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Black *Aspergillus* species as ochratoxin A producers in Portuguese wine grapes

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Abstract

To evaluate the incidence of fungi producing ochratoxin A (OA) in Portuguese wine grapes, a survey was conducted in 11 vineyards, from four winemaking regions each with distinct climatic conditions. From setting to the harvesting period, a total of 1650 berries were sampled by plating methods. Out of 370 aspergilli and 301 *Penicillium* strains isolated, 14% of the aspergilli were OA-producing strains. None of the penicillia were OA-producing strains. The black aspergilli were predominant (90%). All *Aspergillus* strains were tested in vitro for OA production and all were preserved in the Micoteca da Universidade do Minho (MUM) culture collection. Most of the *Aspergillus carbonarius* (97%) and 4% of the *Aspergillus niger* aggregate strains were OA producers. Almost all ochratoxigenic strains were isolated at harvest time, mainly in the regions with a Mediterranean climate. In the vineyards sampled, the percentage of colonized berries with ochratoxigenic strains was up to 38%. The vineyards from the region with Atlantic influences, with high rainfall, exhibited the lowest occurrence of *Aspergillus* and ochratoxigenic strains, 0% to 10% and 0% to 2% colonized berries, respectively. Data obtained here supports the hypothesis that *A. carbonarius* and occasionally *A. niger*, are the main producers of OA in grapes. In this study, the highest incidence of these fungi occurred in vineyards with a Mediterranean climate.

Keywords: *A. carbonarius*; Black aspergilli; Grapes; Ochratoxin A; Wine

1. Introduction

Ochratoxin A (OA) is a mycotoxin with nephrotoxic, nephrocarcinogenic, teratogenic and immunosuppressive properties, which has received growing interest from the scientific community and food committees in the last few years (Battaglia et al., 1996; Walker, 1999). It has been detected in different kinds of foods and beverages, including grape juice and wine, where it was reported for the first time by Zimmerli and Dick (1995). Since then, surveys conducted in different countries have revealed the presence of OA in these foodstuffs (Burdaspal and Legarda, 1999; Pietri et al., 2001; Sage et al., 2002). The only reported species capable of producing OA belong to the genera *Aspergillus* and *Penicillium*. Some species of black aspergilli (*Aspergillus niger* group, Raper and Fennell, 1965; *Aspergillus* section *Nigri*, Gams et al., 1985) have been described as capable of producing OA (Abarca et al., 1994; Téren et al., 1996). These species are commonly

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present in the vineyards and have the ability to cause rot in berries, known as *Aspergillus* rot (Snowdon, 1990). Among the species of this group, *Aspergillus carbonarius* shows the highest ochratoxigenic potential, with most of the isolates having the ability to produce OA in media (Heenan et al., 1998). It has been proposed that *A. carbonarius* is the fungus responsible for OA production in grapes (Pitt, 2000; Cabañes et al., 2002). A survey conducted in the year 2000 in a Portuguese winemaking region, Vinhos Verdes, did not reveal the presence of this species, and the only ochratoxigenic strain found belonged to the *A. niger* aggregate (Serra et al., 2001). Nevertheless, Portugal shows a considerable climatic diversity between regions. The Portuguese climate is dominated by Atlantic and Mediterranean influences. Thirty-two winemaking regions are defined, spread across the whole country. They possess distinct climatic conditions, which determine the grape varieties cultivated and influence the wine properties. The presence of OA-producing fungi in the vineyards may lead to contamination of grapes with OA before harvest. In order to investigate the main ochratoxigenic species present in grapes, four winemaking regions were studied.

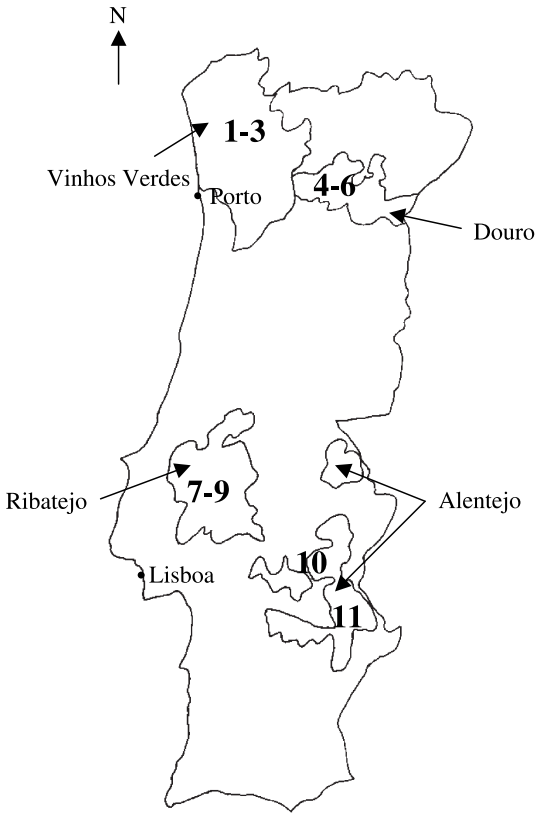

Fig. 1. Location of the regions and vineyards studied in Portugal.

2. Materials and methods

2.1. Study area

Four winemaking regions were chosen for this study, based on their climatic differences and national economical importance: Vinhos Verdes (vineyards 1 to 3), Douro (4 to 6), Ribatejo (7 to 9) and Alentejo (10 and 11). A total of 11 vineyards were studied, distributed as indicated in Fig. 1. The Portuguese mainland has a rectangular form with a North–South direction with 88,607 km^2 area and is located between the parallels 36°57′39″ and 42°9′8″ (latitude North) and the meridians 6°11′10″ and 9°22′5″ (longitude West).

2.2. Sample collection

Grapes were collected from July to September 2001, at three developmental stages of the berries—green berry, early veraison and ripe berry (harvest time). At each sampling time in each vineyard, 10 bunches were collected along two crossing diagonal transects. The samples were taken to the laboratory in closed paper bags, transported in cooled boxes, and analysed in the shortest time possible, usually within 6 h, with those collected from further distances analysed within 24 h of collection.

2.3. Mycological analysis of the grapes

From each bunch, five berries were randomly selected, cut in half and aseptically plated on DRBC medium (Oxoid). Therefore at each sampling period, a total of 50 berries were collected per vineyard. Additionally, a whole bunch was pressed against a plate of the same medium, in order to compare the fungal species isolated using both techniques. The plates were incubated for 7 days at 25 °C. From the second day of incubation, the plates were monitored in the stereomicroscope for the presence of *Aspergillus* and *Penicillium* species. The aspergilli and penicillia

present in each grape were re-isolated onto CYA containing yeast extract (Difco) and MEA (Blakeslee formula) media, respectively (Pitt, 1979). The identification of *Penicillium* was made according to Pitt (1979) and that of *Aspergillus* according to Raper and Fennell (1965).

2.4. Preservation of the isolated strains

All the aspergilli and representative strains of the penicillia found in this study were preserved by a suspension of spores in glycerol solution at 10% and maintained at −80 °C. The strains were deposited in the Micoteca da Universidade do Minho (MUM) culture collection (Santos and Lima, 2001).

2.5. Ochratoxigenic ability of the isolates

The strains were tested for OA production in the CYA plate where they were isolated, according to Bragulat et al. (2001): after 7 days growth, three agar plugs were removed from a colony, and placed into a 4 ml amber vial, and 500 μl of methanol were added. After 60 min, the extract was filtered and injected in the HPLC. When it was not possible to test the strains in situ (as above), they were grown on MEA from the frozen spore suspension for 4 days and then inoculated onto CYA and tested after 7 days growth.

2.6. OA detection by HPLC

The samples were analysed using a reverse phase HPLC equipped with a Jasco FP-920 fluorescence detector (330 nm excitation wavelength; 460 nm emission wavelength). Chromatographic separations were performed on a C18 column (Waters Spherisorb ODS2, 4.6 × 250 mm, 5 μm), fitted with a precolumn with the same stationary phase. The mobile phase used was pumped at 1.0 ml/min and consisted of an isocratic programme as follows: acetonitrile/water/acetic acid (99:99:2, v/v). The injection volume was 100 μl.

The OA standard was supplied by Sigma (St. Louis, MO). Samples were taken as positive for OA presence if they yielded a peak at a retention time similar to the OA standard peak (approximately 12 min), with a height five times higher than the baseline noise.

3. Results and discussion

Red wines originating from southern Europe and North Africa, with Mediterranean climates, are more contaminated than those originating from more temperate regions of central Europe (Zimmerli and Dick, 1996; Otteneder and Majerus, 2000). The higher levels of OA in red wines were interpreted as due to differences in the processing of red compared with white wines, whilst a stronger presence of OA-producing fungi in grapes grown in the south was considered the probable cause for the higher incidence of OA in these regions.

Penicillia and aspergilli were isolated from 17% and 22% of the total sampled grapes, respectively. The species are listed in Tables 1 and 2. The main *Penicillium* species isolated were *Penicillium aurantiogriseum*, *Penicillium brevicompactum*, *Penicillium citrinum*, *Penicillium crustosum*, *Penicillium expansum*, *Penicillium simplicissimum*, *Penicillium spinulosum* and *Penicillium thomii*, constituting 86% of the total *Penicillium* strains isolated. None of the 301 penicillia strains were ochratoxigenic species. In previous studies, several *Penicillium* species have been referred to as ochratoxigenic, however, it is now accepted that *Penicillium verrucosum* is the only species of *Penicillium* capable of ochratoxin A production (Pitt, 1987; Frisvad and Filtenborg, 1990). *P. verrucosum* was not isolated in this study. Therefore, penicillia were not tested for OA production.

Black aspergilli (*A. carbonarius*, *Aspergillus japonicus*, *A. niger* aggregate) constituted 90% of the 370 *Aspergillus* strains isolated (Table 1). Except for *A. japonicus*, all these species are biseriate. Most of them were identified as belonging to the *A. niger* aggregate. *A. carbonarius* was also found, and was readily recognized. All black biseriate aspergilli strains other than *A. carbonarius* will be referred to as the *A. niger* aggregate. The percentage of ochratoxigenic strains detected is also shown in Table 2. *A. carbonarius* was the main OA producer isolated from grapes, with 97% of the strains having the ability to produce this mycotoxin, followed by the *A. niger* aggregate, in which only 4% of the tested strains showed ochratoxigenic ability by the method used. *A. ochraceus* and *A. alliaceus* also showed a high ochratoxigenic ability (Table 2). However, they constituted less than 1% of the strains isolated.

Table 1
Penicillium species isolated from grapes in the Portuguese studied vineyards

Species	Number of isolated strains
Penicillium aurantiogriseum	9
Penicillium brevicompactum	88
Penicillium chrysogenum	2
Penicillium citrinum	20
Penicillium corylophilum	4
Penicillium crustosum	10
Penicillium echinulatum	3
Penicillium expansum	7
Penicillium fellutanum	1
Penicillium funiculosum	2
Penicillium glabrum	1
Penicillium implicatum	4
Penicillium janczewskii	1
Penicillium miczynskii	2
Penicillium minioluteum	4
Penicillium oxalicum	2
Penicillium pinophilum	1
Penicillium purpurogenum	4
Penicillium raistrickii	1
Penicillium restrictum	1
Penicillium roquefortii	4
Penicillium sclerotiorum	2
Penicillium simplicissimum	13
Penicillium spinulosum	47
Penicillium thomii	65
Penicillium variabile	2
Penicillium verruculosum	1
Total	301

The isolation rate for black aspergilli found in the sampled berries was 98%. The distribution of the *A. niger* aggregate and *A. carbonarius* strains isolated during the development of the berry is given in Fig. 2. *A. niger* aggregate strains were present at all three sampling times, increasing their presence during the development of the berry, and achieving the highest levels (38%) at harvest time. In contrast, *A. carbonarius* was only detected at harvest time, when nearly all the ochratoxigenic strains were detected (Fig. 2). This same trend was observed for all four regions studied.

The distribution of the ochratoxigenic species found at harvest time in all vineyards studied is presented in Table 3. The incidence of black aspergilli differed among the vineyards. Black aspergilli, in the Vinhos Verdes region, were never present in more than 10% of the sampled grapes; in Ribatejo they ranged from 18% to 48%; in Douro they ranged from 20% to 92%; and in Alentejo they ranged from 72% to 100%. The latter two regions, where the highest incidence of black aspergilli in grapes was recorded, have Mediterranean climates, very hot and dry during summer time, frequently achieving temperatures around 40 °C. The incidence of black aspergilli is apparently related to the climatic conditions of each

Table 2
Aspergillus strains isolated from grapes in the Portuguese studied vineyards and the percentage of OA-producing strains of each species

Species	Number of isolated strains	Ochratoxigenic strains (%)
Aspergillus alliaceus	1	100
Aspergillus carbonarius	39	97
Aspergillus clavatus	1	0
Aspergillus flavus	11	0
Aspergillus fumigatus	5	0
Aspergillus japonicus	1	0
Aspergillus niger aggregate	294	4
Aspergillus ochraceus	2	50
Aspergillus terreus	3	0
Aspergillus ustus	4	0
Aspergillus versicolor	5	0
Aspergillus wentii	4	0
Total aspergilli isolated	370	14

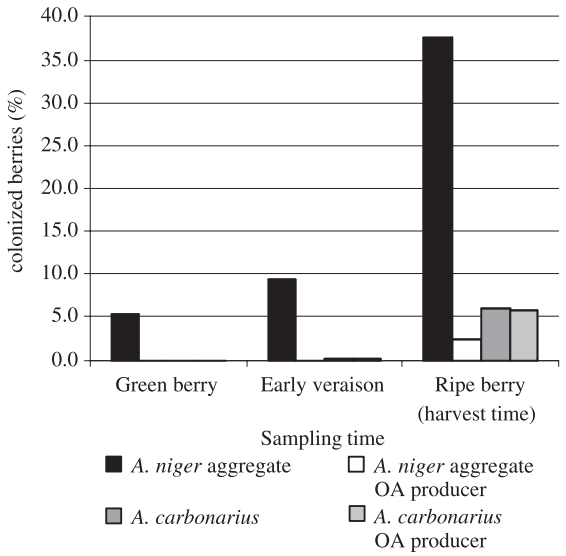

Fig. 2. Comparison between berries colonized with *A. niger* aggregate, *A. carbonarius* and respective ochratoxin A (OA)-producing strains, in the three sampling times studied.

Table 3
Fungal incidence overall and OA-producing biseriate black *Aspergillus* strains in grapes from the Portuguese studied vineyards, at harvest time

Vineyard	Colonized berries (%) overall		Colonized berries (%) with OA producing	
	A. carbonarius	*A. niger* aggregate	*A. carbonarius*	*A. niger* aggregate
1	ND	ND	–	–
2	ND	ND	–	–
3	2	8	2	0
4	8	56	8	0
5	ND	92	–	20
6	ND	16	–	2
7	12	26	12	0
8	2	46	2	2
9	ND	18	–	0
10	40	32	38	2
11	2	100	2	0

ND—not detected.

region. The Alentejo region has the highest values of sun exposure in the summer compared with the other regions studied. Ribatejo region also has Mediterranean influences, but has higher humidity levels than those of Douro and Alentejo. The region of Vinhos Verdes, located in the northwest of Portugal, has distinct climatic conditions. It is dominated by an Atlantic influence, and the temperatures in the summer are usually more moderate. It has the lowest sun levels of all the regions studied, and it is also more humid. Black aspergilli are very resistant to sun exposure and to hot and dry environments. They are adapted to the conditions in the vineyards with Mediterranean climates, constituting a marked presence in the grapes of these locations. The highest incidence of ochratoxigenic fungi observed was in vineyard 10, in Alentejo region, where 38% of the grapes were colonized with these fungi. In Douro and Ribatejo regions, the maximum incidence of grapes colonized with OA-producing strains in the vineyards did not exceed 20% and 12% of the sampled grapes, respectively. *A. carbonarius* is the major ochratoxin A-producing species found in the vineyards of southern Portugal, but in Douro region, *A. niger* aggregate strains were the dominant OA producers. *A. carbonarius* was also found in this region, in vineyard 4. It is worth noting that this vineyard was one with grapes in a very poor condition, due to an infection with powdery mildew. The highest incidence of OA-producing strains belonging to *A. niger* aggregate were found in vineyard 5.

It is only recently that studies on OA formation in grapes have been conducted, and few data are available. However, comparing the incidence of ochratoxigenic fungi in Portuguese grapes grown in distinct climates with the pattern of OA distribution in wines, Portuguese data supports the hypothesis that black aspergilli, in particular *A. niger* aggregate and *A. carbonarius*, are the fungal species responsible for OA presence in grapes and wine from winegrowing areas with warm and dry conditions.

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