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Isolation and characterization of ochratoxin A and aflatoxin B1 producing fungi infecting grapevines cultivated in Tunisia

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A survey was conducted to access mycotoxin-producing fungi and to evaluate the ochratoxin A (OTA) and the Aflatoxin B1 (AFB1) potential production for fungal strains contaminating table grapes in different Tunisian vineyards. Among 100 *Aspergillus* isolates, *Aspergillus niger* aggregate were the most frequent (70%) followed by *Aspergillus carbonarius* (7%) and *Aspergillus flavus* (23%). The mycotoxigenic capacity of the isolates was tested in culture media revealed that the highest levels of OTA production were obtained with strains of *A. carbonarius* (80% of them) whereas only 5% of *A. niger* aggregate were OTA producers. Furthermore, 39% of the *A. flavus* produced aflatoxin B1 (between 21 and 54 µg/g). Finally, we demonstrated, for the first time, that 3% of the *Penicillium* isolates (especially *Penicillium expansum*) were OTA producers in grapes.

Key words: *Aspergillus*, *Penicillium*, grape, ochratoxin A, aflatoxin B1**.**

INTRODUCTION

Mycotoxins are fungal secondary metabolites that are harmful to animals and humans as they may cause serious diseases (Smith and Moss, 1985; Bauerand and Gareis, 1987; Essono et al., 2007). Among these toxins, ochratoxin A (OTA) is considered as nephrotoxic, neurotoxic, teratogenic and immunosuppressive (Bacha et al., 1986; Boorman, 1989; Walker 1999; Battilani et al., 2003). In Tunisia, a correlation between the presence of ochratoxin A in blood and nephropathy has been established (Marquardt and Frohlich, 1992; Zimmerli and Dick, 1996). The tolerated threshold of its presence in wine was fixed to 2 µg/l by the "Organisation Internationale de la Vigne et du Vin (OIV)". Several reports revealed the presence of OTA in grapes as consequence of contamination with *Aspergillus ochraceus* and *Penicillium verrucosum* (Bacha et al., 1986; Maaroufi et al., 1995; Otteneder and Majerus, 2000; Pardo et al., 2005). However, production of aflatoxin B1 by ochratoxygenic fungi are still not common in grapes (Serra et al., 2003; Gonzalez et al., 2005; Gomez et al., 2006). *A. flavus* Link and *Aspergillus parasiticus* are often reported as the main producers of such toxins (Asao et al., 1963; Bankole, 1993).

In this study, the main objective was to identify the mycotoxigenic fungal flora, contaminating grapes from different Tunisian vineyards and to monitor their OTA and AFB1 production. Since, such data is not yet available; this study is of great interest, especially for viticulturists as it would allow a better management of ochratoxigenic fungi by using specific and preventive treatments.

MATERIAL AND METHODS

Fungal isolation and growth conditions

Fungal strains were isolated from grape berry samples collected from different representative vineyards in Tunisia according to Warcup (1996). During the season (June-September), samples were collected at 3 stages of the development of the berry: setting, veraison and maturity. At each sampling, 180 berries were collected from each of the vineyards located in the North (especially from the

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Table 1. Fungal distribution according to geographical sampling area**.**

Strains		Origin	Number of isolates
Black aspergilli	*A. niger* aggregate	North	60
		Centre	7
		South	3
	A. carbonarius	North	4
		Centre	2
		South	1
A. flavus		North	23
		Centre	0
		South	0
Penicillium spp	*Penicillium expansum*	North	4
		Centre	2
		South	1
	Other *Penicillium* strains	North	85
		Centre	8
		South	0

main growing are off "Cap- Bon", characterized by temperature season average of 30°C and annual rainfall of 400 - 500 mm), the Centre, Regueb characterized by an average temperature of 35°C, 200 - 300 mm of rainfall and the South (Rjim-Maatoug) (39°C, < 100 mm) of the country. Fungal cultivation was carried out by incubation of fresh berries (cut into 2 halves) for 7 days at 25°C in MEA medium (Malt Extract Agar) (Pitt and Hocking, 1997).

Fungal sampling

In each parcel, 90 grapevine plants (distributed in 6 blocks of 30 plants) were targeted for sampling. 2 bunches were tagged on each plant. At each developmental stage, 02 Berries were taken from each of the tagged bunches and transferred to the laboratory in a refrigerated box.

OTA and AFB1 extraction from fungal cultures

OTA and AFB1-producing ability of the isolates were performed by cultivating the fungal strains in Czapek Yeast extract agar medium (1 g of K_2HPO_4, 10 ml of Czapek concentrate, 1 ml of trace metal solution, 5.0 $g.l^{-1}$ of yeast extract, 30 g. $l^{-1}$of sucrose and 15 g. $l^{-1}$of agar) at 25°C for 7 days. For each strain, three agar plugs were picked up from the central area of the colony. Each plug was weighted, mixed in 1 ml of methanol and incubated for 1 h at 25°C before to be centrifuged at 1300 rpm for 10 min. The supernatants were recovered and filtered through 0.45 µm PVDF filters (Supelco) for HPLC analysis of both mycotoxins.

HPLC analysis of OTA and AFB1 production

HPLC monitoring of OTA was carried out according to Sage et al. (2002) using analytical column (150 x 4˙6 mm Uptisphere 5 µm C18 ODB) fitted with a guard column of 10 x 4 mm. The excitation and emission wavelengths were: λ_{ex} = 332 nm and λ_{em} = 466 nm. The mobile phase consisted in a mixture of water:acetic acid:acetonitrile (57:41:2, v/v/v). The flow rate was of 1.0 ml min^{-1}. The injection volume was of 80 µl and the retention time was of around 15 min. The OTA and the AFB1 levels were obtained on the basis of the HPLC fluorimetric response compared to standards (OTA and AFB1 standards were provided from Sigma Aldrich-Germany).

Identification of toxinogenic isolates

Aspergillus isolates were identified using morphological features (Raper and Fennell, 1995; Bragulat et al., 1998) by comparison to reference strains of *A. niger* aggregate, *Aspergillus carbonarius* and *Aspergillus japonicus.* However, the identification of ochratoxigenic *Penicillium* strains was carried out by PCR amplification of the ITS1-5.8-ITS2 ribosomal sequence (Dao et al., 2005). PCR was performed in 50 µl reaction mixture containing: 5 µl of *Taq* Polymerase buffer 10X, 1.5 µl of 50 mM $MgCl_2$, 1 µl of dNTPs 10 mM (Promega), 1 µM of each primer, 1.5 U of *Taq* recombinant polymerase (Invitrogen, USA). The method of Klich and Mullaney (1987) was used to purify total cellular DNAs from 0.5 g of freeze-dried mycelia.

45 ng of genomic DNA was used to carry out the PCR reaction in the presence of ITS primers (ITS4: 5'-TCCTCCGCTTAT-TGATATGC-3' and ITS5: 5'-GGAAGTAAAAGTCGTAACAAGG-3').

The PCR reaction program was: 1 cycle (95°C for 10 min) for the initial denaturation and 35 sequential cycles for the DNA amplification (95°C for 45 s, 55°C for 45 s and 72°C for 1 min) followed by a final extension step at 72°C for 10 min. The amplified products (600 bp) were separated on agarose gel (1%) and purified from the gel using specific columns (Qbiogene) before sequencing. The alignment of the resulting sequences was carried out using the database located at the website http:// prodes.toulouse.inra.fr/mutalin/mutalin.html (Bragulat et al., 2001).

RESULTS AND DISCUSSION

Phenotypic characterization of the strains

Since *Aspergillus* and *Penicillium* are known as the main producers of OTA or AFB1, we limited our identification to these 2 genera.

Aspergillus identification

Based on morphological features, 100 strains were identified as biseriate species (*A. niger* aggregate, *A. carbonarius* and *A. flavus*). The *Aspergillus* strains were classified in 3 groups as specified in Table 1.

70% of the total *Aspergillus* isolates consisted in *A. niger* aggregate among which 60% were isolated from material sampled in vineyards located in the North (Cap Bon), 7% from the Centre (Regueb area) and 3% from the South (Rjim Maatoug). For *A. carbonarius* (representing 7% of total isolates), 97% of them were found in samples coming from the North (especially from Belli). The remaining 3% were from the Centre (Regueb)**.** Finally, the 23% of isolates identified as *A. flavus* were obtained exclusively from samples harvested in the North (Baddar).

The results demonstrated that fungal microflora isolated from grapes was composed of black *aspergilli* (*A. niger*

Table 2. Production of OTA by *Aspergillus* and *Penicillium* species isolated from representative Tunisian vineyards.

Fungal species	Number of OTA producing strains	Geographical origin	Amount of OTA produced (µg/g of medium)
A. niger	05	North	$6.53\ 10^{-3}$ - $2.78\ 10^{-2}$
A. carbonarius	07	North (5 strains))	1.18 - $1.07\ 10^{-2}$
		Centre (2 strain))	6. 82
P. expansum	03	North	0.24 - 1.53

aggregate and *A. carbonarius*) and *A. flavus* (Table 1). In agreement with previous studies, the high frequency of the genus *Aspergillus,* section *Nigri,* detected in Tunisian vineyards was also reported in many Mediterranean countries such as Portugal and Spain (Sage et al., 2002; Belli et al., 2004). In this work, the *A. niger* aggregate was a major component in the mycobiota of grapes.

No strain belonging to uniseriate species, *A. japonicus* was found in the collection of *Aspergillus* isolates obtained from grapes at harvest time. This species is usually found in foods (Pitt and Hocking, 1997) and could be isolated from soils, plant rhizospheres or leaf litter predominantly in tropical zones (Klich, 2002). For the moment, none of the *A. japonicus* species is considered to be OTA producing (Parenicova et al., 2001; Leong et al., 2004)

Penicillum identification

The identification of the 100 *Penicillium* isolates was performed morphologically, based on the typical shape of the mycelium. Further identification of the species was carried out by PCR using specific primers of the ITS4-ITS5 ribosomal sequence. The molecular analysis revealed that the *Penicillium* flora was composed of *Penicillium chrysogenum* (58%) , *Penicillium brevicompactum* (13%), *Penicillium glabrum* (5%), *Penicillium expansum* (4%) and *Penicillium minioluteum* (2%) and all of them were isolated from the North. The remaining was composed of *P. verruculosum* (18%), 10% of them were isolated from the North and 8% from the Centre.

Ochratoxin A and aflatoxin B1 producing potential of *Aspergillus* and *Penicillium* strains

Among the strains isolated, 80% of the *A. carbonarius* and 5% of the *A. niger* aggregate were OTA producers (Table 2). OTA production ranged from $6.53\ 10^{-3}$ to 6.82 µg for *Aspergillus* species and from 0.24 to 1.53 µg for *P. expansum* (Table 2). This situation corroborates previous reports from Mediterranean countries where *A. carbonarius* species was the most frequent species with a high ochratoxigenic potential (Singh et al., 1991; Heenan et al., 1998; Stroka et al., 2000; Rosa et al., 2002; Sage et al., 2002; Serra et al., 2003). Moreover, in accordance with Abarca et al. (2004) the percentages of ochratoxigenic isolates in *A. carbonarius* ranged from 25 to 100% and from 0.6 to 50% in A. *niger* aggregate strains.The majority of these ochratoxinogenic strains were isolated from the north where ecophysiological growth and OTA synthesis conditions are favorable.

Concerning the OTA production for the *Penicillium* genus, next to the strain *Penicillium verrucosum*, no other strains are known to be able to produce OTA in grapes, especially in the temperate climate areas. Our analysis, revealed, for the first time, that 3 isolates of *P. expansum* were able to produce OTA in grapevine. However, this original finding has to be confirmed by analyzing a larger population of *Penicillium* through several years. This contrasts with Pitt (1987) who states that only *P. verrucosum* is OTA producer as well as with Larsen et al. (2001) who found OTA production only in *P. verrucosum* and *P. nordicum*.

Generally speaking, *P. expansum* is considered to be mainly responsible for patulin production in fruits. It may cause rot in grapes but usually does not attack grapes before harvest (Serra et al., 2005).

Among, strains isolated from grapes, potentially AFB1 producers were *A. flavus* responsible of 39% of AFB1 production. The quantification of this mycotoxin shows concentrations between 0.021 and 0.054 mg/g (Table 3). This study shows the presence of *A. flavus* with a high capacity to produce AFB1 in grapes. According to joint FAO/WHO Expert Committee on Food Additives (JECFA), it was already concluded that a level of 0 - 20 ng/g of aflatoxin B1, a level of 0 - 50 ng/g of total aflatoxins in food (Stoloff, 1989; Stroka et al., 2000). As shown in Table 3, the amounts of AFB1 produced by *A. flavus* were important. In disagreement with the results obtained in Lebanese wine–grapes by El Khoury et al. (2008), the aflatoxigenic ability of the *A. flavus* isolates which represents 43.4% of the *Aspergillus* isolates, did not exceed 22.6 g/g of medium (CYA).

The present method of quantification provides a rapid, clean way for detecting production of OTA or AFB1 by fungi, quantifies their production in pure culture. This method was optimized by Bragulat et al. (2001) and Abrunhosa et al. (2001). It has several advantages over

Table 3. Production of AFB1 by *Aspergillus* species isolated from representative Tunisian vineyards.

Fungal species	Geographical origin	Amount of AFB1 produced (µg/g of medium)
A. flavus	North	21
A. flavus	North	23
A. flavus	North	25
A. flavus	North	27
A. flavus	North	30
A. flavus	North	32
A. flavus	North	35
A. flavus	North	40
A. flavus	North	54

other conventional methods which use large amounts of natural substrates or culture media and subsequently, are quite costly in terms of solvents. Aflatoxin content of foods and feeds is regulated in many developed countries and in some products including maize, aflatoxin levels below 20 ng are mandated (Serafini et al., 1991; Stoloff et al., 1991).

Controlling toxigenic fungi in the vineyard throng the implementation of objective agricultural practices is an efficient way to protect the consumer against hazards that might be generated by the presence of ochratoxin A and AFB1 in grape and derivatives (Abarca et al., 2001). This paper reports the results of an extensive survey on the occurrence of filamentous fungi isolated from grapes in Tunisia and to test their ability to produce ochratoxin A (OTA) and aflatoxin B1 (AFB1) on CYA culture medium, in order to assess their potential for producing these mycotoxins on grapes.

This report represents a first step towards the goal. Indeed, we identified the ochratoxigenic and the aflatoxigenic species among the fungal flora present on grape culivated in Tunisia. Furthermore, the OTA and the AFB1 were monitored and allowed the classification of these pathogenic specimens according to their OTA and AFB1 producing potential.

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