Black aspergilli and ochratoxin A production in French vineyards

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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% ($\pm\sigma = 1.26$) of the fungal isolates were OTA producers and 93% ($\pm\sigma = 2.65$) 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 (Sweeny 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

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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 km^2 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.

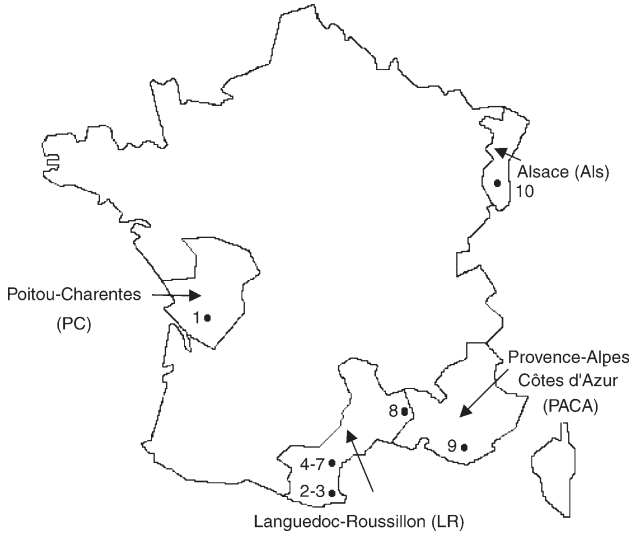

Fig. 1. Location of the regions and vineyards studied in France. (Each number represented one vineyard).

2.3. Mycological analysis of grapes

Five randomly choosen 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 milliliter 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
Ochratoxin A production by *Aspergillus* Section *Nigri* deposited isolates in international collections

Isolate N°	Identification	IMI N°	ITEM N°	OTA production (μg/g CYA)
A2215	*Aspergillus japonicus*	389196	5322	ND
A411	*A. japonicus*	389195	5321	ND
AX35	*A. japonicus*	389197	5323	ND
B111	*A. japonicus*	*389198*	5325	ND
B712	*A. japonicus*	389199	5326	ND
C518	*A. japonicus*	389201	5328	ND
C513	*A. japonicus*	389200	5327	ND
CA122	*A. japonicus*	389206	5333	ND
CA223	*A. japonicus*	389207	5334	ND
G132	*A. japonicus*	389213	5339	ND
G221	*A. japonicus*	389210	5336	ND
G231	*A. japonicus*	388506	4814	ND
CN311	*A. japonicus*	389205	5332	ND
G522	*A. japonicus*	389211	5337	ND
CA924	*A. japonicus*	389208	5335	ND
G921	*A. japonicus*	389212	5338	ND
G936	*A. japonicus*	389214	5340	ND
Mu212	*A. japonicus*	389202	5329	ND
Mu541	*A. japonicus*	389204	5331	ND
SA411	*A. japonicus*	389209	5324	ND
CA924	*A. japonicus*	389208	5335	ND
Mu412	*A. japonicus*	389203	5330	ND
2A215	*A. japonicus*	390080		ND
2G221	*A. japonicus*	390083		ND
2G621	*A. japonicus*	390082		ND
2SA 511	*A. japonicus*	390081		ND
C133	*Aspergillus carbonarius*	388568	4781	13,0
C134	*A. carbonarius*	388490	4796	10,6
C135	*A. carbonarius*	388569	4782	17
C232	*A. carbonarius*	388478	4791	3,5
C234	*A. carbonarius*	388488	4797	8,2
C235	*A. carbonarius*	388458	4810	1,2
C336	*A. carbonarius*	388479	4792	3,5
C338	*A. carbonarius*	388459	4811	ND
C536	*A. carbonarius*	388570	4783	10,9
C633	*A. carbonarius*	388486	4809	ND
C637	*A. carbonarius*	388480	4793	6,8
C933	*A. carbonarius*	388484	4798	4,0
CA332	*A. carbonarius*	388572	4789	25,3
CA532	*A. carbonarius*	388496	4805	26,0
G731	*A. carbonarius*	388573	4790	2,8
G732	*A. carbonarius*	388494	4806	2,6
G738	*A. carbonarius*	388495	4807	37,5
Mu141	*A. carbonarius*	388574	4785	4,0
Mu245	*A. carbonarius*	388489		ND
Mu247	*A. carbonarius*	388481	4794	8,8
Mu441	*A. carbonarius*	388575	4786	15,0
SA332	*A. carbonarius*	388497	4799	25,0
Mu543	*A. carbonarius*	388491	4801	6,0
Mu642	*A. carbonarius*	388576	4787	13,2
Mu644	*A. carbonarius*	388492	4802	14,0
Mu649	*A. carbonarius*	388577	4788	9,4
Mu7412	*A. carbonarius*	388485	4804	7,4
Mu746	*A. carbonarius*	388493	4803	10,9
MuX42	*A. carbonarius*	388482	4795	6,5
SA436	*A. carbonarius*	388500	4808	ND
SA636	*A. carbonarius*	388571	4784	1,6
2A235	*A. carbonarius*	390090		15,0
2B437	*A. carbonarius*	390093		3,6
2C236	*A. carbonarius*	390094		1,8
2CA332	*A. carbonarius*	390092		4,9
2MU531	*A. carbonarius*	390091		2,0
C535	*Aspergillus niger*	388560	4815	0,1
CA932	*A. niger*	388483	4830	ND
CaX34	*A. niger*	388505	4828	ND
CAX38	*A. niger*	388566	4821	ND
CX33	*A. niger*	388499	4824	0,1
G431	*A. niger*	388507	4829	ND
GX312	*A. niger*	388567	4822	ND
Mu143	*A. niger*	388561	4816	ND
Mu148	*A. niger*	388502	4826	ND
Mu246	*A. niger*	388487	4817	ND
Mu249	*A. niger*	38562	4818	ND
Mu343	*A. niger*	388563	4819	ND
Mu747	*A. niger*	388564	4831	ND
Mu848	*A. niger*	388503	4832	ND
Mu943	*A. niger*	388504	4827	ND
MuX44	*A. niger*	388565	4820	ND
SA731	*A. niger*	388501	4825	ND
2A138	*A. niger*	390084		ND
2CA331	*A. niger*	390089		ND
2CA432	*A. niger*	390087		ND
2CN331	*A. niger*	390088		ND
2MU135	*A. niger*	390086		ND
2SA331	*A. niger*	390085		ND

*ITEM = Institute of Sciences of Food Production, CNR, Bari and IMI= CABI Bioscience Bakeham Lane, Egham, respectively.
ND = not detected.

rate of 1 ml min^{-1}. Analysis of OTA in grape juices was done over 45 min with a linear gradient from 10% to 50% of *B* over the first 30 min followed by a linear gradient to 90% of *B* from 30 to 35 min, then a steady flow of 90% of *B* for 8 min finally reduced to 10% for 2 min. Analysis of OTA produced by grape isolates was done using a run time of 20 min and an isocratic method [*A*(59%) − *B*(41%)]. 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 brevicompactum*, *Penicillium oxalicum*, *Penicillium citrinum*, *Penicillium glandicola* and *Penicillium adametzoides*.

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

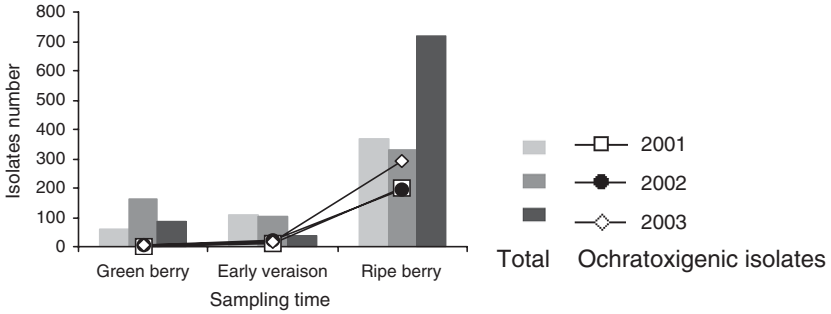

Fig. 2. Evolution of total and ochratoxigenic isolates of black aspergilli during growing season.

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

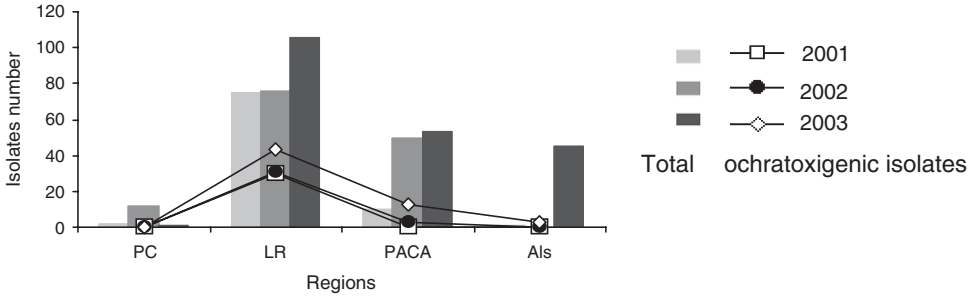

Fig. 3. Distribution of total and ochratoxigenic black aspergilli isolates in different regions during 2001, 2002 and 2003 (fungi isolates number represented in LR are mean values calculated on 7 vineyards).

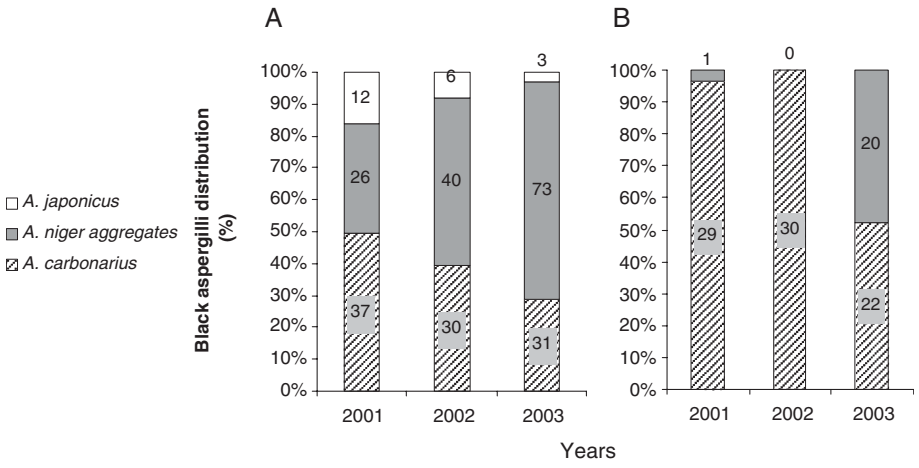

Fig. 4. Black aspergilli species distribution among total (A) and ochratoxigenic (B) isolates in Languedoc-Roussillon region and during 2001, 2002 and 2003. (Values mentioned on the graphics are average isolate numbers calculated on 7 vineyards of LR region).

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; Pittet, 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 *A. carbonarius*. 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, *A. carbonarius* was absent. Ochratoxigenic fungi isolated in those regions were also not relevant and only 3 *A. niger* 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, *A. carbonarius* followed by *A. niger* 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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