International Journal of Food Microbiology 132 (2009) 14–23

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Study of the phenotypic and genotypic biodiversity of potentially ochratoxigenic black aspergilli isolated from grapes

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ARTICLE INFO

Article history:
Received 22 April 2008
Received in revised form 6 March 2009
Accepted 17 March 2009

Keywords:
Aspergillus niger aggregate
Aspergillus carbonarius
RAPD-PCR
ITS-PCR
Ochratoxin A
Grapes

ABSTRACT

Ochratoxin A (OTA) is a mycotoxin with nephrotoxic, carcinogenic, teratogenic and immunotoxic effects, naturally found in agricultural products including grapes and wine. Black *Aspergillus* species (Section *Nigri*) are mainly responsible for OTA accumulation in wine grapes and in particular *Aspergillus carbonarius* and *Aspergillus niger* aggregate. The biodiversity of potentially ochratoxigenic strains of black aspergilli from different French vineyards in the southern Mediterranean region of Languedoc–Roussillon was studied. One hundred and eighty nine black strains were isolated from grapes and studied according to harvest year, production zone, grape variety and pre-harvest treatment of grapevines. The strains were identified and classified in two groups according to macroscopic and microscopic characters; these were called the *A. carbonarius* representative group and the *A. niger* aggregate representative group. Members of each group were classified in subgroups based on macroscopic morphological colony characters. Strain biodiversity was studied according to phenotypic and genotypic characterization and to the OTA production of selected strains on PDA medium. After identification was confirmed by specific PCR using primer pair ITS1/CAR and ITS1/NIG, 24 potential ochratoxigenic strains belonging to *A. carbonarius* and *A. niger* aggregate were discriminated by RAPD-PCR using 8 different OPC primers. The use of specific primers supported the identification based on phenotypic and morphological characters. RAPD-PCR patterns demonstrated a considerable diversity among the strains. Clustering among *A. niger* aggregate strains was associated with production zone and harvest year, but not grape variety or pre-harvest treatment. Clustering among *A. carbonarius* strains was not associated with any of the above parameters. OTA production of strains on culture medium seemed to correlate better with morphological characters than with genotypic profiles. No clear relation could be established between phenotypic and genotypic characters of the studied black aspergilli.

1. Introduction

Ochratoxin A (OTA) is a mycotoxin which shows a potent nephrotoxic effect (Vrabcheva et al., 2000) and also exhibits immunosuppressive (Petzinger and Weidenbach, 2002), teratogenic (Castegnaro and Pfohl-Leszkowicz, 2002) and carcinogenic (2B group) (IARC, 1993) effects. OTA is one of the most common naturally occurring mycotoxins, contaminating a wide range of different plant products, including cereals, coffee beans, cocoa, nuts, spices, dried fruits, beer and wine (SCOOP, 2002). In Europe, wine and especially red wine has been determined as the second major source of human exposure to OTA following cereals and preceding coffee and beer (SCOOP, 2002; Walker, 1999). Thus the European Commission has fixed the maximum limit for OTA in wine and grape juice at 2.0 µg/kg (Regulation (CE) N° 123/2005, 2005).

Aspergillus section *Nigri* (black aspergilli) is responsible for OTA production on grapes. (Abarca et al., 1994; Samson et al., 2004; Téren et al., 1996; Varga et al., 2004). A comprehensive molecular characterization of black aspergilli from grapes was performed in Europe by the EU project Wine-Ochra Risk (QLK1-CT-2001-01761) using representative strains from 107 vineyards in the Mediterranean basin most affected by the OTA problem. AFLP, RFLP and sequence analysis delineated 4 main populations of *Aspergillus* from grapes: *A. carbonarius*, *A. tubingensis*, *A. niger* and uniseriate isolates such as *A. japonicus* and *A. aculeatus*. (Bau et al., 2005; Perrone, 2007; Perrone et al., 2006; Varga et al., 2000). Within the *Aspergillus* section *Nigri*, *A. carbonarius* and the species included in the *A. niger* aggregate were considered as the principal sources of OTA contamination in grapes, dried grapes and wine (Abarca et al., 2001; Esteban et al., 2004; Perrone et al., 2006; Samson et al., 2004). *A. carbonarius* is the most frequently observed and isolated OTA producer (Bau et al., 2005; Belli et al., 2004; Cabañes et al., 2002; Delage et al., 2003; Esteban et al., 2004; Serra et al., 2003; Varga and Kozakiewicz, 2006).

Various molecular methods have been used for phenotypic and genotypic studies of aspergilli (Rinyu et al., 1995; Varga et al., 2000) and for the detection of these strains from agricultural samples (Sartori et al., 2006). The main targets for genus level detection of

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doi:10.1016/j.ijfoodmicro.2009.03.011

Aspergillus include the 18S rRNA gene, mitochondrial DNA, the intergenic spacer region, and the internal transcribed spacer (ITS) regions. The ITS region, located between the 18S and 28S rRNA genes, is an area of particular importance in discriminating between closely related species or at intraspecific level, because it has areas both of high conservation and high variability. ITS has been used to identify *Aspergillus* species (Accensi et al., 1999; Henry et al., 2000) and to discriminate possible OTA-producing *Aspergillus niger* and other *Aspergillus* species belonging to section *Nigri* by PCR assays (González-Salgado et al., 2005; Patiño et al., 2005).

The control of grape diseases still depends largely of the use of chemical fungicides applied as pre-harvest sprays during the period of berry development. These products have various levels of toxicity for the user, the consumer and the environment. Plant treatment with signaling molecules such as elicitors stimulate their natural defense mechanisms. For example, methyl jasmonate was used as an exogenous elicitor against fungal pathogens of grapevine like powdery mildew (Belhadj et al., 2006). Chitosan also showed potency in inducing some responses in grapevine leaves that might improve resistance to grey mould (Trotel-Aziz et al., 2006). FEN 560, a treatment derived from fenugreek, also showed efficiency against grape downy mildew (Martinez et al., 2002).

This project was thus devoted to the study of the biodiversity of potentially ochratoxigenic strains of *Aspergillus* section *Nigri* group, isolated from grapes according to different parameters; harvest year, production zone, grape variety and pre-harvest treatment of grapevines. A classical chemical treatment and a natural treatment with plant extract called "biological treatment" were compared. The study was done on a sampling pattern of grapes harvested from different French vineyards in the southern Mediterranean region of Languedoc–Roussillon, where most of them are contaminated by ochratoxigenic fungi and have the highest concentration of OTA in France (Bejaoui et al., 2006). Strain biodiversity was studied according to phenotypic and genotypic characterization of selected strains and to their OTA production. The genetic variability within potential ochratoxigenic strains belonging to *A. carbonarius* and *A. niger* aggregate was examined by RAPD-PCR after their discrimination by specific PCR.

2. Materials and methods

2.1. Vineyard and strain origins

All the chosen parameters for the study of black aspergilli diversity are listed in Table 1. The study was carried out over three years (2004–2006) at two different vineyards located at Vauvert and Béziers in the Languedoc–Roussillon region in the south of France. Several grape varieties were included; Carignan from Vauvert and Syrah from Béziers in 2004, Cabernet and Grenache from Béziers in 2005, Cabernet and Grenache from Béziers and Syrah, Grenache and Carignan from Vauvert in 2006. One hectare at each vineyard was treated with the biological treatment FEN 560 (Soft, Port La Nouvelle, France; 4.5 kg/ha), with three separate treatments at two week intervals before harvesting, while a nearby reference hectare was treated by the traditional chemical treatment of a mixture of anti-oidium Sofral® (10 kg/ha) and anti-mildew Copral® (3 kg/ha) sprayed in seven treatments every two weeks from April to July.

Table 1
Samples analyzed in 2004–2006 indicating origin: production zone, grape varieties, and treatment.

Harvest year	Production zone	Grape varieties	Treatment	
			Chemical[a]	Biological[b]
2004	Béziers	Syrah	x	x
	Vauvert	Carignan	x	x
2005	Béziers	Cabernet	x	x
		Grenache	x	x
2006	Béziers	Cabernet	x	x
		Grenache	x	x
	Vauvert	Carignan	x	x
		Grenache	x	x
		Syrah	x	x

[a] A mixture of anti-oidium, Sofral® (10 kg/ha) and anti-mildew Copral® (3 kg/ha) with seven treatments every two weeks from April to July.
[b] FEN 560 (SOFT, Port La Nouvelle, France) (4.5 kg/ha) with three treatments every two weeks before harvest.

2.2. Samples

Grapes were harvested in September each year. Samples of 3 kg of bunches were randomly collected for each sampling time in each vineyard. The samples were transported to the laboratory in closed plastic bags, protected in cool boxes. Mycological analyses were done immediately and remaining samples kept frozen at −20 °C.

2.3. Mycological analysis of grapes

2.3.1. Media

Because of the macroscopic similarities of the black mould colonies isolated from grape, the first step of this work was to select a culture medium that could facilitate colony differentiation. Five media were selected for their suitability for macroscopic differentiation of aspergilli (Samson et al., 1995): PDA (potato–dextrose agar) (Biokar Diagnostics, Beauvais, France), YM (Yeast malt agar), DRBC (Dichloran rose Bengal chloramphenicol agar), DRYES (Dichloran rose bengal yeast extract sucrose agar), CZ (Czapek agar) and DG18 (Dichloran 18% Glycerol agar) (Pitt and Hocking, 1999; Samson et al., 1995).

2.3.2. Enumeration

To estimate the total and black aspergilli fungal populations, 50 g of grapes were randomly selected in triplicate, mixed with 450 mL of sterile water and homogenized with a blender for 1 min (Lab-Blender 400, Seward Medical UAC House, London, UK). Then, 1 mL of the suspension was diluted in 9 mL sterile physiological water (NaCl 8.5 g/L) containing 0.01% Tween 80 (Merck Germany). Fungal populations were quantified by plating a 6-fold dilution series of each suspension by spreading 0.1 mL of each dilution on PDA plates and then incubating at 25 °C for 5 days. The results were expressed in CFU/g (colony forming units) (ISO 7218:1996/Amd.1: 2001(F)) (AFNOR, 2002).

2.3.3. Isolation and identification

Five berries were randomly selected from each grape sample and aseptically put directly onto the surface of culture medium (PDA). The plates were incubated at 25 °C for 7 days. Black spore-producing filamentous fungi were isolated and identified to genus and species level by morphological criteria; color, density and colony appearance (layer color, wrinkled, umbilical, thick or flat) and microscopic observation (conidial head, conidiophore and conidia characters) in accordance with appropriate keys (Pitt and Hocking, 1999; Samson et al., 1995, 2007). The main black aspergilli found during the process of enumeration were also isolated and identified.

2.4. OTA production and extraction from mould culture

Conidia suspensions of each isolate were prepared in 1% Tween 80 in physiological water (v/v). They were adjusted to 10^6 conidia per mL and enumerated with a Thoma haemocytometer (0.0025 $mm^2 \times 0.1$ mm). Five microliters of the adjusted suspension were point inoculated at the center of PDA and DG18 media and incubated at 25 °C (Chulze et al., 2006; Suarez-Quiroz et al., 2004a).

After 3, 5, 10, 15 and 20 days of incubation, colony diameters were measured and three agar plugs of 5 mm in diameter were removed from the middle of the colonies. The plugs were weighed, introduced into 3 mL vials and extracted with 2.5 mL of solvent (methanol/formic