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ORIGINAL ARTICLE

Survey of mycobiota, black *Aspergillus* and ochratoxin A occurrence on Brazilian wine grapes

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Abstract The southern state of Brazil is responsible for 90 % of the wine produced in the country; however, the mycobiota and the occurrence of ochratoxin A (OTA) on wine grapes produced in this region has never been fully characterized. To evaluate the fungal and OTA contamination in wine grapes of two varieties cultivated in southern Brazil, a survey was conducted in three regions with slightly different climates. From each region, samples were obtained at early ripeness and at harvest stage. Eight genera were isolated; *Alternaria* and *Trichoderma* were the most prevalent. Within the *Aspergillus* genus, the section *Nigri* was predominant. One uniseriate black *Aspergillus* was able to produce OTA under the conditions tested but no detectable levels of OTA were found in the grape samples. The low overall incidence of black *Aspergillus* and the absence of OTA on the grapes may be explained by the meteorological conditions at the analyzed regions, and characterizes a high quality of this product regarding mycotoxigenic contamination. This is the first study to fully describe the mycological biota of two wine grape varieties produced at three different regions of Rio Grande do Sul state.

Keywords *Alternaria* · Black *Aspergillus* · Wine grapes · Ochratoxin A · Brazil · Uniseriate black *Aspergillus*

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Introduction

Viticulture is an important activity in many countries. In Brazil, the economic importance of viticulture is increasing every year. Rio Grande do Sul, the southern state of Brazil, is the main producer, responsible for almost 90 % of the wines in the country (Loiva 2010).

Contamination of grapes with molds can generate significant economic losses, reducing the productivity and quality of these products. Fungi may change the chemical composition of grapes, altering wine flavor and color (Fleet 2002; Magnoli et al. 2003). A variety of fungal genera, mainly *Botrytis*, *Alternaria*, *Aspergillus*, *Penicillium* and *Cladosporium*, can contribute to grape spoilage before harvest (Magnoli et al. 2003; Medina et al. 2005; Belli et al. 2006). *Aspergillus* section *Nigri* (black *Aspergillus*) can be highlighted due to its high frequency as a grape spoiler, and also as it has the ability to produce different mycotoxins, mainly ochratoxin A (OTA) (Medina et al. 2005; Belli et al. 2006; Logrieco et al. 2011).

OTA is a mycotoxin with nephrotoxic, teratogenic, immunosuppressive and carcinogenic properties (Bondy and Armstrong 1998; Creppy et al. 1991; Mateo et al. 2007) and has been classified as Group 2B by the International Agency for Research on Cancer as a possible human carcinogen (WHO 1993). It is also associated with Balkan Endemic Nephropathy and iron deficiency anemia (Smith and Solomons 1994; Abouzied et al. 2002). This mycotoxin has been detected in grape must and wine products worldwide, and the black *Aspergillus* are the main responsible for the contamination, in particular, *Aspergillus carbonarius* and species belonging to *Aspergillus niger* aggregate (Battilani et al. 2003, 2006; Khoury et al. 2006; Ponsone et al. 2010; Teixeira et al. 2011; Spadaro et al. 2012; Chiotta et al. 2013).

The occurrence of *Aspergillus* section *Nigri* in grapes is determined by many factors, including vineyard humidity

and temperature, grape variety, and chemical fungicides, and it is known that its frequency increases from setting to harvest (Bau et al. 2005; Medina et al. 2005). *Aspergillus* section *Nigri* has been isolated in high frequencies, mainly in Europe, but also in South American countries like Argentina, Chile and Brazil (Battilani et al. 2001; Rosa et al. 2002; Bau et al. 2005; Bejaoui et al. 2006; Diaz et al. 2009; Chiotta et al. 2009). In Brazil, a few studies have been conducted in order to characterize grape quality. Rosa et al. (2002) were the first to report the occurrence of ochratoxigenic fungi on Brazilian grapes. Data on fungal contamination are important to characterize product quality and assure consumer safety. The aim of this study was to characterize the fungal and OTA contamination of two varieties of wine grapes cultivated in three different regions of Rio Grande do Sul state during two maturation stages.

Material and methods

Grape sampling

Grape samples of Cabernet Sauvignon and Merlot varieties were obtained in three vineyards with slightly different climate conditions : (1) Serra do Nordeste located in the northeast; (2) Serra do Sudeste, located in the southeast; and (3) Campanha, located in the southwest of the state of Rio Grande do Sul, which is a southern state in Brazil. The climatic characteristics of the three regions are summarized in Table 1. Samples were collected at different stages: (A) early ripeness and (B) harvest. The sampling was conducted at the 2011 vintage, between January and March of that year. At each sampling time, two diagonals crossing the vineyards were delimited, and five healthy bunches from each diagonal were obtained. Each bunch was collected in a sterilized plastic bag and sent to the laboratory under refrigerated conditions to be analyzed.

Mycological analysis

Ten berries were selected randomly from each bunch (totaling 1200 berries), surface disinfected with 0.01 % sodium hypochlorite for 1 min, and inoculated directly into the surface of Dichloran Rose Bengal Chloramphenicol (DRBC) agar and malt extract agar (MEA) supplemented with chloramphenicol (Pitt and Hocking 2009). Plates were incubated at 25 °C for 7 days in darkness.

After incubation, all colonies from different genera and within *Aspergillus* section *Nigri* morphology were isolated to Czapek Yeast Extract agar (CYA) and incubated at 25 °C for 7 days in darkness. Genera identification was conducted according to microscopic and macroscopic criteria using the keys of Pitt and Hocking (2009) and Pitt et al. (2009). The *Aspergillus* colonies were identified to species level also according to micro and macroscopic criteria, using the keys of Klich (2002).

Ochratoxin A production ability

OTA production was tested in 22 *Aspergillus* strains belonging to the section *Nigri*. Qualitative analysis was conducted according to the method proposed by Bragulat et al. (2001) using thin layer chromatography (TLC) with charged coupled device (CCD) (Welke et al. 2009; Teixeira et al. 2011). In this technique, the isolates were grown in CYA plates for 10 days at 30 °C. For each isolate, agar plugs were removed from central and border areas of the colony and extracted with 1 mL chloroform. Extracts were analyzed using the TLC-CCD technique.

Quantitative analysis was conducted according to the method proposed by Ponsone et al. (2007) with a few modifications, using TLC-CCD. Briefly, 10^6 spores from a suspension prepared previously were grown in 20 mL CYA medium for 10 days at 30 °C in darkness. After the incubation period, 1 mL medium was mixed with 1 mL chloroform and centrifuged at 4000 *g* for 10 min. Then, 0.5 mL of the chloroform was transferred to a clean vial, evaporated and redissolved in 0.5 mL diluent (PBS). The PBS solution employed was prepared following the VICAM manual (VICAM, St. Louis, MO). The mobile phase was added to an immunoaffinity column OchraTest (VICAM), which was cleaned up with 10 mL PBS and 10 mL distilled water. OTA was eluted with methanol, evaporated to dryness and redissolved in 100 μL chloroform and applied to the TLC plate. Plates with samples were eluted with a toluene/ethyl acetate/formic acid solution (60:30:10, v/v) and then analyzed and quantified through

Table 1 Geoclimatic characteristics of the three main wine producing regions of Rio Grande do Sul, Brazil[a]

	Altitude (m)	Annual temperature range C°)	Mean temperature C°)	Mean summer temperature C°)	Mean winter temperature C°)	Annual rainfall (mm)	Air relative humidity (%)
Serra do Nordeste	640	12.5–26	17.3	21.4	13.2	1739.3	76.2
Serra do Sudeste	410	13–25.4	19.2	23.7	14.6	1504.8	80
Campanha	208	12.7–23.5	17.8	22.8	12.4	1467.0	71.6

[a] Adapted from: Czermainski and Zat 2011; Matzenauer et al. 2011

CCD using ImageJ Software (Welke et al. 2009; Teixeira et al. 2011). The confirmation of the compound identity was conducted according to the methodology of Hunt et al. (1980), using boron trifluoride.

Ochratoxin A occurrence on grape samples

The methodology was divided into two steps: extraction and clean-up. For extraction, the collected grapes were crushed with a blender and OTA occurrence and content was determined according to the methodology proposed by Zimmerli and Dick (1996). Briefly, the blended grapes were filtered to remove particulate matter, and 5 mL of the filtrate was mixed with 10 mL aqueous solution (3.4 % orthophosphoric acid, 1.18 % sodium chloride) in a clean vial. The OTA content was extracted with 10 mL chloroform, shaken three times and transferred to a clean vial. To perform the clean-up, 10 mL chloroform extract was evaporated to dryness in a bath at 65 °C and redissolved in 5 mL PBS that was added to an immunoaffinity column OchraTest (VICAM) pre-washed with 20 mL PBS. The column was washed with 10 mL distilled water, and OTA was eluted from the column with 3 mL methanol/acetic acid (98:2). The extract was evaporated to dryness in nitrogen and redissolved in toluene:acetic acid (99:1) and applied to TLC plates. The plates with the samples were eluted in a glass chamber containing toluene/ethyl acetate/formic acid solution (60:30:10, v/v) and then analyzed with CCD, using the computer software ImageJ for Windows (https://imagej.nih.gov/ij/).

Recovery

Grape samples were contaminated with an OTA standard, at concentrations of 7.5 ng mL^{-1}, 10 ng mL^{-1} and 20 ng mL^{-1}, in triplicate. The OTA extraction and determination analysis was conducted three times according to the description above.

Statistical analysis

To compare the means of fungal incidence of the two groups (grape varieties and cultivation stage) the Mann-Whitney test was used, and to compare the means of more than two groups the Kruskal-Wallis test was conducted. These non-parametric tests were chosen due to the reduced sample size.

Results

Common mycobiota

Eight fungal genera were identified from the grape samples: *Alternaria*, *Aspergillus*, *Trichoderma*, *Nigrospora*, *Penicillium*, *Fusarium*, *Cladosporium* and *Curvularia*, highlighting the first four genera (Table 2). About 3 % of the isolated fungi did not produce conidiophores or conidia on the tested conditions, and were nominated “non-sporulated fungi”.

The number of isolates within the several genera found on grapes from different regions and varieties are shown in Table 2. In the Campanha region, 785 isolates were found, followed by Serra do Sudeste and Serra do Nordeste with 554 and 474 isolates identified, respectively.

Ochratoxigenic mycobiota

Twenty two *Aspergillus* section *Nigri* were found, distributed between the varieties, grape growing periods and geographical regions (Fig. 1). Two subgroups within the *A*. section *Nigri* were identified, uniseriate aspergilli and *Aspergillus niger* aggregate. The section *Nigri* was predominant within the *Aspergillus* genus, representing 88 % of fungi isolated from this genus. No statistical differences were found between the varieties, grape growing period or geographical regions

Table 2 Number of isolates of different fungal genera from 2011 vintage grapes from three different regions and two varieties

Fungi	Campanha		Serra do Sudeste		Serra do Nordeste	
	Cabernet Sauvignon	Merlot	Cabernet Sauvignon	Merlot	Cabernet Sauvignon	Merlot
Alternaria	386	334	174	224	243	183
Aspergillus	12	3	1	6	3	0
Cladosporium	0	0	0	0	1	7
Curvularia	0	11	0	0	0	0
Fusarium	0	0	0	0	4	0
Nigrospora	35	0	0	0	1	0
Penicillium	10	1	0	1	0	2
Trichoderma	7	14	85	63	20	10
Total	450	336	260	294	272	202

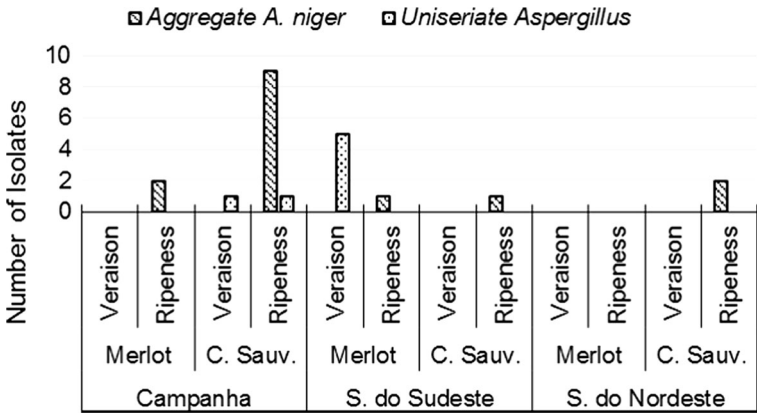

Fig. 1 Distribution of black *Aspergillus* isolates among varieties of wine grapes, growing periods and different regions of Rio Grande do Sul, Brazil

regarding the number of *Aspergillus* section *Nigri* isolated ($p > 0.05$).

Ochratoxin A producing ability

One black *Aspergillus* isolate, identified as a uniseriate *Aspergillus*, found on Serra do Sudeste at ripeness period was able to produce OTA under the conditions tested. The concentration produced was 148 ng mL^{-1}.

Ochratoxin A content on grapes

The samples used for fungal isolation were also analyzed to investigate OTA occurrence. The method recovery was 97 %, and the detection limit and quantification limit were 0.4 ng mL^{-1} and 0.8 ng mL^{-1}, respectively. No detectable levels of OTA were found for any sample.

Discussion

The predominance of *Alternaria* sp. was observed both at the beginning of the ripeness and during harvest, but the genera prevalence decreased at harvest (data not shown). This result agrees with the results of Magnoli et al. (2003), Medina et al. (2005) and Serra et al. (2006). It is known that the frequency of ambient genera decreases as the ripeness advances, being gradually substituted by degrading fungi (Serra et al. 2006). The predominance of *Alternaria* genus may indicate a toxicological hazard, since some species may produce several mycotoxins, e.g., *Alternaria alternata*, a very common food contaminant, which is potentially producer of Alternariol, tenuazonic acid, altertoxin and other less relevant mycotoxins (Ostry 2008).

The *Botrytis* genus, which is regarded as the main spoilage cause in wine grapes, was not isolated in this study, but the absence of this genus has already been reported by Magnoli et al. (2003) in Argentina, and Medina et al. (2005) in Spain.

Aspergillus was the fourth most common genera (1.3 % of all fungi). These results differ from those obtained by other authors, who reported a much higher frequency from this genus, ranging from 70 % to 95 % (Magnoli et al. 2003; Medina et al. 2005; Khoury et al. 2008). Magnoli et al. (2003), when analyzing wine grapes in Argentina in a region that is geographically very close to Rio Grande do Sul, found similar results to this study, probably due to the geographical proximity of the analyzed regions.

The *A. niger* aggregate was predominant within the *Aspergillus* section *Nigri*, representing 78 % of the isolates, which agrees with previous publications by Battilani et al. (2003), Khoury et al. (2006) and Ponsone et al. (2010). The species that belong to this aggregate are considered the main *Aspergillus* contaminant in wine grapes produced even in regions with climate conditions that are completely different to those reported for our study (Table 1), probably because these fungi are great competitors, and extremely adapted to the ecosystem present in the vineyards. The aggregate *A. niger* species represent 80–85 % of the contamination, mainly during harvest period (Visconti et al. 2008).

The occurrence of uniseriate black *Aspergillus* is also consistent with other published studies (Battilani et al. 2003; Khoury et al. 2006; Belli et al. 2006). This group is considered the third most important black *Aspergillus* species contaminating wine grapes, immediately above the *A. niger* aggregate and *A. carbonarius* (Visconti et al. 2008). Battilani et al. (2003) reported that about 23 % of the black aspergilli isolated correspond to uniseriate species, which is similar to the results of our study, where these fungi represented 32 % of the *Aspergillus* section *Nigri*. According to Visconti et al. (2008), the frequency of uniseriate black aspergilli is higher in colder regions, probably due to cold resistance adaptation, which agrees with results observed in our study. Rio Grande do Sul is located in southern Brazil, and is characterized by a temperate climate (average temperature between 15 °C and 19 °C) with a particularly harsh winter (Table 1) (SEPLAG 2012).

It is interesting to note the absence of isolates belonging to the species *A. carbonarius*, which is considered the predominant species responsible for the occurrence of OTA in wine

grapes and derivatives (Visconti et al. 2008; Khoury et al. 2008; Ponsone et al. 2010). A low occurrence of this fungus was previously reported in Argentina (Chiotta et al. 2009; Ponsone et al. 2010) and in Lebanon (Khoury et al. 2006), and the absence of this fungus was observed in cold regions, like Germany, North Hungary, Czech Republic and Portugal (Abrunhosa et al. 2001; Ostry et al. 2007; Varga et al. 2005). The occurrence of *A. carbonarius* is closely related to high temperatures not only during the growing season, but throughout the year (Battilani et al. 2006; Chiotta et al. 2009, 2013; Barberis et al. 2014). The absence of this species, like the frequency of uniseriate black aspergilli, may be attributed to the climate conditions of Rio Grande do Sul.

Although this work has identified potentially ochratoxigenic species in all regions of the state, the numbers of isolates identified was lower than those observed in other studies conducted in different countries: Belli et al. (2006) isolated about 1100 black *Aspergillus* in Spain, Khoury et al. (2008) found 487 in Lebanon, Serra et al. (2003) found 333 in Portugal, Chiotta et al. (2009) found 284 in Argentina, and Diaz et al. (2009) found 77 in Chile.

When the *Aspergillus* section *Nigri* and fungal genera contamination was compared between the regions surveyed, no significant difference was found in spite of differences in the climate conditions of the three regions regarding altitude and annual rainfall levels. According to Battilani et al. (2006), the black *Aspergillus* requires less moisture for optimal growth, and the lower frequency of these fungi in all regions may be attributed to the high rainfall rate observed during the grape-growing months, with means between 111 mm and 218 mm (Berlato and Fontana 2002; Leivas et al. 2006; INMET 2011; SEPLAG 2012; Chiotta et al. 2013).

Fourteen black aspergilli were isolated from the Cabernet Sauvignon variety and eight from Merlot. No statistical differences were found between the varieties, which is in contrast with previous studies that indicated that Cabernet Sauvignon was more susceptible to these fungi (Battilani et al. 2004; Visconti et al. 2008). According to Lasram et al. (2007), cropping factors such as training systems, irrigation and phytosanitary treatments directly influence the ecosystem of the vine and thus the fungal population. The fields where the grapes were cultivated were very close and the cropping factors for both varieties were almost the same; this proximity could be related to the similarity of our results regarding grape varieties.

One uniseriate black *Aspergillus* was able to produce OTA at a concentration of 148 ng mL^{-1} under the conditions tested. The occurrence of a uniseriate black aspergilli OTA producer is rare but was previously reported by Battilani et al. (2003) in Italy and by Dalcero et al. (2002) and Ponsone et al. (2007) in Argentina. Spadaro et al. (2012) also found a uniseriate black *Aspergillus* (*A. japonicus*) identified by ITS-RFLP method that was able to produce OTA. The ability of uniseriate black *Aspergillus* to produce OTA is a controversial idea and is not accepted by many mycologists, which suggests that this isolate may be a new species with a particular niche of growth.

OTA was not found in samples of Cabernet Sauvignon grapes cultivated in Uruguay (Chulze et al. 2006), which agrees with the present results, especially considering that Uruguay is bordered by the state of Rio Grande do Sul and has climatic conditions that are similar to those of the Brazilian state.

This is the first study to investigate fungal and OTA contamination in wine grapes produced at different regions in the southern Brazil. Considering the importance of the state in marketing noble wines, the data presented are extremely relevant to characterizing the quality of grapes and grape derivatives. The geographical location, climate and, consequently, the non-occurrence of *A. carbonarius*, probably explain the absence of OTA on the samples analyzed. The low overall occurrence of potentially toxigenic fungi and the complete absence of OTA on the analyzed samples indicate the high quality of the wine grapes produced in Brazil.

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