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Ochratoxin A-producing fungi in Spanish wine grapes and their relationship with meteorological conditions

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Abstract

Forty vineyards from four wine making regions of Spain were sampled at three different growth stages in 2002 and 2003. The aim was to study the fungi associated with grapes and their ability to produce ochratoxin A (OTA) on synthetic media. Among the total mycoflora, 464 (7.7%) and 648 (10.8%) *Aspergillus* section *Nigri* (black aspergilli) strains were isolated in 2002 and 2003, respectively, and were classified into three groups: isolates with uniseriate heads, *A. niger* aggregate and *A. carbonarius*. The latter presented the highest percentage of OTA-positive strains (82% in 2002 and 76% in 2003) and produced the highest levels of toxin (2.5–25 μg g^{-1}). The sampling year, sampling date, the region and their interactions presented significant differences in the number of black aspergilli isolated. Most black aspergilli were found in 2003 and at harvest. A positive correlation between the number of black aspergilli found in grapes and the temperature in the field was found. Grapes from 2003, the warmest year, and from Costers del Segre, the warmest region, were significantly the most contaminated. No significant correlation between black aspergilli presence and other meteorological factors such as relative humidity or rainfall could be established. Musts from all the vineyards were also analysed in both years, although no OTA was found in either year.

Introduction

Ochratoxin A (OTA) is a toxic secondary metabolite naturally occurring in a wide range of foods both of vegetable and animal origin (van Egmond and Speijers, 1994). OTA has been reported in wine since 1996 (Zimmerli and Dick, 1996). Wine is estimated to be the second source of OTA in the diet after cereals in Europe, as it can represent up to 15% of the total OTA intake (Codex Alimentarius Commission, 1999). OTA possesses teratogenic, nephrotoxic and immunotoxic properties and has been classified as a possible human carcinogen (Group 2B) (IARC, 1993).

OTA is produced by species in *Aspergillus* sections *Nigri* (black aspergilli) and *Circumdati*, commonly found in warm and tropical climates, with *Penicillium verrucosum* being the main source in temperate climates and more frequently associated with cereals (Pitt and Hocking, 1997). Recent studies have suggested that black aspergilli, essentially *A. carbonarius*, are the main species producing OTA in grapes (Cabañes et al., 2002; Abarca et al., 2003; Battilani et al., 2003; Bellí et al., 2004b). With growing concern over European exposure to OTA, the European Union legislation authorities have recently introduced a limit of 2.0 μg l^{-1} wine, must or grape juice (European Commission, 2005).

Little information exists on mycoflora and potential OTA-producing fungi in Spanish wine grapes. This study focused on the identification of the common mycoflora in wine grapes from four important grape growing regions of Spain to study their progression during grape ripening in 2002 and 2003, with particular interest in ochratoxigenic species and their ability to produce OTA. An initial study was carried out by our team in 2001, and the results have been recently published (Bellí et al., 2004a). In the present study the objective was to correlate the fungal populations isolated over the 3 years with the meteorological conditions in the vineyards.

Materials and methods

Field sampling

Four wine-producing regions representing a cross section of five important Designations of Origin of Spain (La Rioja, Costers del Segre, Utiel-Requena, Penedés and Conca de Barberà) were chosen for the study. Ten fields were selected in each region ($n = 40$) covering a range of foreign and regional grape varieties, both red and white. Samples were taken at three growth stages (1 month after setting, veraison and harvest time) in 2002 and 2003. Ten vines were chosen along the diagonals of each vineyard and a bunch of grapes was randomly collected from each vine. Bunches were collected in paper bags to reduce handling and prevent external contamination, and kept at 4 °C until laboratory analysis. Meteorological data of each sampled region was obtained from the Spanish National Institute of Meteorology database (INM, 2003).

Mycoflora determination

Five grapes were randomly chosen from each bunch and plated directly in Petri dishes containing Dichloran Rose Bengal Chloramphenicol medium (DRBC) (Pitt and Hocking, 1997) under sterile conditions. Plates were incubated for 7 days at 25 °C and colonies of developing fungi were examined and classified into genera according to Pitt and Hocking (1997). Most of the potential OTA producers were isolated onto Czapek Dox agar (CZ) (Pitt and Hocking, 1997) for classification, and onto Czapek Yeast Extract agar (CYA) (Pitt and Hocking, 1997) for OTA production; both media were incubated at 25 °C for 7 days. As morphological identification of black aspergilli is time-consuming and due to the high number of strains isolated in this study, they were classified according to the morphology of their spores and conidial heads into three groups: uniseriates, *A. niger* aggregate (biseriates excluding *A. carbonarius*) and *A. carbonarius*, as recommended by Dr. Z. Kozakiewicz (CABI Bioscience, UK) and Dr. J. Cabañes (Autonomous University of Barcelona, Spain).

Screening of fungi for OTA production

The method used was adapted from Bragulat et al. (2001). Three agar plugs, 6 mm in diameter, were extracted in 1 ml of methanol for 1 h. The extracts were filtered (Millex^R SLHV 013NK, Millipore, Bedford, Massachusetts, USA) before chromatographic analysis. A HPLC system with a fluorescence detector (Waters 474, Milford, Massachusetts, USA) (λ_{exc} 330 nm; λ_{em} 460 nm) and a C18 column (Waters Spherisorb 5 μm, ODS2, 4.6 × 250 mm) were used. Mobile phase (acetonitrile–water–acetic acid, 57:41:2) was pumped at 1 ml min^{-1}. The ochratoxin standard was from *A. ochraceus* (Sigma-Aldrich, Steinheim, Germany). Recovery of added OTA to the media ranged from 80 to 100%. The retention time was 7.1 min and the detection limit was 0.01 μg OTA g^{-1} of CYA, based on a signal-to-noise ratio of 3:1.

OTA in musts

At the last sampling time of both years, the same ten bunches collected from each vineyard for the mycoflora study were crushed and the resulting musts ($n = 40$) were analysed for OTA using the method of the Office International de la Vigne et du Vin (Bezzo et al., 2002). Briefly, 100 ml of each sample (pH 7.4 with NaOH 4 M) were centrifuged (3830 g, 15 min) and filtered (Whatman No. 1). Afterwards, they were passed through an immunoaffinity column (Ochraprep, Rhône Diagnostics Technologies, Glasgow, UK) at 2–3 ml min^{-1}. The column was then washed with 20 ml of distilled water (5 ml min^{-1}) and finally dried in an air stream (2 min). Desorption was carried out with 1.5 ml of methanol/acetic acid

(98/2) solution. The eluate was evaporated to dryness at 40 °C under a stream of nitrogen and redissolved in 2 ml of mobile phase (acetonitrile 48%-sodium acetate 4 mM/acetic acid (19/1) 52%). About 25 µl of each final sample were injected into a HPLC system equipped with a fluorescence detector (Waters 474) (λ_{exc} 230 nm; λ_{em} 458 nm) and a C_{18} column (Waters Spherisorb 5 µm, ODS2, 4.6 × 250 mm). The analysis was performed under isocratic conditions at a flow rate of 1 ml min^{-1}. Detection limit and retention time were 0.05 µg l^{-1} and 11.5 min, respectively.

Statistical analysis

The percentages of infection of common mycoflora, black aspergilli species and OTA-producing isolates, were analysed by the General Linear Model Procedure of SAS (version 8.02, SAS Institute, Inc., Cary, N.C., U.S.A.) with Student-Newman-Keuls (SNK) test ($P < 0.05$). The significance of the correlation between maximum, mean and minimum temperatures, relative humidity, rainfall, number of rainy days, number of black aspergilli isolates, number of OTA positive isolates, and number of uniseriates, *A. niger* aggregate and *A. carbonarius* isolates in 2001, 2002 and 2003, was assessed with the same programme using the Pearson coefficients at $P < 0.05$.

Results

The colonisation of grapes by fungi occurred rapidly in the field and increased from setting (75–85%) to harvest (100%) in all regions and in both years. The most common mycoflora isolated from grapes, in decreasing order, were: *Alternaria*, yeasts, *Aspergillus*, *Botrytis*, *Epicoccum*, *Cladosporium*, *Rhizopus*, *Penicillium*, *Fusarium*, *Mucor*, *Phoma*, *Trichoderma* and *Ulocladium*. No statistically significant differences were found between years or regions. Therefore, as an example, the fungi infecting grapes at each sampling date in 2003 in La Rioja region are shown in Figure 1. *Alternaria* was the highest component of the natural flora on the surface of fresh grapes, followed by yeasts. The number of *Aspergillus*, *Botrytis*, *Epicoccum*, *Rhizopus* and yeasts were statistically higher at harvest. The exception was *Alternaria*, which decreased from 95 to 70% in the later growth stages. The remaining genera were rarely isolated and did not follow any trend.

According to analysis of variance, all single factors: year, sampling date and region and their interactions, presented significant differences in the number of black aspergilli isolated ($P < 0.0001$). A total of 464 (7.7%) and 648 (10.8%) black aspergilli were isolated in 2002 and 2003, respectively, distributed in the four regions sampled. The number of these moulds found at harvest was significantly higher than were found in the first or second sampling for the four regions (Figure 2). Grapes from Costers del Segre were significantly the most contaminated every year, with approximately 300 strains isolated in 2003 and around 180 in 2002 at harvest, followed by those from Utiel-Requena with around 100 isolates in both years at harvest. However, no statistical differences were

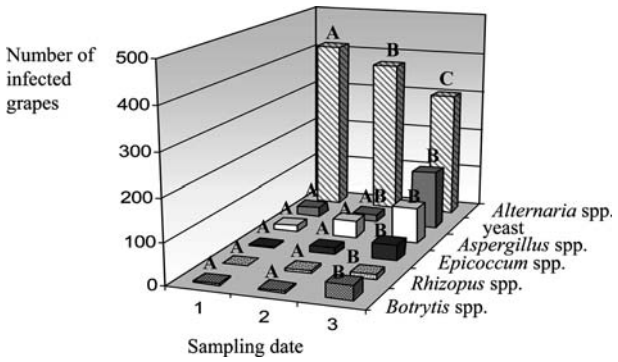

Figure 1. Number of grapes infected by different fungi from a total of 500 grapes plated on DRBC, at each sampling date: (1) 1 month after setting, (2) veraison and (3) harvest, in 2003 in La Rioja region. Different letters over bars indicate significant differences in the number of these fungi between sampling periods.

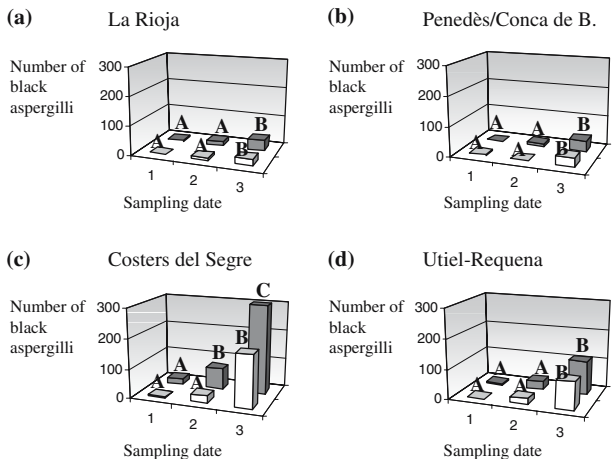

Figure 2. Number of black aspergilli found on grapes plated on DRBC in □ 2002 and in ■ 2003 in four wine-making regions of Spain, at three sampling periods (2000 grapes per sampling period): (1) 1 month after setting, (2) veraison and (3) harvest. Different letters over bars indicate significant differences in the number of these fungi between sampling periods.

found between Penedés/Conca de Barberà and La Rioja regions.

Figure 3 shows the distribution of the black aspergilli isolates that were classified, with *A. niger* aggregate the most common species (75% in 2002 and 53% in 2003) followed by *A. carbonarius* (7% in 2002 and 29% in 2003). In contrast, the percentage of isolates with uniseriate heads was similar in both years (18%). No isolates of *P. verrucosum* were detected, while few *A. ochraceus* isolates were found (19 in 2002 and 31 in 2003), most at harvest and representing approximately 15% of strains which were OTA-producers.

In 2002, 7% of the total number of black aspergilli strains isolated produced detectable levels of OTA in culture, whereas 25% were detected in 2003. Among the black aspergilli groups, *A. carbonarius* presented the highest percentage of OTA-positive strains (82% in 2002 and 76% in 2003). A low percentage of *A. niger* aggregate isolates produced OTA (2% in 2002 and 5% in 2003) and no toxin was detected in any of the uniseriate strains in either year (Table 1). Furthermore, more than 95% of the total number of positive isolates produced low amounts of OTA (< 2.5 μg g^{-1}), although some isolates were found to produce OTA at higher levels (2.5–25 μg g^{-1}). The most toxigenic ones were *A. carbonarius* isolates in both years. However, subsequent analysis of musts detected no OTA. Fungi isolated from grapes were correlated to the meteorological data in each sampling month for each region in 2002 and 2003 detailed in Table 2. A positive correlation between the number of black aspergilli isolated and the temperature in the field in the months preceding harvest was found (Table 3). High relative humidity (R.H.) also contributed to the infection of these fungi, mainly for the uniseriate group.

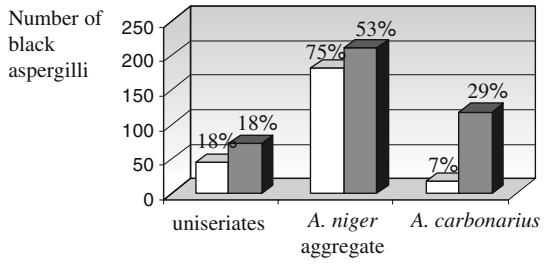

Figure 3. Number of black aspergilli isolated from grapes in □ 2002 and in ■ 2003, classified into three groups and percentage of each group among all black aspergilli isolated.

Discussion

The main moulds causing secondary rots of grapes are black aspergilli, *Alternaria*, *Rhizopus*, *Cladosporium* and *Penicillium*. These are generally associated with vine trash on soil, leaves, leaf buds and other residues in the field (MAPA, 1998). These fungi were the dominant genera isolated from grapes in the present survey as well as in another study in Argentina and Brazil by Da Rocha Rosa et al. (2002). They found that yeasts were a major

Table 1. Percentage of black aspergilli isolates of each group found in each region at each sampling date in 2002 and 2003 among the total number of grapes analysed

S[a]	Region	Uniseriate group		*A. niger* aggregate		*A. carbonarius*	
		2002	2003	2002	2003	2002	2003
1	P/CB[b]	0% (0/0)	0% (0/0)	0.01% (1/0)	0.03% (2/0)	0% (0/0)	0% (0/0)
	UR	0% (0/0)	0% (0/0)	0.10% (6/0)	0.18% (11/0)	0% (0/0)	0% (0/0)
	R	0.06% (4/0)	0.06% (4/0)	0.20% (12/0)	0.46% (28/0)	0% (0/0)	0% (0/0)
	CS	0% (0/0)	0.20% (12/0)	0.03% (2/0)	0.10% (6/0)	0% (0/0)	0% (0/0)
2	P/CB	0.25% (15/0)	0.56% (34/0)	0.16% (10/0)	0.25% (15/1)	0% (0/0)	0.01% (1/1)
	UR	0.40% (24/0)	0.23% (14/0)	1.18% (71/0)	0.55% (33/2)	0.01% (1/1)	0% (0/0)
	R	0% (0/0)	0% (0/0)	0% (0/0)	0.06% (4/2)	0% (0/0)	0% (0/0)
	CS	0% (0/0)	0% (0/0)	0.25% (15/0)	0.26% (16/1)	0% (0/0)	0.10% (6/0)
3	P/CB	0.01% (1/0)	0% (0/0)	0.88% (53/0)	0.96% (58/5)	0.01% (1/0)	0% (0/0)
	UR	0% (0/0)	0% (0/0)	0.05% (3/1)	0.03% (2/–)[c]	0.11% (7/6)	0.11% (7/7)
	R	0% (0/0)	0.03% (2/0)	0.03% (2/1)	0.13% (8/–)	0% (0/0)	0% (0/0)
	CS	0.01% (1/0)	0.10% (6/0)	0.10% (6/1)	0.46% (28/–)	0.13% (8/7)	1.73% (104/82)
Total		0.75% (45/0)	1.2% (72/0)	3.0% (181/3)	3.5% (211/11)	0.28% (17/14)	1.96% (118/90)

Numbers in brackets are the total number of black aspergilli isolated / the number of isolates producing OTA above the detection limit (0.01 μg g^{-1} CYA).
[a]S, sampling (1, 1 month after setting; 2, veraison; 3, harvest).
[b]P/CB, Penedès/Conca-Barberà; UR, Utiel-Requena; R, La Rioja; CS, Costers del Segre.
[c](–) not tested for OTA.

component of the fungal population, and *Alternaria*, *Aspergillus* and *Botrytis* were frequently isolated. *Alternaria* and *Aspergillus* were also the most frequent moulds of the mycoflora of Argentinean grapes isolated by Magnoli et al. (2003). *Penicillium*, *Cladosporium* and *Botrytis* prevailed in Portuguese grapes (Abrunhosa et al., 2001). Da Rocha Rosa et al. (2002) suggested that the diversity of grape mycoflora depends on grape variety, degree of berry maturity, physical damage, viticulture practices and climatic conditions.

A positive correlation between the number of black aspergilli isolated from grapes and temperature was found (Table 3). This correlation was mainly due to *A. niger* aggregate. It is known that optimum temperatures for growth of *A. niger* aggregate *in vitro* are between 30–37 °C; meanwhile the optimum for *A. carbonarius* and

Table 2. Mean of 2001, 2002 and 2003 meteorological data of each region at each sampling period (1, June; 2, July; 3, August)

Region	Sampling	*T* max[a] (°C)	*T* mean[b] (°C)	*T* min[c] (°C)	R.H. (%)	Rainfall (mm)	Rain (days)
Utiel-Requena	1	30.3	22.9	15.5	62.1	10.2	1.3
	2	32.4	25.0	17.5	63.4	0.0	0.0
	3	32.3	25.3	18.2	65.5	21.7	2.0
Rioja	1	27.2	20.5	13.7	52.9	37.4	7.0
	2	27.6	20.9	14.3	52.5	23.8	5.0
	3	29.1	22.3	15.4	55.3	29.3	7.3
Penedés/ Conca de Barberà	1	29.2	22.6	16.5	62.2	20.8	4.3
	2	26.7	23.5	17.3	67.7	31.1	6.7
	3	30.7	24.0	18.2	68.9	21.8	4.7
Costers del Segre	1	30.9	23.1	15.8	58.2	11.5	3.0
	2	30.8	23.6	17.0	64.7	61.1	9.0
	3	32.4	25.1	18.2	62.6	20.5	4.7

(INM, 2003)
[a]*T* max: mean daily maximum temperature for each sampling stage.
[b]*T* mean: mean daily mean temperature for each sampling stage.
[c]*T* min: mean daily minimum temperature for each sampling stage.
R.H.: mean daily R.H. for each sampling stage.

Table 3. Correlation between meteorological parameters with ochratoxigenic fungi isolated from grapes using the coefficients of Pearson

	Black aspergilli	Black aspergilli OTA+	Uniseriates	*A. niger* aggregate	*A. carbonarius*
T max	0.40*	0.26	0.23	0.44*	0.25
T mean	0.46**	0.28	0.21	0.51*	0.26
T min	0.48**	0.26	0.30	0.55**	0.27
R.H.	0.20	0.09	0.36	0.14	0.10
Rainfall	0.10	0.17	0.06	−0.02	0.15
Num. rainy days	−0.01	0.01	0.22	−0.22	−0.02

*significant $P < 0.05$;
**significant $P < 0.001$.

uniseriate strains are between 25–30 °C (Mitchell et al., 2003; Bellí et al., 2004b). In addition, water activity (a_w) has been demonstrated to have an effect on *in vitro* growth of strains of black aspergilli, with the highest levels (0.98–0.995 a_w), similar to that of grapes, being the optimum in most cases (Bellí et al., 2004a).

The highest number of black aspergilli were detected at harvest in the four regions and in both years. The same trend was found in the sampling carried out in 2001 (Bellí et al., 2004a), which suggests that late ripening marks a profound change in the ecological factors affecting fungal sporulation, dissemination of spores as well as microbial growth. External factors such as air movement, cultural practices and insect damage would disseminate spores to the surface of berries and start fungal infection. Moreover, grapes are more susceptible to fungal infection when approaching harvest as sugar content increases and the berry texture softens (MAPA, 1998). All of this, together with the increasing temperatures in the month preceding harvest, sometimes above 30 °C (Table 2), could influence black aspergilli development. The general pattern of colonisation by fungal species of grapes, was not significantly different in 2001, 2002 and 2003; thus results can be considered representative of the situation in the sampled areas. However, more black aspergilli were isolated in 2003 than in the 2 previous years, probably because 2003 was an extremely hot year in Spain. High temperatures could also explain the higher number of black aspergilli found in Costers del Segre in 2002 and 2003.

Percentages of uniseriate isolates, *A. niger* aggregate and *A. carbonarius* (21, 60 and 19%, respectively) found in a survey of Italian grapes in 1999–2000 (Battilani et al., 2003), were very similar to those found in the present study. High incidence of *A. niger* aggregate was also found by Da Rocha Rosa et al. (2002) in a mycofloral survey of wine grapes from Argentina and Brazil. Less *A. carbonarius* were found, but 25% of these were OTA producers (18–234 μg g^{-1} on CYA). OTA was not detected in any of the must samples analysed, although in 2001, 15% of the musts contained low amounts of OTA: five samples contained 0.091–0.293 ng ml^{-1} and one 0.813 ng ml^{-1} (Bellí et al., 2004b). Similar results for OTA in some years but not others has also emerged from an Italian study of OTA content in grapes, which concluded that temperature, rain and relative humidity are the main factors that influenced OTA production in grapes (Battilani and Pietri, 2002). Due to the absence of OTA in the musts analysed, no correlation between the incidence of OTA-producing strains in grapes and OTA in musts could be established from the present study. In contrast, Sage et al. (2002) found a strong correlation between these factors, as eight of eleven must samples were found to be contaminated with OTA (10–461 ng l^{-1}) and a significant number of *A. carbonarius* strains were previously isolated from grapes.

Ecophysiological studies with black aspergilli, and in particular *A. carbonarius*, are needed to determine the conditions that favour growth and toxin production. Moreover, it would be interesting to study the infection process of black aspergilli in grapes and the role of grape skin damage, in order to determine preventive actions that minimise OTA content in grapes. Further investigations on the mechanisms of interactions and dominance of the fungi commonly isolated from grapes could be also developed.

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