white Garnacha grape variety that they studied. Their samples were from Tarragona, an area that is near to Cuenca and Valencia, sampled in the present study. However, the occurrence of *Penicillium* spp. in our samples was generally low (Table 2), and *P. purpurogenum* was not isolated in any sample. Obviously, there is a great complexity with regard to the native mycobiota in different grape varieties.

It is interesting that there are not significant differences in ochratoxinogenic fungi occurrence on samples of the same grape variety when grown in vineyards in different areas. Studies of OTA incidence in wines carried out in various countries point out that the number of samples contaminated with this toxin and their levels are higher in wines from southern than from northern regions of Europe. These levels ranged from 0.01 to 3.5 μg/liter (9, 40, 41, 47, 48, 51). However, the grape variety used to produce wines might have a strong influence on these differences. It has been reported (9, 40, 41, 47, 48, 51) that wines from red berries exhibit higher OTA levels than wines from pink and white ones, in this sequence. Four of the grape varieties analyzed by us (Bobal, Garnacha, Tempranillo, and Monastrell) have red berries (Table 1), and there were significant differences among them in the occurrence of ochratoxinogenic fungi. This result is very compatible with the idea that OTA levels in wines depend on the grape variety from which they are produced, regardless of the color of berries. The higher OTA content of red wines might be associated with the grape variety used to produce them and with the winemaking process, especially with the contact time between grape juice (must) and berry skins. However, more studies are needed to assure differences in grape varieties with regard to susceptibility to fungal contamination.

The taxonomy of black aspergilli is far from clear. It has long been studied by means of morphological and cultural criteria. Whereas *A. carbonarius* can be microscopically distinguished by conidial size and ornamentation, all the taxa in the *A. niger* aggregate are morphologically indistinguishable. This problem has been the origin of misidentifications and discrepancies in the physiological characteristics of the species included in this aggregate. The results from the present study agree with those from Accensi et al. (7). A target for endonuclease RsaI was detected in the rRNA gene ITS1 of *A. niger*. It does not exist in the sequence of *A. tubingensis*. The PCR-amplified-5.8S rRNA gene of *A. niger* was digested into two fragments of 519 and 76 bp. The 76-bp fragment was too small to remain on the gel (Fig. 1).

Before this report, *A. tubingensis* had not been found to be able to produce OTA (2, 9, 14, 56). However, in this work, three OTA-producing isolates (two from the Bobal and one from the Monastrell grape varieties) were found. The classifications of these isolates as *A. tubingensis* based on RFLP were confirmed by sequencing the ITS1-5.8S rRNA gene-ITS2 region. Two of the isolates (Bo56 and Bo66) matched at 99% (592 of 593 bp) with *A. tubingensis* CBS 643.92 and *A. tubingensis* CBS 127.49; 100% identity was found for the third isolate (Mn24). According to the NCBI Nucleotide Database, the difference in the sequence of this region between type strains of *A. tubingensis* CBS 643.92 and CBS 134.48 (EMBL accession number AJ223853.1) is one nucleotide (the last strain has C instead of G) at position 16 (99% identity). There are various possible reasons for the disagreement in results from different researchers. The difficult differentiation between *A. niger* and *A. tubingensis* based on morphological characteristics may have provided misidentifications. In other cases (2, 14), failing to detect OTA in cultures of *A. tubingensis* might be due to culture medium and/or incubation time. In these reports, the authors used YES broth, incubation time was only 7 days, and sampling sizes for LC analysis were small. This accumulation of events could have led to negative results. Current research is being carried out in our laboratory to study the influence of culture medium on OTA production by *Aspergillus* section *Nigri* isolates (unpublished data). It has been shown that accumulations of OTA in YES broth and YES broth supplemented with 5% grape must are 80% and 92%, respectively, of the levels reached with YES broth supplemented with 5% bee pollen. The capacity of bee pollen to stimulate biosynthesis of OTA by *A. ochraceus* has been previously reported (42), but it has not been studied until now in *Aspergillus* section *Nigri*.

P. verrucosum was not detected in any sample. According to several authors (10, 26, 55), ochratoxicoses in southern Europe seem to be connected with the presence of *Aspergillus*, while in Germany and in Scandinavia, they would be connected with the presence of penicillia. Owing to their different temperature needs (26) ochratoxinogenic penicillia grow well over a range of temperatures (4 to 31°C), whereas aspergilli that produce OTA require higher temperatures (12 to 39°C). Temperatures in the Spanish grape crop areas are generally high, especially during summer when berry ripening and harvest take place.

The high occurrence of *Alternaria* spp. in the analyzed samples is noteworthy. Species of this genus have been reported as producers of some mycotoxins such as alternariol, alternariol monomethyl ether, tenuazonic acid, and zinniol, among others (18, 20, 65). According to Miller (45), the cooccurrence of several mycotoxins can influence each other's production levels, as well as the toxicity of contaminated material. To date, research on this topic has not been carried out either with grapes or, consequently, with wine. In any case, grape selection avoiding the use of decaying berries constitutes a critical con-

trol point that should be exhaustively monitored. A suitable selection of clusters rejecting the rotten ones could reduce the OTA content of wines by 98% (36, 44).

On the basis of our results, it can be deduced that the fungi responsible for OTA occurrence in wines made with the five grape varieties studied here and grown in Spain belong mainly to the species *A. carbonarius* and to a lesser extent to species from the *A. niger* aggregate, specifically, *A. tubingensis*, a species never previously reported as an OTA producer.

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