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OCHRATOXIN A AND OCHRATOXIGENIC FUNGI IN TUNISIAN GRAPES AND WINE

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Abstract

This work summarizes the results of a large study on the occurrence of ochratoxigenic fungi and Ochratoxin A (= OTA) from wine and table grapes in Tunisia.
Black aspergilli were the dominant genus among the filamentous fungi isolated from grapes and were the only potential OTA-producing fungi found. The most abundant species were member of *Aspergillus niger* aggregate than *Aspergillus carbonarius*. Uniseriate aspergilli were rarely present. Of the *A. carbonarius* isolates, 97% were OTA positive but only 3% of the *A. niger* aggregate isolates produce this toxin. During grape maturation, the frequency of black aspergilli increased due to increase of the number of *A. carbonarius*.
Thereafter musts produced from mature grapes were analysed for their OTA content. More than the half of the samples contained detectable levels of OTA, (between 0.01 and 5.85µg OTA l^{-1}). The most contaminated musts were obtained from the region of Raf-Raf located in the North-Est and characterized by a humid climate, however, musts obtained from the region of Regueb located in the center, which is a new area for the grapevine cultivation and characterized with an arid climate were rarely contaminated.
For the contamination of tunisian wine, OTA was detected 85% of the analyzed samples. The results show OTA levels ranged between 0.09 and 1.5 µg/L. Neither of the studied samples shown levels above the European regulatory limit (2 µg/L).
Keywords: Black aspergilli, grape, Ochratoxin A; Tunisian wine

Introduction

Ochratoxin A (OTA) is a toxic secondary metabolite produced by moulds belonging to several species of the genera *Aspergillus* and *Penicillium*. It is a potent nephrotoxin and hepatoxin with teratogenic, mutagenic and immunosuppressive effects (Pitt et al., 2001). Ochratoxin A was first detected in wine by Zimmerli and Dick (1996). Since then, presence of OTA in grape and its derived products, such as dried vine fruits, grape juices and wine has been worldwide reported (Varga et al. 2007). The European Union legislation authorities have introduced an OTA limit of 2 µg/L in wine, must or grape juice (European Commission, 2005). Ochratoxin A is produced by species in *Aspergillus* sections *Nigri* (black aspergilli) and *Circumdati*, commonly found in warm and tropical climates. *Penicillium verrucosum* is considered as the main source in temperate climates and is more frequently associated with cereals (Pitt and Hocking, 1997). These authors found that among black aspergilli, *A. carbonarius* was the main species producing OTA in grapes. Some members of the *A. niger* aggregate have also been found to produce OTA. It was observed that wines from Southern Europe and North Africa, with Mediterranean climates, contained more OTA than those originating from the more temperate regions of central Europe (Zimmerli and Dick, 1996; Otteneder and Majerus, 2000). In fact, it was found that climate has an effect on the occurrence of OTA and OTA-producing fungi in grapes (Battilani et al., 2006a, 2006b; Serra et al., 2006). After cereals, the wine is the second food with the greatest contribution in the daily intake of OTA by the population of European Union. In order to reduce consumers' exposure, EFSA recommended maximum tolerable daily intakes of 120 ng/kg.bw/week. Wine production in Tunisia

is estimated to 300.000 Hectoliters per year. The annual exportation overtakes 140.000 Hectoliters, essentially, to European countries. Beer production in Tunisia is estimated to 1.000.000 Hectoliter intended exclusively to local consumption. The aim of this work was to survey the contamination of Tunisian grapes and wines with OTA

Materials and methods

Sampling

The survey was carried out over 3 years and involved four vineyards located in four viticultural regions of Tunisia (Belli: Grombalia region, Baddar: Cap bon region, Regueb: Center of Tunisia, Raf-Raf: North east of Tunisia) with specific climate. Six grape varieties in each vineyard, representative of their grape-growing area were selected for further study. The varieties included wine and table grapes. Grape samples were harvested at maturity stage (End of July/August) depending on the region and variety. Ten plants along diagonal transects were chosen in each vineyard and one bunch of grapes was taken from each plant. Bunches were kept in sterile bags and transported in cooled boxes (4 °C) to the laboratory for analysis. A total of 34 wine samples (18 red, 9 rosé and 7 white) produced in Tunisia were also purchased, in commercially available size, from different supermarkets in Tunis (Tunisia). The wine samples were produced between 2004 and 2006 and consisted of various varieties from different vintages (designation of origin). The samples were stored in their original containers in fridge at 4°C until OTA analysis.

Isolation and identification of fungal flora

Berries were surface-disinfected with hypochlorite (15%) Afterwards, they were plated in Petri dishes containing Malt Extract Agar medium (MEA) with Chloramphenicol (100 $mg.l^{-1}$). Plates were incubated at 25°C for 10 days. All the colonies of potential OTA- producing fungi (*Aspergillus* and *Penicillium*) were classified into genera. For species identification, the *Aspergillus* isolates were cultivated in Czapek Yeast Agar medium (CYA) and incubated at 25°C for 5 days. Black *Aspergillus* species were classified into three groups (uniseriates, *Aspergillus niger* aggregate and *Aspergillus carbonarius*) in accordance with Dr. Kozakiewicz guidelines (CABI Bioscience, Egham, UK).

Analysis of OTA in grapes and wines

After selecting berries for fungal isolation, the ten bunches collected from each vineyard and variety were manually crushed and the resulting musts (n=72) were analyzed for OTA content according to the method of Bezzo et al. (2002). Musts (100 ml) were first centrifuged (2500 g, 20 min), liquid phase was, brought to a pH 7.4 (NaOH 4M), filtered and applied to an immunoaffinity column (Ochraprep, Rhône Diagnostics Technologies Ltd, Glasgow, UK). For wine samples no centrifugation was realized. The column was then washed with 20 ml of distilled water and dried up with an air stream. Elution was realized with 1.5 ml of methanol/acid acetic (98/2) solution. The eluate was evaporated to dryness, dissolved with 0.8 ml of mobile phase and injected into the HPLC system equipped with a fluorescence detector ($_{exc}$ 230 nm; $_{em}$ 458 nm) and a reversed-phase column C18 (Waters Spherisorb 5 µm, ODS 2, 4.6 × 250 mm, Milford, MA, USA). The mobile phase was composed by a sodium acetate 4 mM/acetic acid (19:1: v/v) solution (52%) and acetonitrile (48%). Detection and quantification limits (LOD and LOQ) were 0.03 µg OTA/l and 0.05 µg OTA/L, respectively. The recovery for OTA on grape juice samples was 95 ± 4% (mean ± SD., $n = 3$).

Results and discussion

Distribution of black Aspergillus species

A total of 1242 black aspergilli were isolated along the three sampling three years distributed in the four regions from Tunisia (Belli, Baddar, Raf-Raf and Regueb). The analysis of variance revealed

that the single factor region and the interaction year and region presented significant differences in the number of black aspergilli isolated ($P < 0.01$). The sampling year
does not affect the fungal colonisation grapes ($P > 0.05$). Grapes from Regueb were significantly the most contaminated with black aspergilli each year. No statistical differences were found between the number of these moulds in the regions of Baddar, Belli and RafeRaf (P ¼ 0.05). The distribution of black aspergilli isolates is presented in Table 1.

Table1: Percentage of black aspergilli isolates. Numbers in brackets are the number of black aspergilli isolates of each group isolated/the total number of black aspergilli.

Group		Region			
		Baddar	Belli	Regueb	Rafraf
Uniseriate	1st Year	2.9 (4/136)	1.0 (1/101)	1.9 (2/104)	0 (0/101
	2nd Year	4.8 (4/84)	1.1 (1/94)	10.0 (13/130)	0 (0/115)
	3rd Year	0 (0/77)	1.6 (1/63)	0.7 (1/150)	2.3 (2/87)
		Total			
		2.5 (8/297)	1.2 (3/258)	4.2 (16/384)	0.7 (2/303)
A. carbonarius	1st Year	19.9 (27/136)	24.8 (25/101)	2.9 (3/104)	20.8 (21/101)
	2nd Year	23.8 (20/84)	41.5 (39/94)	0 (0/130)	22.6 (26/115)
	3rd Year	58.4 (45/77)	74.6 (47/63)	0.7 (1/150)	18.4 (16/87)
		Total			
		30.9 (92/297)	43.0 (111/258)	1.0 (4/384)	20.8 (63/303)
A. niger aggregate	1st Year	76.5 (104/136)	76.2 (77/101)	94.2 (98/104)	79.2 (80/101)
	2nd Year	60.7 (51/84)	57.4 (54/94)	90 (117/130)	77.4 (89/115)
	3rd Year	98.7 (148/150)	30.2 (19/63)	36.4 (28/77)	79.3 (69/87)
		Total			
		61.6 (183/297)	58.1 (150/258)	94.5 (363/384)	78.5 (238/30)

Isolates of *A. niger* aggregate formed the dominant group in the four regions, especially in the region of Regueb where they represent 94.5% of the isolated black aspergilli. In contrast, uniseriate isolates were recovered in small numbers in the different regions. At each sampling year, the highest level of *A. carbonarius* infection was observed in the region of RafeRaf where it represented a mean of 43% of black aspergilli isolated. The level of contamination by this specie was lower in the regions of Baddar and Belli with, respectively, 30.9% and 20.8% of black aspergilli. The incidence of *A. carbonarius* was very low in Regueb region where only four *A. carbonarius* (1% of black aspergilli) were isolated during the three sampling years.

OTA contamination of grapes

The data related to the OTA concentrations in musts are presented in table Table 1.

The results showed that at maturity stage, 54% (39/72) of analyzed samples were contaminated with 10% (4/39) of OTA positive samples exceeding the EU restrictive limit (> 2 µg/L). These samples came from Raf-Raf (5.44 and 5.84 µg OTA/L) and Baddar (3.27 and 2.23 µg OTA/L) regions. Eighty three percent (15/18) of grapes from Raf-Raf were OTA contaminated in the range of 0.06-5.85 µg/L. In Baddar, 83% (15/18) of grape samples contained between 0.06 and 3.27 µg OTA/L. Forty four percent (8/18) of grapes from Belli contained between 0.09 and 0.96 µg OTA/L. Among the grape samples originating from Regueb, only one sample was contaminated with OTA at a very low concentration (0.05 µg/L). The multiple comparison test (P=0.05) showed that the OTA contamination of grape is significantly different between Regueb region and the regions of Baddar and Raf-Raf. A significant difference for OTA contamination was also found between Belli and Raf-Raf regions. The distribution of the OTA concentrations in the grapes showed that higher OTA concentrations were detected in grapes from Raf-Raf with 40% of samples containing between 0.5 and 2 µg OTA/L and 13% containing more than 2 µg OTA /L. For grapes originating from Baddar,

20% of samples were contaminated in the range of 0.5-2 µg OTA/L and 13% of samples exceeded 2 µ OTA /L. This level of contamination is higher than observed in Belli were 75% of samples contained less than 0.5 µg OTA/L and 25% were contaminated with concentrations ranging between 0.5 and 1 µg OTA/L.

Table 2**:** OTA concentrations in grapes collected from the four regions in the different years (µg/L).

Region	**Variety**	**Sampling year**		
		1st year	**2nd year**	**3rd year**
Baddar (Est part of Tunisia Cap bon region)	Muscat Alexandrie	0.97	3.27	0.25
	Cabernet Sauvignon	0.14	1.53	<LQ*
	Ugni Blanc	0.06	1.21	0.11
	Italia	0.17	0.24	0.38
	Syrah	0.38	0.12	<LQ
	Carignan	<LQ	0.16	2.23
Belli (North part of Tunisia)	Chardonnay	0.18	0.13	0.96
	Muscat Alexandrie	<LQ	0.35	<LQ
	Cabernet Sauvignon	<LQ	0.10	<LQ
	Italia	<LQ	0.77	<LQ
	Syrah	0.25	<LQ	<LQ
	Carignan	0.13	<LQ	<LQ
Raf-Raf (North-Est part of Tunisia)	Rezeki	0.1	1.49	0.96
	Bezoul Khadem	0.12	<LQ	5.85
	Akhal Raf-Raf	<LQ	0.15	0.27
	Marsaoui	0.18	0.55	<LQ
	Bid Hmam	1.88	0.07	0.85
	Muscat Raf-Raf	0.36	5.45	1.09
Regueb (Center part of Tunisia)	Italia	<LQ	<LQ	<LQ
	Victoria	<LQ	0.05	<LQ
	Sultanine	<LQ	<LQ	<LQ
	Superior Seedless 1	<LQ	<LQ	<LQ
	Matilde	<LQ	<LQ	<LQ
	Superior Seedless 2	<LQ	<LQ	<LQ

*LQ (0.05 µg/L)

These results showed the presence of the toxin in both wine and table grapes at the three sampling years. Almost half of samples were contaminated with OTA which is similar to the level of contamination detected in Southern Italy (Lucchetta et al., 2010). Among the OTA positive samples, 4 out of 39 exceeded 2 µg/l. However, it’s important to note that grape collected at maturity will undergo several stages (transport, storage, vinification, drying) which may influence

the OTA final concentration of grape product. Several factors affect the grape colonization by ochratoxigenic moulds and the OTA contamination, in particular, geographic and climatic conditions. In several Europeans countries, southern regions located in the Mediterranean basin, were found to be particularly affected (Battilani et al., 2006a; Battilani et al., 2006b). Tunisia is a small country with an extended Mediterranean coast; however, areas of grape planting and production are characterized by different climatic characteristics. In Regueb region characterized by an arid climate vineyards grapes were rarely contaminated with OTA and the level detected was very low. The region of Raf-Raf is a coastal region located in the North-Est of the country. It is characterized by a sub-humid climate. These climatic conditions seem to be very favorable to OTA accumulation Baddar and Belli are located in Cap-Bon region (North) which is the main viticulture area in Tunisia and especially for wine grape. This area has a typically semi-arid Mediterranean climate.

For OTA contamination, no difference was found between the two regions, thus the incidence of OTA in grapes depend not only on climatic conditions but also on other factors related to the variety and crop management, such as training system, irrigation and phytosanitary treatments, which influence on the ecosystem of the vine.

3.2. Occurrence of OTA in wine

In the present study, a total of 34 wine samples from designation of origin from Tunisia were analyzed and the results are shown in Table 3.

Table 3: Occurrence and OTA levels in wines produced in Tunisia.

Wine sample	Vintage	No. of samples	No. of samples with OTA level > LQ* (µg/L)
Red	1st year	8	8 (0.5, 0.51, 0.94, 0.44, 0.28, 0.3, 0.38, 0.55)
	2nd year	2	1 (0.53)
	3rd year	8	5 (0.18, 0.43, 0.29, 0.19, 0.09)
Rosé	1st year	1	1 (0.64)
	2nd year	1	1 (0.12)
	3rd year	7	6 (0.22, 0.20, 0.09, 0.15, 0.22, 0.25)
White	1st year	1	1 (0.48)
	2nd year	2	2 (0.92, 1.50)
	3rd year	4	3 (0.39, 0.12, 0.11)

*LQ (0.05 µg/L)

Eighty-five percent (85%) of the samples contained detectable amounts of OTA ranged from 0.09 to 1.50 µg/L and a mean value of 0.38 ± 0.31 µg/L. The European Union has proposed 2 µg/L of OTA in wine as a maximum residue level. Neither of the studied samples shown levels above the European regulatory limit. The incidence of OTA-positive samples for red, rosé and white sample was 78%, 100% and 86%, respectively. Mean OTA levels and range of contamination in red, rosé and white wines were 0.40 (0.09–0.94 µg/L), 0.22 (0.09–0.64 µg/L) and 0.59 µg/L (0.11–1.5 µg/L), respectively. Different studies in Europe have shown that the wines from the Southern regions usually contain higher OTA concentrations than from the North due to the climate being characterized by high humidity and high temperature (Battilani et al., 2006a). The wines produced in Tunisia come mostly from vineyards located in the North-Est of the country, which is characterized by Mediterranean weather with a semi-arid to semi-humid climate. In several studies conducted in different European countries, red wines were usually found more contaminated with OTA than rosé and white wine (Mateo et al., 2005). Although the number of samples analyzed in our study is low for a relevant discussion about differences in OTA concentrations between the type of wine OTA was found with high concentration in both white and red wines. The higher values of OTA were found in white wines (0.9 and 1.5 µg/L) and in a red wine (0.94 µg/L). Several studies reported that dessert wines are quite prone to be contaminated with OTA (Mateo et al., 2007). No dessert wine is produced in Tunisia.

Conclusion

The occurrence of OTA has been determined in grapes and wines produced in Tunisia. A high frequency of OTA contaminated sample was also found in Tunisian wines; however, none of the analyzed wines contained OTA at levels above the limit fixed by the European Union. Thus, there is no risk for the Tunisian wine exportations. For grape contamination; the results of the present work demonstrated the high risk of OTA in Northern Tunisia, especially for coastal regions.

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