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Ochratoxin A-producing fungi from grapes intended for liqueur wine production

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Summary

The ochratoxigenic mycobiota of grapes intended for liqueur wines from four Spanish vineyards were studied. The specific wine-making technology of these wines requires overripening of the grapes on the vine or extended post-harvest exposure of the grapes in the sun. In every vineyard, samples were taken at three different developmental stages: veraison, harvesting time and after overripening. With the maturation of the berries there was a clear increase of *Aspergillus* spp. In the last sampling time studied, they were isolated from the 90.3% of the plated berries. Black aspergilli (mainly *A. niger* aggregate and *A. carbonarius*) were predominant among the different *Aspergillus* spp. isolated and constituted 98.5% of the total *Aspergillus* strains isolated. At harvesting time and after overripening, the percentage of colonized berries with *A. carbonarius* exceeded that of *Aspergillus niger* aggregate. Due to their low frequency of isolation, *Penicillium* spp. and *Aspergillus* spp. outside black aspergilli are not an important source of ochratoxin A in grapes for liqueur wine production. On the contrary, 98.5% of the *A. carbonarius* isolates screened were able to produce ochratoxin A. Although the possible participation of different ochratoxin A-producing species may occur, our results confirm that *A. carbonarius* is the most important source of ochratoxin A in liqueur wines, increasing its occurrence along the ripening of grapes.

Keywords: *Aspergillus carbonarius*; Black aspergilli; Ochratoxin A; Grapes; Liqueur wine; Mycobiota

1. Introduction

Ochratoxin A (OTA) is a mycotoxin with nephrotoxic, carcinogenic, teratogenic, genotoxic and immunotoxic properties (Creppy, 1999). It occurs in a variety of plant products all over the world contaminating a diversity of foods in the normal diet. Following cereals, wine has been identified as the second major source of human exposure to OTA in Europe (Anonymous, 1998). The occurrence of OTA in wine and grape juice has been attracting considerable attention in many countries since the first results were published in 1996 (Zimmerli and Dick, 1996). The European Union has set limits on OTA concentration in some foods. The last regulation which took effect on April 1, 2005, established a maximum OTA level for wine (red, white and rosé) at 2 μg/kg (Anonymous, 2005). Before 30 June 2006, the Commission will set maximum level for OTA in liqueur wines among other food products.

The presence of OTA in must and wine is due to fungal contamination of grapes. Until recently, *Aspergillus ochraceus* and related species of section *Circumdati* and *Penicillium verrucosum* were considered the main OTA-producing species. Nevertheless, since the first description of OTA production by the black aspergilli *A. niger* var. *niger* (Abarca et al., 1994) and *A. carbonarius* (Horie, 1995), these species have been recognized as the main OTA contamination in grapes in many reports, mainly from European countries (Cabañes et al., 2002; Sage et al., 2002; Battilani et al., 2003; Serra et al., 2003, 2005; Bellí et al., 2004b; Tjamos et al., 2004; Bau et al., 2005) and dried vine fruits (Heenan et al., 1998; Abarca et al., 2003; Magnoli et al., 2004).

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While considerable research is in progress on fungi responsible for formation of OTA in grapes for table wines, there is a lack of data concerning grapes for liqueur wines. Several studies have pointed out that liqueur wines contained higher amount of OTA than table wines (Zimmerli and Dick, 1996; Burdaspal and Legarda, 1999; Pietri et al., 2001; Soufleros et al., 2003; Stefanaki et al., 2003; Bellí et al., 2004a; Ng et al., 2004). The specific wine-making technology of these wines requires overripening of the grapes on the vine or extended post-harvest exposure of the grapes in the sun. Such practices will influence the susceptibility of grapes to fungal attack (Soufleros et al., 2003).

The objective of this study was to identify the OTA-producing mycobiota of grapes intended for liqueur wines.

2. Materials and methods

2.1. Samples

During 2003 season, a total of 4 Spanish vineyards belonging to 3 grape-growing regions: Cádiz (one vineyard), Córdoba (one vineyard) and Girona (two vineyards) were studied. Three grape varieties were included: Moscatel (vineyards from Cádiz and Girona), Pedro Ximénez (Córdoba) and Garnatxa rojada (Girona). The vineyards of Cádiz and Córdoba are in the South of Spain. Cádiz's warm southern climate is significantly influenced by the Atlantic Ocean. In the vineyard of Córdoba, the climate is hot and semi-arid, in the continental style. The vineyards of Girona are in the Northeast of Spain. Their climate is Mediterranean, with the influences of wet winds from the south and cold winds from the north.

In every vineyard, from August to September 2003, samples were taken at three different times, coinciding with the following developmental stages of the grape: veraison, harvesting time and after overripening. At each sampling time, 10 bunches were obtained from 10 different plants located approximately along two crossing diagonals of the vineyard. Every bunch was collected in a separate paper bag. Samples were sent to the laboratory as soon as they were collected and they were analysed within 24–48 h maximum.

2.2. Mycological study

From each bunch, five berries were randomly selected and plated directly onto malt extract agar (MEA) (Pitt and Hocking, 1997) supplemented with 100 ppm of chloramphenicol and 50 ppm of streptomycin and five berries more onto dichloran rose bengal chloramphenicol agar (DRBC) (Pitt and Hocking, 1997). In total, 1200 berries were analysed. Plates were incubated at 25 °C for 7 days.

On the last day of incubation, all fungi considered to represent different species were isolated and transferred to slants and then to plates for identification. Taxonomic identification of different isolates was made using macroscopic and microscopic morphological criteria in accordance with appropriate keys. Only the *Aspergillus* spp. isolates were identified to species level (Raper and Fennell, 1965; Klich and Pitt, 1988). Mucorales and yeasts were not identified and they were considered as unique groups. The remaining molds were identified to genus level.

2.3. OTA-producing ability

Isolates belonging to *Aspergillus* spp. and *Penicillium* spp. were evaluated using a previously described HPLC screening method (Bragulat et al., 2001). Briefly, the isolates were grown on Czapek yeast extract agar (CYA) and on yeast extract sucrose agar (YES) (Pitt and Hocking, 1997) and incubated at 25 °C for 7 days. Isolates identified as *A. carbonarius* were grown on CYA for 10 days at 30 and 35 °C. From each isolate, three agar plugs were removed from different points of the colony and extracted with 0.5 ml of methanol. The extracts were filtered and injected into the HPLC. OTA detection and quantification was made by a Waters LCM1 chromatograph with a fluorescence detector Waters 2475 (excitation wavelength: 330 nm/emission wavelength: 460 nm), and with a column C18 Spherisorb S5 ODS2, 250 × 4.6 mm. Twenty microliters of each extract were applied. The mobile phase, with a flow rate of 1 ml/min, consisted of an isocratic program of 57% acetonitrile, 41% water and 2% acetic acid (Bauer and Gareis, 1987). The extracts with the same retention time as OTA (around 6.8 min), were considered positive. Confirmation was made through derivatization of OTA in its methyl-ester (Hunt et al., 1980). The detection limit of the extraction procedure and the HPLC technique was 0.02 ng OTA and the quantification limit of HPLC technique with the extraction procedure was 0.01 μg/g for this mycotoxin.

2.4. Data analysis

Data obtained were analysed statistically by means of Student's test, and χ^2 test. All statistical analyses were performed using SPSS software (version 12.0).

3. Results and discussion

3.1. Mycobiota determination

Predominant mycobiota belonged to *Aspergillus* spp., followed by *Alternaria* spp., *Penicillium* spp. and *Cladosporium* spp. Taking into account the total number of sampled grapes ($n = 1200$), these four genera were isolated from 79.7%, 13.4%, 8% and 4.4% of plated berries, respectively. Mucorales were isolated from 18.1% of berries, and yeasts from 12.8%. The frequency of occurrence of the predominant genera at each sampling time and in the total grapes plated is shown in Fig. 1. While the incidence of *Penicillium* spp. and *Cladosporium* spp. remains similar with the maturation of the berries, there is