Food and Chemical Toxicology 46 (2008) 2244–2250

Contents lists available at ScienceDirect

Food and Chemical Toxicology

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

Fungal contamination and Aflatoxin B1 and Ochratoxin A in Lebanese wine–grapes and musts

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ARTICLE INFO

Article history:
Received 14 May 2007
Accepted 29 February 2008

Keywords:
Aflatoxin B1
Ochratoxin A
Black aspergilli
Aspergillus flavus
Grapes
Must
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ABSTRACT

Five hundred and ten strains of filamentous fungi were isolated from Lebanese grapes during 2005 at veraison and harvesting periods. Four hundred eighty-seven isolates belonged to the *Aspergillus* spp. (95.5%) and 23 belonged to the *Penicillium* spp. (4.5%). Black aspergilli constituted 56.9% (52.2% *Aspergillus niger* aggregates, 2.9% *Aspergillus japonicus and* 1.8% *Aspergillus carbonarius*) while the isolation rate of *Aspergillus flavus* the none habitual member of grape mycobiota was 43.1% of the total *Aspergillus* spp. isolated. All isolates were tested for the ability to produce the Ochratoxin A (OTA) and the Aflatoxin B1 (AFB1). *A. carbonarius* showed that it is the only species able to produce the OTA with a production ability of 100% and a maximum concentration reaching 8.38 μg/g CYA. As for the aflatoxigenic ability, 43.4% of *A. flavus* isolates produced this mycotoxin with a maximum production reaching 22.6 μg/g CYA while none of the other isolates showed a production capacity of this mycotoxin. Forty-seven samples of must produced from the collected grapes were also analyzed. None of these samples was contaminated by OTA at a detectable limit while 40% of these same samples were found to contain AFB1 with concentrations ranging from 0.01 to 0.46 μg l^{-1}.

1. Introduction

Ochratoxin A (OTA) is a mycotoxin with nephrotoxic, teratogenic, and immunosuppressive properties (Pitt et al., 2001). It has been classified in 1993 by the International Agency for Research on Cancer (IARC) as a possible human carcinogen (group 2 B) (IARC, 1993). OTA is suspected to be involved in the Balkan endemic nephropathy (BEN) (a fatal kidney disease occurring in some areas of south-eastern Europe) and in the high frequency of urinary tract tumors observed in some Balkan areas (Pfohl-Leszkowicz et al., 2000).

Surveys conducted in many different countries revealed the presence of OTA in different foodstuffs (Burdaspal and Legarda, 1999; Pietri et al., 2001; Sage et al., 2002) such as cereals, coffee beans, pulses (Jørgensen, 1998; Kuiper-Goodman and Scott, 1989; Micco et al., 1989; Pohland et al., 1992) wheat, barley, maize, and oats (Speijers and Van Egmond, 1993; Trucksess et al., 1999), spices (Hubner et al., 1998), meat and cheese products (Gareis and Scheuer, 2000).

Aflatoxins (AFs) are a group of a highly toxic secondary metabolites produced by the filamentous fungi *A. flavus* and *A. parasiticus* (Deiner et al., 1987; Kutrzman et al., 1987) and to a lesser extent *A. nominus*, *A. tamarii* (Goto et al., 1997) and *A. pseudotamarii* (Ito et al., 2001). Under favorable conditions of temperature and humidity, these fungi grow on certain foods and feeds, resulting in the production of these metabolites.

Aflatoxins are considered as natural contaminants of a variety of agricultural products. The most pronounced contamination has been encountered in corn, peanuts, cottonseed, and other grain crops being most frequently contaminated (Gourama and Bullerman, 1995; Jelinek et al., 1989). Diet is the major way through which humans as well as animals are exposed to these mycotoxins. Apart from this, exposure to aflatoxins can be through ingestion of contaminated food and feed.

The major AFs of concern are designated as B1, B2, G1 and G2. However, Aflatoxin B1 (AFB1) is the most predominant and the most toxic aflatoxin with a toxicity demonstrated in all species of

Abbreviations: OTA, Ochratoxin A; AFB1, Aflatoxin B1; AF, Aflatoxin; BEN, Balkan endemic nephropathy; *A.*, *Aspergillus*; HPLC-FLD, high performance liquid chromatography with fluorescence detector; CYA, Czapek yeast extract agar; DRBC, dichloran rose Bengal chloramphenicol agar; IARC, International Agency for Research on Cancer; Ex, excitation; Em, emission; MRLs, maximum residue limits.

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doi:10.1016/j.fct.2008.02.026

animals tested, resulting in LD_{50} values ranging from 0.3 to 9.0 mg/kg body weight. AFB1 is also known as being one of the most potent genotoxic agent and hepatocarcinogen identified (IARC, 1993; Busby and Wogan, 1984; Sharma and Salunkhe, 1991; Miller and Trenholm, 1994; Wang et al., 1998).

The European Commission set the maximum level for AFB1 in foods to 2 ng/g, although new limits are likely to be established at 1 ng/g (Stroka and Anklam, 2002).

Grapes can be contaminated with a wide variety of moulds including *Aspergillus* and *Penicillium* genera. Among *Aspergillus* genus, black aspergilli seemed to be the most common contaminant of grapes namely *A. niger* aggregates and *A. carbonarius* the most known Ochratoxin A-producing species. Reports by several authors showed that grape and its derived products such as dried vine fruits (MacDonald et al., 1999), grape juices and wines (Burdaspal and Legarda, 1999; Jørgensen, 1998; Zimmerli and Dick, 1996; Visconti et al., 1999; Otteneder and Majerus, 2000) were highly contaminated by the ochratoxin A.

However, until now, there is no available information on the occurrence of the AFB1 on grapes and its derived products since the studies conducted in other Mediterranean countries revealed a very low occurrence of *A. flavus* in the vineyards (Martinez-Culebras and Ramon, 2007; Medina et al., 2005; Melki Ben Fredj et al., 2007). However, in these studies, no quantification of the AFB1 in grapes or its products was performed.

This current document provides, in a timely manner, data on the occurrence of the AFB1 and the OTA-producing species on Lebanese grapes and on the levels of these two mycotoxins in the derived musts.

2. Materials and methods

2.1. Study area

Lebanon is located between 35° and 36°40′ longitude east and between 33° and 34°40′ latitude north on the eastern Mediterranean shores. It has a rectangular shape with 10.452 km^2 area. Seven winemaking regions were chosen for this study in the center and the south of the Bekaa plain since it is a good representative sample for the whole situation in Lebanon according to the study (Ksara, Tanayel, Tal Dnoub, Itany, Mansoura, Kefraya and Kanafar) (Fig. 1).

2.2. Grape samples

Twenty-seven vineyards belonging to the study area were chosen. From each vineyard, Grapes were collected from July to October at 2 developmental stages of the berries: early veraison (mid-July, end of August) and ripe berry (September, mid-October) during the year 2005. The sampling protocol consists of taking from each vineyard a number of 10 bunches along two crossing diagonal transects. Bunches were kept in sterile bags and transported within 2–3 h in cooled boxes (4 °C) to the laboratory for analysis.

2.3. Culture media

The culture media used for fungi isolation and identification, were Dichloran Rose Bengal Chloramphenicol (DRBC) agar (Oxoid, Basingstoke, Hampshire, England), which contained per litre of distilled water: 10 g Glucose (Fisher Labosi Elancourt cedex, France); 5 g meat peptone (Fisher Labosi Elancourt Cedex, France); 1 g KH_2PO_4 (Acros Geel, Belgium); 0.5 g $MgSO_4$–$7H_2O$ (Acros Geel, Belgium); 25 mg Rose Bengal; 2 mg Dichloran; 100 mg Chloramphenicol and 15 g agar (Difco, Fisher Labosi) (Pitt and Hocking, 1997). Czapek Yeast extract (CYA) (CZAPEK Yeast extract Agar, Oxoid) agar which contained per litre of distilled water: 30 g sucrose (Fisher Labosi Elancourt cedex, France); 1 ml trace metal (Cu + Zn) solution (Fisher Labosi); 1 g K_2HPO_4 (Acros Geel, Belgium); 10 ml Czapek concentrate; 5 g yeast extract (Difco, Fisher Labosi) and 15 g agar (Difco, Fisher Labosi) (Pitt and Hocking, 1997).

2.4. Fungi isolation

Five berries were randomly taken from each bunch and directly plated onto (DRBC) medium in Petri dishes. All plates were incubated for 7 days at 25 °C. After which all species belonging to the genera *Aspergillus* and *Penicillium* were isolated. For identification and morphological observations, isolated species were cultured on (CYA) medium pH 6.7 and the identification was performed according to standard taxonomic systems based on the shape of conidiophores and conidia's dimension as observed with a binocular microscope with 100× magnification.

2.5. AFB1 and OTA-producing ability

Aspergillus and *Penicillia* isolates were grown in Petri dishes containing (CYA) medium pH 6.7 for 7 days at 25 °C. Three agar plugs of the solid medium were removed from different point of the colony for each culture, weighted and collected into small tubes. A volume of 0.9 ml of methanol was added to each tube, and the tubes were left stationary for 1 h. Extraction was done after 20 min of centrifugation at 13,000 rpm for each tube and the obtained extracts were filtered through a 0.45 μm Millipore filter into small vials then analyzed and quantified by HPLC/FLD. The quantification of OTA and AFB1 was compared with that of standards of these two mycotoxins.

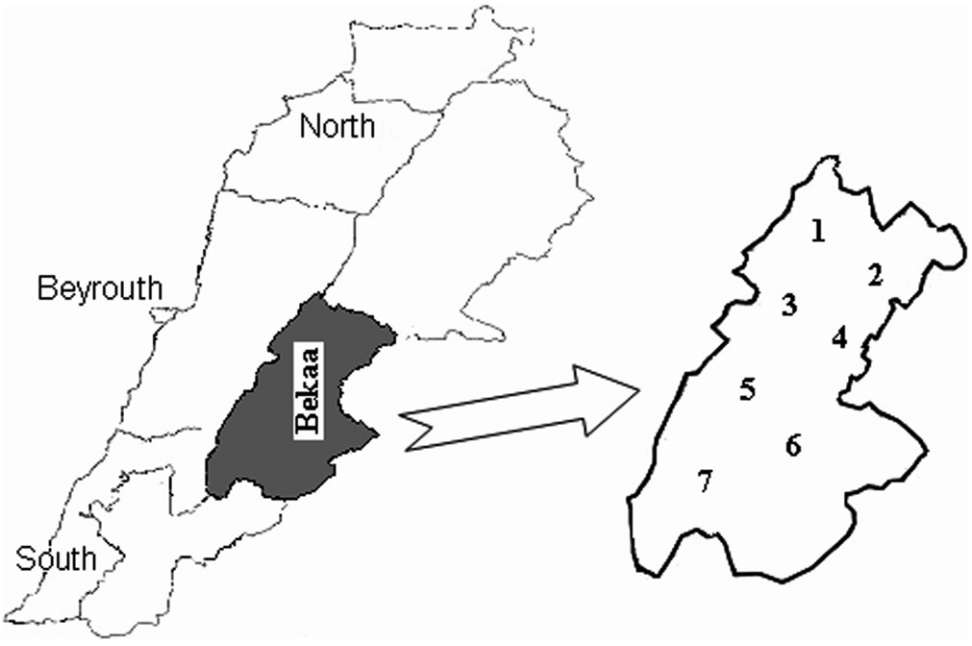

Fig. 1. Origin of samples from Bekaa valley. [1]ksara, [2]Tanayel, [3]Itany, [4]Mansoura, [5]Tal Dnoub, [6]Kefraya, [7]Kanafar.