Black aspergilli and ochratoxin A production in French vineyards

H. Bejaoui a, F. Mathieu a, P. Taillandier b, A. Lebrihi a,*

a Laboratoire de Génie Chimique UMR5503 (CNRS-INPT-UPS), ENSAT/INPT: 1, Av. de l’Agrobiopôle BP12607, Auzeville-Tolosane, 31326 Castanet-Tolosan, Cedex, France

b Laboratoire de Génie Chimique UMR5503 (CNRS-INPT-UPS), ENSIACET/INPT: 5, rue Paulin Talabot, BP 1301, 31106 Toulouse Cedex 1, France

Abstract

A survey on the occurrence on grape of black Aspergillus species and their capability to produce ochratoxin A (OTA) was conducted in France over three years (2001–2003) in 10 vineyards from four winemaking regions with different geographical locations and climatic conditions. During 2001 and 2002, from setting to harvest, the total numbers of fungal isolates were respectively 721 and 711 increasing in 2003 to reach 1035. The Aspergillus genus was essentially represented by Section Nigri (99%) and it was predominant (80%± 4.6) when compared to Penicillium (20%± 4.6).

Regardless of sampling year, 32.5% (±σ=1.26) of the fungal isolates were OTA producers and 93% (±σ=2.65) belonging to black aspergilli. The ochratoxigenic potential of the isolates and their occurrence on grapes revealed that Aspergillus carbonarius was the main OTA producer (up to 37.5 μg/g). At harvest time, the fungal population was maximal and this was the most critical period influencing OTA contamination. Grapes from Languedoc-Roussillon region were most infested with ochratoxigenic fungi and had the highest concentrations of OTA (up to 2.8 ng/g).

Keywords: Ochratoxin A; Grapes; Black aspergilli; A. carbonarius; A. niger aggregate

1. Introduction

In the last few years, ochratoxin A (OTA) has received increasing interest from both scientific communities and food committees because of its nephrotoxic (Krogh et al., 1974; Mortensen et al., 1983) teratogenic (Arora and Fröelén, 1981; Mayura et al., 1989), genotoxic (Dirheimer, 1998), immunosuppressive (Haubeck et al., 1981; Creppy et al., 1983) and carcinogenic (Boorman, 1989) properties. Its ingestion by humans, which occurs mainly through different plant-based foods and beverages, could lead to deterioration of liver or kidney function (Sweeney and Dobson, 1998).

Grapes and derived products such as dried vine fruit (MacDonald et al., 1999), grape juices and wines (Scott and Kanhere, 1995; Zimmerli and Dick, 1996; Jørgensen, 1998; Burdaspal and Legarda, 1999; Visconti et al., 1999; Otteneder and Majerus, 2000) have been reported as potentially contaminated with OTA. Provisional estimates of the Codex Alimentarius Commission, based on limited European data, suggested that red wine is the second major source of human exposure to OTA, following cereals and preceding coffee and beer (Walker, 1999). Swiss authors were the first to detect OTA in table wines collected from various European countries (Zimmerli and Dick, 1996) and red wines had higher concentrations than white ones (Otteneder and Majerus, 2000). Genera Penicillium and Aspergillus raised particular attention as the source of OTA (Varga et al., 2001). Among the Aspergillus, the Section Nigri was responsible for OTA production (Cabañes et al., 2002; Sage et al., 2002; Pechavy et al., 2003) and the species Aspergillus niger aggregate and Aspergillus carbonarius were considered to be particularly important (Abarca et al., 2001; Cabañes et al., 2002). According to different surveys grape products from the Mediterranean regions of South Europe (Burdaspal and Legarda, 1999; Battilani et al., 2003; Belli et al., 2004) and North Africa (Filali et al., 2001) were the most contaminated by OTA. In France, two preliminary studies revealed the presence of OTA in samples of grapes, musts and wines recovered from the South (Ospital et al., 1998; Sage et al., 2002).

This study was intended to assess the potential for ochratoxin A contamination of French grapes. It was done on a large sampling pattern of grapes recovered from different French vineyards in four winemaking regions with a variability of

* Corresponding author. Tel.: +33 5 62 193 944; fax: +33 5 62 193 901.
E-mail address: lebrihi@ensat.fr (A. Lebrihi).
climatic conditions during three years. The principle objectives of this survey were: a) screening the presence of OTA-producing species in *Penicillium* and *Aspergillus Section Nigri*, b) the dynamic of these fungi during growing season and c) determining OTA contents in grapes.

2. Materials and methods

2.1. French study area

The French mainland has almost a regular hexagonal form with 551,602 km² area. From North to South, it extends between the parallels 51°5'27″ and 42°20′ (latitude North) and from West to East between the meridians 5°56′ (longitude West) and 7°9′ (longitude East). This large sweep shelters a big variety of climatic zones.

Ten vineyards located in 4 French winemaking regions with different climatic conditions were chosen in this study (see Fig. 1): Poitou-Charentes (PC) (vineyard 1), Languedoc-Roussillon (LR) (vineyards 2 to 8), Provence-Alpes Côtes d’Azur (PACA) (vineyard 9), and Alsace (Als) (vineyard 10). Eight varieties were analysed: Ugni Blanc (UB) from PC, Riesling (R) from Als, Cinsault (CN) from PACA and 5 varieties from LR were considered: Sauvignon (SA), Muscat (Mu), Syrah (S), Carignan (CA) and Grenache (G). For the Syrah variety, three areas were considered (a, b and c). All the varieties analysed except Ugni Blanc, Riesling and Muscat were red vines.

2.2. Samples collection

Samples were taken during three growth stages: green berry, early veraison and ripe berry (harvest time). For each vineyard and each stage, 10 bunches were collected by following the two diagonals. After their rapid transport to the laboratory in cool boxes, mycological analyses were immediately done. The remaining samples were frozen at −20 °C for subsequent analysis.

2.3. Mycological analysis of grapes

Five randomly chosen berries from each bunch were put onto the surface of the DRBC (Dichloran Rose Bengale Chloramphenicol) agar (Oxoid, Basingstoke, Hampshire, England) (Cahagnier, 1998) and plates incubated at 25 °C for 7 days. Samples were examined daily with a stereomicroscope and all *Aspergillus* and *Penicillium* species isolated on CZ (CZAPEK Agar, Oxoid) (Cahagnier, 1998) medium and purified on CYA (CZAPEK Yeast extract Agar, Oxoid). All fungi isolated were identified by morphological characters according to the most accepted criteria of classification (Raper and Fennell, 1965; Klich and Pitt, 1988; Pitt and Hocking, 1997).

2.4. Ochratoxigenic ability of the isolates

OTA production ability of 2467 isolates was tested on CYA using the method of Bragulat et al. (1998). OTA was analysed by HPLC and ochratoxigenic potential was expressed as µg g⁻¹ CYA. Eighty-six isolates were deposited with an ITEM and IMI accession number in the collection of the Institute of Sciences of Food Production, CNR, Bari (http://www.ispa.cnr.it/Collection) and CABI Bioscience Bakeham Lane, Egham, respectively.

2.5. Ochratoxin A analysis in grapes

Each year, at harvest time, the 10 samples recovered from each vineyard were weighed and crushed for 2 min at room temperature. The juice was recovered after centrifugation and its exact volume was measured. Ten milliliters were adjusted to pH 7.8 with KOH 2 M and diluted to make a final volume of 20 ml with PBS buffer (r-Biopharm, St Didier Au Mont D’Or, France). The diluted sample was loaded onto an OchraPrep (r-Biopharm, St Didier Au Mont D’Or, France) immunoaffinity column operating at a steady flow rate of 2 ml min⁻¹. The immunoaffinity column was washed with 20 ml of sterile distilled water, and then dried with an air stream. OTA was eluted by applying successively 1.5 ml of methanol/acetic acid (98:2) and 1.5 ml of sterile distilled water. The eluted extract was further analysed by HPLC and OTA concentrations expressed in ng/g grapes.

2.6. High performance liquid chromatography (HPLC)

OTA was detected and quantified by reversed-phase HPLC. The analysis was performed using a BIO-TEK HPLC System (Milan, Italy) equipped with a solvent delivery system 525, column thermostat 582, autosampler 465, diode array detector 545V, acquisition data Kroma System KS3000 and Spectra System FL3000 fluorescence detector (λ_ex=332 nm; λ_em=466 nm). The analytical column was a 150×4.6 mm, 5 μm, C18 reversed-phase ODB Uptisphere fitted with a guard column (10×4 mm) having the same stationary phase. During analysis, the column was maintained at 30 °C, and OTA was eluted with a mobile phase consisting of a mixture of HPLC grade acetic acid in water 0.2% (A) and acetonitrile (B) at a flow
### Table 1

**Table 1 (continued)**

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<th>Isolate No</th>
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*ITEM = Institute of Sciences of Food Production, CNR, Bari and IMI= CABI Bioscience Bakeham Lane, Egham, respectively.

ND = not detected.

Ochratoxin A was identified by its retention time according to a standard (Sigma Aldrich, Steinheim, Germany) and quantified by measuring peak area according to a standard curve. The detection limit was 0.025 μg/1 μg l⁻¹. All analysis were done in triplicate.

### 3. Results

#### 3.1. Total fungi isolates

During three years, genera of penicillia and aspergilli on wine French grapes were systematically isolated, counted and identified. In 2001 and 2002, the total fungal isolates were respectively 721 and 711 increasing in 2003 to reach 1035. Penicillia represented only 20% (±4.6) and the species included *Penicillium expansum*, *Penicillium spinulosum*, *Penicillium glabrum*, *Penicillium crustosum*, *Penicillium brevicompectum*, *Penicillium oxalicum*, *Penicillium citrinum*, *Penicillium gladiola* and *Penicillium admetzoides*.

The *Aspergillus* genus was predominant (80% ±4.6) and especially those belonging to the Section *Nigri* (Black aspergilli) (99%). Among the remaining aspergilli (1%), isolates of
Aspergillus flavus, Aspergillus parasiticus and Aspergillus fumigatus were sometimes found. Black aspergilli were represented by three populations: A. carbonarius, A. japonicus and A. niger aggregate. A. carbonarius was microscopically recognised and distinguished by conidial size and ornamentation. All the other black biseriate aspergilli isolates will be referred as A. niger aggregate. Of the total black aspergilli isolated, A. japonicus was in the minority, representing 16%, 8% and 2% respectively in 2001, 2002 and 2003. From 2001 to 2003, A. niger aggregate increased (36%, 56% and 72%) whereas A. carbonarius decreased (48%, 36% and 26%).

3.2. Ochratoxigenic fungal isolates

For all isolates, the capacity for producing ochratoxin A was determined on a solid laboratory medium (CYA) after 7 days at 25 °C. Whatever the sampling year, 32.5% (±1.26) of the total fungal isolates were ochratoxigenic. The OTA producing capacity of the isolates are represented in Table 1.

Over the three years a total number of 1974 black aspergilli were isolated. 745 of them were found to produce OTA (i.e. 37% regardless of the year). Whatever the year, among 148 A. japonicus tested, OTA producers never exceeded 20% (up to 0.01 μg/g). No strain deposited in international culture produced detectable amount of OTA. For 691 isolates of A. carbonarius, OTA producers represented respectively 72% and 80% in 2003 and 2001 and reached 100% in 2002, (up to 37.5 μg/g ). Among 1102 of A. niger aggregate, ochratoxigenic isolates never exceeded 20% (up to 0.1 μg/g).

3.3. Fungi population evolution

3.3.1. During the growing season

Temporal changes in total and ochratoxigenic black aspergilli during the growing season are shown in Fig. 2. At the green berry stage, no OTA producer isolates were found regardless of year. In early veraison, a few OTA producer fungi were recovered and predominantly A. carbonarius. At harvest time, in all three sampling years, fungal contamination highly increased. About 66% (±11) of the total isolates and 90% (±3.1) of the ochratoxigenic ones were isolated. At this time, 96% (±0.5) of these ochratoxigenic isolates were black aspergilli. A. carbonarius species were 98% (±0.67) in 2001 and 2002 and 48% in 2003. Ochratoxigenic A. niger aggregates were only found in 2003 and reached 50% of the total ochratoxigenic black aspergilli isolates.

3.3.2. Regional variations and varieties

Eight grape varieties from four winemaking regions were analysed. The most fungal isolates were from the Languedoc-Roussillon region (ranged between 91 and 132), followed by CN from Provence-Alpes Côte d’Azur (between 17 and 56), R from Alsace (between 0 and 56) and finally UB from Poitou-Charentes (between 2 and 17) (Fig. 3).

No ochratoxigenic fungi were isolated on the UB variety and only three on Riesling one. On those two grapes varieties OTA was never detected.

On CN variety, no ochratoxigenic isolates were found in 2001, 3 A. carbonarius in 2002 and 13 A. niger aggregates in
2003. OTA content in this variety was about 0.18 ppb in 2002 and 0.11 ppb in 2003.

On Languedoc-Roussillon varieties, ochratoxigenic isolates were in majority represented by *A. carbonarius* (98% ± 2%) in 2001 and 2002 and by 52% *A. carbonarius* and 48% *A. niger* aggregate in 2003. The highest OTA concentrations in grapes from this region were observed in 2001 in CA (0.38 ng/g), in 2002 in S (2.78 ng/g) and in 2003 in SA (0.46 ng/g) (Fig. 4).

4. Discussion

In our study, *Aspergillus* and *Penicillium* genera were isolated as they are potential OTA producers in various foodstuffs (Varga et al., 1996; Abarca et al., 1997; Sweeny and Dobson, 1998). In grapes, the predominance of *Aspergillus* compared to *Penicillium* was observed among total (80% versus 20%) and ochratoxigenic (93% versus 7%) isolates. It is known that *Penicillium* species appear more often in temperate and cold climates such as in northern Europe; whereas *Aspergillus* species are commonly associated with warmer and tropical regions (Pitt and Hocking, 1997; Pitt, 1998; Frisvad et al., 1999). Creppy et al. (1991) also outlined that ochratoxicosis in France was connected to aspergilli whereas in Germany or Scandinavia it could be linked to penicillia. Although commonly reported in cereal and cereal products as principle OTA producers (Pitt, 1987; Frisvad, 1989; Frisvad and Filtenborg, 1990), *Penicillium verrucosum* and *Aspergillus ochraceus* have never been isolated on French grapes. This observation was already demonstrated for grapes from south of France (Sage et al., 2002) and from Argentina (Da Rocha Rosa et al., 2002). *P. verrucosum* has never been mentioned as part of the normal microbiota of grapes (Cabañes et al., 2002; Sage et al., 2002; Serra et al., 2003; Da Rocha Rosa et al., 2002), whereas, *A. ochraceus* was already isolated among *Aspergillus* genus, on Brazilian (6%) (Da Rocha Rosa et al., 2002), Spanish (2.5%) (Belli et al., 2004), Portuguese (<1%) (Serra et al., 2003) and Italian (<0.6%) (Battilani et al., 2003) berries.

In our study, among *Aspergillus* species, the black aspergilli were the most common (99%). This group was also predominant on Italian (97%) (Battilani et al., 2003), Spanish (97%) (Belli et al., 2004) and Portuguese (90%) (Serra et al., 2003) grapes. In 2003, the total fungal isolates highly increased (+44.5%) principally due to the increase in *A. niger* aggregate. Their highest growth rate and their tolerance to high temperatures (higher than 37 °C) (Battilani et al., 2003), registered in weather stations in 2003, could explain their tendency to increase.

According to our study, the lower the number of *A. japonicus* species, the fewer ochratoxigenic isolates recovered and their low OTA-producing potential showed that *A. japonicus* was unimportant in OTA French grape contamination.

The number of *A. carbonarius* (230±26/yr), the high frequency of ochratoxigenic isolates (between 70% and 100%) and their potential to produce OTA show that this species is the most OTA producer on grapes in France. Previous studies showed its invasive character to colonise and penetrate berries even without skin damage (Battilani and Pietri, 2002). *A. carbonarius* also reported as the main OTA producer in Spanish (Abarca et al., 2001; Cabañes et al., 2001), Portuguese (Serra et al., 2003) and Italian grapes (Battilani et al., 2003). Its ochratoxigenic potential was not only occasioned by its intrinsic toxigenic character but also due to its aggressiveness.

The consistent presence of *A. niger* aggregate on grapes during the growing season and their important number compared to *A. carbonarius* could explain their importance. However, the lower frequency of ochratoxigenic *A. niger* aggregate (20%) and their lower ability to produce OTA compared to *A. carbonarius*, makes them of second importance in OTA contamination. According to the literature 4%, 16.6% and 30% of *A. niger* aggregate isolated respectively on Portuguese (Serra et al., 2003), Argentinean and Brazilian (Da Rocha Rosa et al., 2002) grapes were potential OTA producers.

During the growing season, the harvest time was considered as a critical period for fungal development. This was also reported on Portuguese grapes (Serra et al., 2003) and on Spanish berries (Belli et al., 2004). Acidity decrease, sugar accumulation and cuticle embrittlement in grapes may explain fungal abundance. At this time, about 90% of ochratoxigenic isolates were from ripe
berries and 92% were <i>A. carbonarius</i>. This has also been observed in Portuguese vineyards (Serra et al., 2003).

According to regions, in the western (Poitou-Charentes) or eastern (Alsace) vineyards, limited fungal population was found. Over the three years, <i>A. carbonarius</i> was absent. Ochratoxigenic fungi isolated in those regions were also not relevant and only 3 <i>A. niger</i> aggregate from Alsace have been confirmed as OTA producers. Moreover, very low concentrations of OTA were measured in grapes from Poitou-Charentes (oceanic climate) and Alsace (continental climate).

French Mediterranean southern vineyards (LR and PACA) had a large fungal flora, especially of black aspergilli. These fungi were reported to be resistant to high sun exposure and to very hot (frequently achieving temperatures of 40 °C during summer time) and dry environments with low rainfall levels characterising this climate (Serra et al., 2003). The presence of OTA on grapes from this area and the important ochratoxigenic isolates confirmed the high contamination in the Mediterranean region. High levels of OTA in wines were previously reported in South Europe (Battaglia et al., 1996; Zimmerli and Dick, 1996) and especially in Mediterranean areas (Zimmerli and Dick, 1996). However samples from PACA were less contaminated by ochratoxigenic fungi than those from LR and presented less OTA concentrations. This difference could not only be due to species involved on grapes but also to variety, soil and growing area.

In conclusion, <i>A. carbonarius</i> followed by <i>A. niger</i> aggregate play a major role in OTA contamination of French grapes. Harvest time is the critical time where almost all ochratoxigenic fungi were detected and the highest amounts of OTA were measured in grapes. Southern Mediterranean regions and especially Languedoc-Roussillon were the most contaminated with OTA.

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