Mycotoxigenic fungi in Portuguese wine regions: fumonisin B_2 and ochratoxin A

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Abstract

Studies were conducted between 2001 and 2003 to evaluate the incidence of ochratoxigenic strains in Portuguese wine-grapes. At that time, the main responsible for ochratoxin A production in grapes were found to be *Aspergillus carbonarius* with a production mean of 1.13 mg/kg and some strains from the *Aspergillus niger* aggregate with a production mean of 0.14 mg/kg. At that time also, all the black aspergilli isolates were preserved in glycerol and stored at -80 ºC. Since recently *A. niger* was reported as a producer of the mycotoxin fumonisin B_2, its levels produced by black aspergilli isolates from the Portuguese grapes were investigated. Approximately 29% of *A. niger* aggregate strains were found to produce it between 0.003–6.0 mg/kg with a mean of 0.66 mg/kg and only 6% produced more than 1 mg/kg. The incidence and distribution of fumonisinogenic strains among five Portuguese wine-regions is also presented and correlated with data of the ochratoxigenic ones.

Resumo

A incidência em Portugal de fungos filamentosos produtores de ocratoxina A em uvas para a produção de vinho foi avaliada entre os anos de 2001 a 2003. Nessa altura, o produtor mais frequente nas uvas foi a espécie *Aspergillus carbonarius*, com uma produção média em laboratório de 1,13 mg/kg, e algumas estirpes do agregado *Aspergillus niger*, com uma produção média em laboratório de 0,14 mg/kg. Todas as estirpes de *Aspergillus* negros então isoladas foram conservadas a -80 ºC. Recentemente, estirpes de *Aspergillus niger* foram descritas como produtores de fumonisina B_2, pelo que a produção desta outra micotoxina foi verificada nas cerca de 700 estirpes conservadas. Aproximadamente 29% das estirpes do agregado *A. niger* produziram fumonisina B_2, com produção em laboratório entre 0,003 a 6,0 mg/kg, sendo que apenas 6% produziram mais de 1 mg/kg. A incidência e a distribuição destas estirpes micotoxigénicas será apresentada e discutida.

Introduction

Grapes are particularly susceptible to mould infections when environmental and climatic factors are favorable and application of recommended fungicides is disregarded. Among main fungi found on grapes, black aspergilli hold an increasing importance since some species of this group are known to produce mycotoxins (Nielsen *et al*., 2009). One of these mycotoxins is ochratoxin A (OTA), which is produced in grapes by *Aspergillus carbonarius* and in less extend by some *Aspergillus niger* isolates (Serra *et al*., 2003). Another one is fumonisin B_2 (FB_2), whose production by *A. niger* has been recently discovered (Frisvad *et al*., 2007).

OTA is a chlorodihydro-isocoumarin derivative linked through an amide bond to a phenylalanine molecule (Fig. 1A). OTA is nephrotoxic and a possible carcinogenic to humans (Pfohl-Leszkowicz, Manderville, 2007). FB_2 is a long-chain polyhydroxyl alkylamine containing

two propane tricarboxylic acid moieties (Fig. 1B). It belongs to a group of fungal secondary metabolites known as fumonisins that are the cause for equine leukoencephalomalacia, porcine pulmonary edema and esophageal cancer in humans.
The presence of OTA in wine-grapes and respective producing fungi has been extensively studied over the last 10 years and several reviews on the subject can be found in the literature (Varga, Kozakiewicz, 2006; Hocking *et al*., 2007). These studies demonstrated that *Aspergillus carbonarius* is the main responsible for OTA presence in grapes and that the severity of infection depends on factors such as the weather conditions during the harvest season, the grapes maturation, the presence of grape caterpillars or other pests, the existence of physical damage on grapes and on the viticulture practices. On the contrary, the presence of FB_2 in wine-grapes is less well studied, since this new issue emerged recently. Nevertheless, FB_2 has already been detected in grapes, raisins, musts and wines (Logrieco *et al*., 2009; Mogensen *et al*., 2010). In Portuguese wines, FB_2 has been detected in one *Vinho do Porto* (2.8 µg/L), among 7 analyzed ones (Mogensen *et al*., 2010).

Aspergillus section *Nigri* in grapes

From studies conducted between 2001 and 2003 in five Portuguese wine-regions, which aimed to study the incidence of ochratoxigenic strains in wine-grapes, approximately 700 black aspergilli were isolated, identified and preserved in glycerol at -80 ℃ (Serra, 2005). Those strains are still representative of the local black *Aspergillus* and were fully characterized in terms of morphological identification and OTA production. Now, in this work, the production of FB_2 by those strains was screened, to evaluate the incidence and distribution of fumonisinogenic strains in each wine-region and, also, to evaluate if its production correlates with the production of OTA.

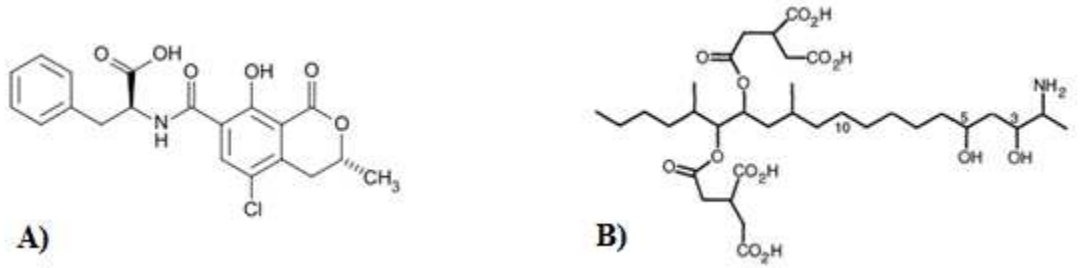

Fig. 1 Molecular structure of **A)** ochratoxin A, and **B)** Fumonisin B_2.

Materials and methods

Chemicals and Reagents

Sodium cyanide, 2,3-naphthalenedicarboxaldehyde (NDA), boric acid and an FB_2 standard (OEKANAL®, 50 mg/L in acetonitrile:water) were obtained from Sigma-Aldrich (Sintra, Portugal). Isocratic grade acetonitrile and methanol, acetic acid and NaOH were obtained from Merck (Lisbon, Portugal). The FumoniTest WB™ immunoaffinity columns (IAC) used in this study were obtained from Vicam (USA).

Biological Materials and Growth Conditions

Strains were isolated between 2001 and 2003 from Portuguese wine-grapes from five different Portuguese wine regions. These strains were identified and preserved at -80 ºC in glycerol by Serra (2005). They include 75 strains of *A. carbonarius*, 9 strains of *A. ibericus* and 597 strains belonging to the *A. niger* aggregate. All of the *A. carbonarius* strains were ochratoxigenic (mean of 1.13 mg/kg), but only 4% of the *A. niger* aggregate strains produce OTA (mean of 0.14 mg/kg). None of the *A. ibericus* strains were found to produce OTA.

A. niger ex-type strain MUM 03.01 and *A. niger* strain MUM 92.13 were used as positive controls. The reference strain *A. tubingensis* MUM 06.152 was used as FB_2 negative control. Strains were revived in MEA (Blakeslee's formulation) for seven days in the dark at 25 ºC and then subcultured into CYA and incubated at 25 ºC for eight days in the dark. Twenty-five strains from the *A. niger* aggregate (with different levels of FB_2 production) were selected to be subcultured into a 50% grape juice medium (GJ50), as described elsewhere (Serra, 2005).

FB_2 Extraction and Determination

FB_2 was extracted from colonies using five plugs that were 7 mm in diameter (mean weight = 0.707 g) and 1 mL of methanol/distilled H_2O (3:1, v/v), as reported previously (Abrunhosa *et al*, in press). Briefly, samples were dried at 50 ºC with a gentle stream of nitrogen, and then the dried residues were derivatized with NDA. After vortexing, samples were heated at 60 ºC for 15 min in a thermostated bath, cooled to room temperature, diluted with 1.4 mL of acetonitrile/distilled H_2O (3:2, v/v) and analyzed by HPLC with fluorescence detection (λ_{exc}= 420 nm and λ_{em}= 500 nm). Batches of 20 samples were prepared and injected within 12 h of derivatization.

The HPLC apparatus was comprised of a Varian 9002 pump, a Marathon Basic autosampler with a 50 µL loop, a Jasco FP-920 fluorescence detector and a Galaxie chromatography data system. The chromatographic separation was a 30 min isocratic run on a C18 reversed-phase YMC-Pack ODS-AQ analytical column (250 x 4.6 mm i.d., 5 µm), fitted with a pre-column of the same stationary phase. The mobile phase was acetonitrile/water/acetic acid (60:40:1, v/v/v) that was filtered and degassed with a 0.2 µm membrane filter (GHP, Gelman). The flow rate was set to 1.0 mL/min and the column temperature to 28 ºC (Technochroma).

FB_2 levels were determined by measuring the peak area and comparing it to the calibration curve from FB_2 standards of 200, 100, 20, 10 and 1 µg/L. Standards were injected regularly and added to the calibration curve (n=10). The limit of detection (LOD) and the limit of quantification (LOQ) were calculated from the residual standard deviation (σ) of the regression line of the calibration curve and its slope (S) using the following equations: LOD = 3.3x(σ/S) and LOQ = 10x(σ/S).

To confirm the identity of FB_2, extracts from 10 strains were submitted for purification using immunoaffinity columns (FumoniTest WB™). Briefly, the dried residues were obtained as described above, re-suspended in 1 mL of methanol and diluted with 10 mL of phosphate buffered saline (PBS). The pH was adjusted to 7.0 with 6N HCl, and the total volume was loaded onto IAC at a rate of one to two drops per second. Then columns were washed with 10 mL of PBS, FB_2 was eluted with 2 mL of methanol into clean vials, and samples were evaporated to dryness, derivatized with NDA and analyzed by HPLC, as previously described.

Association between FB2 and OTA production

To determine whether there was an association between the productions of FB_2 and the production of OTA, a 2x2 contingency table test was used. A low association between variables is indicated when Phi is close to 0, and a strong association is indicated when Phi is close to 1. A p-value ≤ 0.05 (two-tailed) was considered significant. The statistical package SPSS Statistics, version 19.0, was used to perform the statistical analysis.

Results and Discussion

***Aspergillus* section *Nigri* in grapes**

Previous research shown that the main black aspergilli found in Portuguese wine grapes are *A. niger* aggregate species (*ca*. 85%), followed by *A. carbonarius* (*ca.* 11%). Other species, as

A. ibericus and uniseriate ones are less frequently found. Although the *A. niger* aggregate species are the dominant ones, only few isolates produce OTA (*ca*. 4% of strains), making *A. carbonarius* the main responsible for OTA production in grapes. The latter species produce the mycotoxin at high levels (mean of 1.13 mg/kg), while the first species produce OTA at lower amounts (mean of 0.14 mg/kg).
Also, the distribution of black aspergilli among wine regions varies slightly, being more frequent in vineyards with Mediterranean climate. On the whole, their presence is significantly lower in *Vinhos Verdes* wine region and higher in the remaining regions under study. Specifically, the highest incidence of *A. niger* aggregate isolates is observed in the *Douro* region, while the highest incidence of *A. carbonarius* strains is observed in Ribatejo and Alentejo regions.

Fumonisin B_2 production

FB_2 production was screened in all preserved black aspergilli strains. Contrary to OTA production, FB_2 was only detected in the *A. niger* aggregate. It was found that 29% of the *A. niger* aggregate strains produced FB_2 above the detection limit and that only 6% of the fumonisinogenic strains produced more than 1.0 mg/kg. Levels produced were between 0.003–6.0 mg/kg with a mean of 0.66 mg/kg (tab.1). The higher incidence of producing strains (38%) was observed in Douro region while the lower (10%) was obtained in Alentejo region. As in the case of OTA production, in Douro region a higher incidence of producing strains in observed.

Tab. 1. Number of *A. niger* aggregate strains that produce FB_2 when grown on CYA and its distribution and levels in the main Portuguese wine regions.

Wine regions	number of strains	number of strains producing FB_2 (mg/kg)				FB_2 (mg/kg)	
		< 0.1	[0.1-1]	> 1	overall	Mean	Median
Douro	239	56	12	23	91	0.75	0.02
Ribatejo	198	39	19	5	63	0.28	0.01
Alentejo	135	5	3	6	14	1.48	0.75
Vinho Verde	21	0	2	3	5	1.09	1.29
Madeira	4	0	1	2	3	1.39	1.38
All	597	100(17%)	37(6%)	39 (6%)	176(29%)	0.66	0.02

Association between FB_2 and OTA production

Comparing the production of both mycotoxins, an association between FB_2 and OTA production was not found. Strains that produce simultaneously the two mycotoxins were rare (10 strains) and they were mostly from Douro (Tab. 2).
As one can realize from Tab. 2, although no significant difference occur between the number of isolated strains (per sampling places) in Alentejo, Douro and Ribatejo regions, it is clear that the number of *A. niger* aggregate strains producing one or both mycotoxins is lower in Alentejo. Also, this table does not include the number of *A. carbonarius* strains isolated in each region, which will increase the number of ochratoxigenic species in Alentejo and Ribatejo.

Tab. 2. Percentages of OTA and FB_2 producer strains from the *A. niger* aggregate found in each wine region.

Wine regions	Strains (sampling places)	OTA^+ (%)[a]	FB_2^+ (%)	$OTA^+ + FB_2^+$ (%)	*Phi*	*p-value*
Douro	239 (3)	8.4	38.1	3.3	0.012	0.853
Ribatejo	198 (3)	2.5	31.8	1.0	0.028	0.691
Alentejo	135 (2)	0.7	10.4	0	- 0.030	0.732
Vinhos Verdes	21 (3)	4.8	23.8	0	- 0.125	0.567
Madeira	4 (-)	0.0	75.0	0	- [b]	- [b]
Total	597	4.4	28.8	1.7	0.036	0.382

[a] be aware that the 75 *A. carbonarius* strains are not included.
[b] No statistics were calculated because the OTA is a constant.

Conclusions

In average, the levels of FB_2 produced by *A. niger* strains (0.66 mg/kg) are lower than the levels of OTA produced by *A. carbonarius* strains (1.1 mg/kg), but of the same order of magnitude. Moreover, the number of strains producing FB_2 is much higher than the ones producing OTA. Looking only at these data, it is possible to speculate that the probability of detecting fumonisin in wines would be higher. However, this comparison should only be made after taking in consideration the toxicity of both mycotoxins.

The average Tolerable Daily Intake (TDI) for fumonisins is 2 $\mu g/kg_{bw} \cdot day$, 400 times higher than the one for OTA (5 $ng/kg_{bw} \cdot day$). For this reason, our findings confirm a low risk for FB_2 contamination in Portuguese wine-grapes because of the low exposure to FB_2 by the consumption of grape products. These data will be further discussed in the presentation.

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