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RESEARCH PAPER

Aspergillus spp. and their secondary metabolite production in grape berries from Slovakia

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Summary. Occurrence of *Aspergillus* spp. in grapes from Slovak has been surveyed during 2 years, to assess their ability to produce secondary metabolites, *in vitro* and in berries. A large number of *Aspergillus* spp., including *A. flavus, A. japonicus, A. niger, A. carbonarius* and *A.ibericus*, have been identified, and strains randomly selected and analysed to verify their potential ability to produce secondary metabolites. A broad spectrum of fungal metabolites has been observed *in vitro* and have been analysed using HPLC-MS/MS. *Aspergillus niger*, the most frequently isolated species, produced ochratoxin A (in a range from 104 to 1745 μg L^{-1}) and fumonisin B_2 (93 μg L^{-1}). *Aspergillus carbonarius* produced ochratoxin A in the range 138 to 2031 μg L^{-1}. Ochratoxin A and fumonisins were not detected in grape berries. Other secondary metabolites, including emodin, 3-nitropropionic acid, kojic acid and malformin C, were also detected at different concentrations in berries, but their occurrence was dependant on the geographic area of origin and on the year of berry collection.

Key words: microfungi, ochratoxin A, fumonisin B_2, HPLC-MS/MS.

Introduction

The presence of microfungi of the genus *Aspergillus* represents a serious risk for grape, wine and dried fruits because of the possible production of harmful mycotoxins by these fungi (Perrone *et al*., 2007; Somma *et al.*, 2012). The black *Aspergillus* spp. are considered amongst the most widespread contaminants of food and feed. Their interest as potential grape berries contaminants has been highlighted after ochratoxin A (OTA), namely *N*-{[(3*R*)-5-chloro-8-hydroxy-3-methyl-1-oxo-3,4-dihydro-1*H*-isochromen-7-yl]carbonyl}-L-phenylalanine, was detected in wine (Zimmerli and Dick, 1996). Figure 1 shows the chemical structure of OTA, and this mycotoxin is a derivative of an isocoumarin. Ochratoxin A is a potent toxin affecting kidney function in humans and animals (Pfohl-Leszkowicz and Manderville, 2012). The toxin has been identified mainly in cereals and cereal products, but also in meat, in grapes and in many food commodities (Hayat *et al.*, 2012). Ochratoxin A can cause contamination in grape products, including must or wine (Cabañes *et al.*, 2002; Bellì *et al.*, 2004), and represents a risk of exposure to this toxic metabolite. The major risk of mycotoxin contamination in grapes, and the possible presence of toxic secondary metabolites, at a global level, comes from *Aspergillus* rot. Infestation by *Aspergillus* species, depending on the year and the season, can be at high levels. Recently, the presence of fumonisins B_2 (FB_2) and B_4 (FB_4), produced by *A. niger* on grapes and raisins, has also been reported (Logrieco *et al.*, 2009; Mogesen *et al.*, 2012).

Considering the increasing interest in grape berries in diet for their content of antioxidant com-

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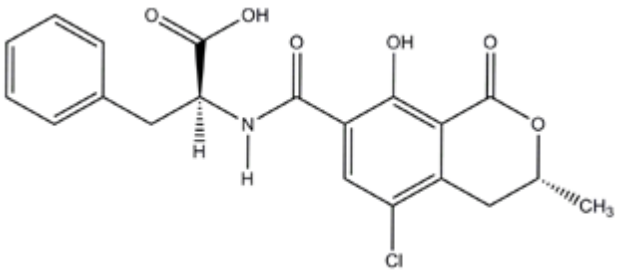

Figure 1. Chemical structure of ochratoxin A.

pounds and nutraceuticals, for prevention and protection from severe pathological conditions (Palomino *et al.*, 2000; Ajila and Brar, 2012), and also the widespread consumption of wine and wine derived beverages, the aim of the present study was to assess the production of secondary metabolites by *Aspergillus* strains isolated from grape berries and the presence of these secondary metabolites *in vitro* and in dry grape berries. Strains were selected from different Slovak grape growing regions, during the years 2008 and 2009, and in two different periods, during veraison and at harvest.

Slovakia is a country with a rich wine production history and good climate conditions, which favour the vine growth. Slovak vineyards are represented, under the European territorial segmentation, by six wine regions for a total of approximately 220 km^2 grapevine cultivated areas and are classified as Zone B according to the Europe Council Regulation (EEC n. 822/87). These regions are defined based on appropriateness of the conditions for achieving the average alcohol percentage formed naturally in usual manufacturing conditions and when usual technology is adopted for wine production, grapes growing and processing. Europe includes three wine-growing zones: A, B, and C. In particular, Zone A includes Luxembourg, Germany Mid-Rhine, the Saar, Moselle, Bavaria, Neckar; Zone B includes France, Champagne and Alsace, Germany, Upper Rhine, Baden, Palatinate, Switzerland, Austria, Czech Republic, Slovak Republic, Hungary, Romania, Bulgaria, Moldova, Ukraine; Zone C1 includes Central France, Northern Italy and Spain; Zone C2 includes Southern France, Italy and Spain, and Zone C3 includes Greece, Portugal and Southern France.

Materials and methods

Three Slovak winemaking regions: Small Carpathians (SC), Nitra (N), and Southern Slovakian (SS) (representing a total grape-growing area of approximately 17,0 km^2) were chosen for their climatic differences and national economic importance. Grapes were collected twice during each year (2008 and 2009) at veraison (in July) and ripening (in September). One sample was collected from each of the three regions. Sample collection and handling was performed as described by Mikušová *et al.* (2012). At each sampling time and in each vineyard, three bunches of grapes were randomly collected and brought to the laboratory in closed on paper bags. Cooled boxes were used for transport of the bunches. Samples were analysed within 24h from the collection. Each sample from different locations represents a single occurrence of fungi. Part of the berries from each bunch were dried in laboratory, in a thermostat (FTC 90 E) at 25°C, for 5 d, until the weight of berries remained constant.

Identification and extraction of secondary metabolites

The inoculum obtained from a suspension of spores was three-point inoculated on malt extract agar (MEA) in 9 cm diam. plastic Petri dishes (PD) and incubated. After incubation, randomly selected strains of *Aspergillus* section *Niger* were inoculated onto selective media: including MEA, Creatine agar (CREA), Aspergillus differentiation agar (ADA) for identification of *Aspergillus* species, and on Czapek yeast agar (CYA) and Yeast extract agar (YES), which are suitable media for identification of extracellular and intracellular metabolites (Samson and Frisvad, 2004). To obtain isolates a dilution method was used. Samples were mixed with sterile distilled water and a series of dilutions were made. From the dilutions, 1 mL volumes were transferred onto suitable *Aspergillus* differentiation media. Fungal colonies were counted and screened for *Aspergillus* species. Identification, using macro and micro morphological characteristics using microscopy, was as described by Mikušová *et al.* (2010). Isolates were lodged in the "Agriculturally important toxigenic fungi and mycotoxins" collection, at ISPA Institute of the CNR in Bari, Italy.

Analysis of secondary metabolites

Fungal cultures were extracted using a micro-scale extraction method based on 6 mm agar plugs from fungal cultures (Smedsgaard, 1997). Two agar plugs were cut out of each colony (CYA, YES) from the centre and from the edge of the colony. The plugs were placed in a 2 mL Eppendorf vessel; 1 mL of solvent solution (chloroform:methanol; 2:1) was added, and the mixture stirred using a Vortex device for 3–5 min; Grape berries were also extracted as follows: 5 g of berries were mixed with 20 mL of a mixture acetonitrile:water:acetic acid (79:20:1) as extraction solvent, and stirred for 1 h. The extracts were filtered using a polytetrafluoroethylene PFTE (0.45 μm) filter, and dried. Subsequently, 500 μL of a solution acetonitrile:water (1:1) were added and the solution obtained was used analysed. HPLC-MS/MS analysis was carried out on a QTrap 4000 (AB Sciex equipped with a TurboIonSpray electro spray ionisation (ESI) source and connected to an Agilent 1100 HPLC device (Agilent). The separation achieved on a 150 × 4.6 mm i.d. Gemini C_{18} column equipped with a 4 × 3 mm security guard cartridge (Phenomenex). The chromatographic approach, as well as chromatographic and mass spectrometric (MS) parameters for 186 of the investigated analytes, was carried out according to Vishwanath *et al*. (2009).

ESI-MS/MS was performed in the time-scheduled multiple reaction monitoring (MRM) mode both in positive and negative polarities in two separate chromatographic runs per sample by scanning two fragmentation reactions per analyte. The MRM detection window of each analyte was set to its expected retention time ± 30 sec in the positive mode, and ± 60 sec in the negative mode. Confirmation of positive analyte identification was obtained by the acquisition of two MRMs per analyte (with the exception of 3-nitropropionic acid, that exhibits only one fragment ion), which yielded 4.0 identification points according to the European Commission decision 2006/657/EC (2006). Liquid chromatography (LC) retention time agreed with the related values of the standard within 0.1 min, and the intensity ratio agreement of the two MRM transition was 30%.

All samples were analysed in triplicate and average results are reported. A detection limit (LOD) and quantification limit (LOQ) of mycotoxins of 5 μg L^{-1} was determined on the basis of a certified standard.

Results and discussion

Table 1 lists the *Aspergillus* species isolated from grape berries at veraison time (July) and at harvest time (September) in 2008 and 2009. The geographic regions from which the fungi were isolated are also indicated.

The greatest number of contaminated samples were obtained from Nitrian and Southern Slovakian regions. These areas are characterized by hot and dry climate, and this confirms the greater presence of *Aspergillus* in areas with high average temperatures (Serra *et al*., 2006a). Species of section *Flavi* and *Niger* were also present in the samples. Table 2 lists selected *Aspergillus* strains isolated from grape berries and their observed metabolites produced *in vitro*. Only one strain from section *Flavi, Aspergillus flavus*, developed an intense yellow orange colour

Table 1. Aspergillus strains isolated from Slovak grape berries. Geographic regions are indicated as: Small Carpathians SC, Southern Slovakia, SS, Nitra, N. The symbols + and – stand for isolated and absent, respectively.

Aspergillus species	Year	Region	July (veraison)	September (harvest)
A. japonicus	2008	N	+	-
A. niger	2008	SS	+	-
A. niger	2008	SS	+	-
A. niger	2008	N	+	-
A. niger	2008	N	+	-
A. niger	2008	N	+	-
A. niger	2008	N	+	-
A. niger	2008	N	-	+
A. niger	2008	N	-	+
A. niger	2009	SS	-	+
A. niger	2009	SS	+	-
A. niger	2009	SC	+	-
A. niger	2009	SC	-	+
A. niger	2009	N	-	+
A. carbonarius	2008	SS	+	-
A. ibericus	2009	SS	+	-
A. flavus	2009	SS	-	+

at the base of the colonies (on ADA), a characteristic considered typical for this species. This strain produced 550817 μg L^{-1} of kojic acid only on YES medium, but no aflatoxins were found. Figure 2 shows the chemical structure of kojic acid together with some of the other metabolites identified in this study. Remaining isolates of *Aspergillus* strains belonged to the section *Niger*. Uniseriate *A. japonicus* Saito, produced 0.655 μg L^{-1} of festuclavine. This species was able to grow at 36°C; grew quite well on CREA, and produced acid. At first observation of the microscopic characteristics, vesicle size ranged from 20 to 30 μm diam. and the conidia were subglobose to globose and 3.0–4.5 μm in diameter. This fungus was identified as *A. uvarum*, a uniseriate species previously isolated from grapes in Europe (Perrone *et al*., 2008). Microscopic observations of specimens stained with cotton blue with lactophenol (Mikušová *et al*., 2010) allowed fungal identification, according to conidium size and shape, as well as the colour of the cultures.

Aspergillus niger van Tieghem was the most frequently isolated species. This biseriate species grew well on ADA, with pale yellow colour on the underside of colonies and black heads on colony upper surfaces. As shown in Table 2, two strains produced 103.5 µg L^{-1} of malformin C *in vitro*, and only one of these produced 1745 μg L^{-1} of OTA and 92.5 μg L^{-1} of fumonisin B_2. This strain was obtained in 2009 only in Southern Slovakia during veraison. Other strains of *A. niger* were isolated mainly in 2008 as reported in Table 1, and mainly at the veraison stage. The most frequent isolations were made from samples

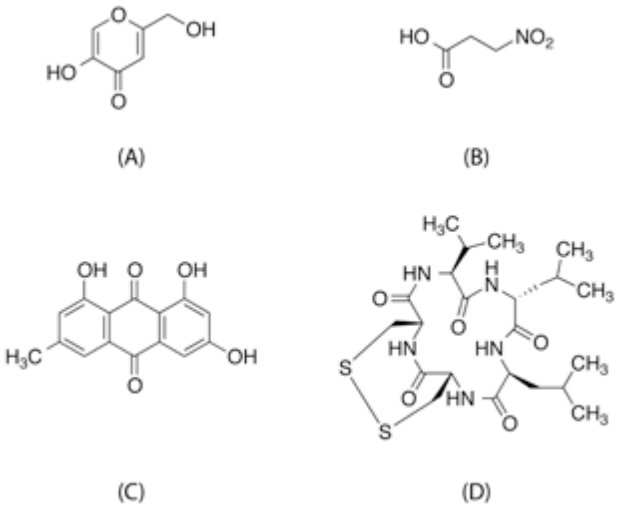

Figure 2. Chemical structures of kojic acid (A), 3-nitro propionic acid (B), emodin (C), malformin C (D).

Table 2. Secondary metabolites produced *in vitro* from randomly selected *Aspergillus* strains isolated from grape berries. OTA (ochratoxin A), FB_2 (fumonisin B_2). Concentration is expressed as μg L^{-1}.

Aspergillus species	Growth media	Kojic acid	Festuclavine	Malformin C	OTA	FB2
A. flavus	CYA	-	-	-	-	-
	YES	550817	-	-	-	-
A. japonicus	CYA	-	0.655	-	-	-
	YES	-	-		-	-
A. niger	CYA	-	-	0.671	-	-
	YES	-	-		-	-
A. niger	CYA	-	-	0.351	-	-
	YES	-	-	-	-	-
A. niger	CYA	-	-	-	103.5	-
	YES	-	-	-	1745	92.5
A. carbonarius	CYA	-	-	-	138	-
	YES	-	-	-	2031	-

originating from Nitrian region, and these did not produce secondary metabolites.

Only one strain of *A. carbonarius* Bainier produced OTA in amounts ranging from 138 to 2031 $\mu g\ L^{-1}$ (Table 2). *Aspergillus carbonarius* is considered the main ochratoxigenic species present on grapes that can result in OTA contamination in the major wine-producing countries (Cabañes *et al*., 2002). This species occurs widely in Mediterranean countries, but with a low production of OTA (Serra *et al*., 2005). The recent observation of secondary metabolites produced by this species has identified the production of fumonisins B_2 and B_4 (Mogensen *et al*., 2010).

Other *Aspergillus* strains of the section *Nigri*, which did not produce OTA, have been identified as *A. ibericus* (Serra *et al.*, 2006b). Both *A. carbonarius* and *A. ibericus* have black biseriate aspergilli with long stipes and relatively large conidia (> 5 μm) when compared to the species of the *A. niger* aggregate (35 μm). Among the 15 species in the section *Nigri* accepted by Samson *et al.* (2007) in the most recent revision, this taxon had conidia of greater than 6 μm diam, namely *A. carbonarius* (7–9 μm) and *A. ibericus* (6–9 μm).

Metabolites produced from *Aspergillus* were also present in dry berries (Table 3). Emodin, malformin C, 3-nitropropionic acid and kojic acid were detected in berries; while three other secondary metabolites, festuclavine, OTA and FB_2, were not observed in dry berries but were only produce *in vitro* by fungi. Emodin was present in all samples of dry berries, but in at lower concentrations than kojic acid. The observed amounts of emodin ranged between 0.5 $\mu g\ kg^{-1}$ and 55.6 $\mu g\ L^{-1}$. This compound can be a metabolite from *A. wentii* (Wells *et al*., 1975; Rodriquez *et al*., 2011) and *A. ochraceus* (Lu *et al*., 2010), but these species were not been isolated from any of the samples analysed in the present study. Kojic acid has been reported as having the ability to reduce inflammation and has been shown to be active against some types of cancer cells (Ghosh *et al*., 2010). Emodin (1,3,8-trihydroxy-6-methylanthracene-9,10-dione) is a natural compound belonging to the anthraquinone family. It occurs naturally either free or combined with a sugar in a glucoside, in rhubarb, cascara sagrada, aloe, and other plants. It has been found to have many activities and antitumour effects on human cells (Ma *et al*., 2012).

Three *Aspergillus* metabolites were isolated from samples during the two seasons for both years 2008 and 2009. Kojic acid was present in all samples, with concentrations ranging from 504 to 13560 $\mu g\ L^{-1}$. Malformin C, a product of *A. niger*, was found in a few samples, with a maximum concentration of 2108 $\mu g\ L^{-1}$ (Table 3). Concentrations observed for 3-nitropropionic acid were between 21.3 and 2128 $\mu g\ L^{-1}$. The metabolites produced from dry grape berries can be associated with *Aspergillus* strains isolated from grape berries. Kojic acid is produced by many strains from the genera *Penicillium* and *Aspergillus*. The production of this extracellular secondary metabolite by *A. flavus* has been known for many years (May *et al*., 1931), but only recently has this compound been recognized as a macrophage activator (Rodriques *et al*., 2011).

Aspergillus flavus strains produced kojic acid; this metabolite was extracted *in vitro* only from YES, a medium shown previously to be suitable for production of extracellular metabolites (Filtenborg and Frisvad, 1980). The presence of 3-nitropropionic acid in dry berries may also derive from *A. flavus*. It has been proposed that this metabolite is either an intermediate or it is in equilibrium with an intermediate in nitrification caused by the fungus (Doxner and Alexander, 1966). Malformin C has been associated with the presence of *A. niger* strains (Samson et al.,

Table 3. *Aspergillus* secondary metabolites isolated from dry grape berries. Concentration is expressed as $\mu g\ kg^{-1}$. SC (Small Carpathians), SS (South Slovakia), N (Nitra).

Region	Year	Emodin	Malformin C	3-nitropropionic acid	Kojic acid
SC	2008	19	-	-	3000
SC	2008	1.7	2108	-	2336
N	2008	1.18	-	2128	1792
N	2008	17.5	-	21.3	504
SS	2008	0.5	-	-	1844
JS	2008	1.18	3.77	-	3756
SC	2009	11.6	-	-	4240
SC	2009	13.6	-	-	13560
N	2009	55.6	2.57	271	1236
N	2009	4	-	239	1760
SS	2009	15.2	-	-	4720
SS	2009	2.42	-	-	3060

2007). The most important secondary metabolite associated with grape berries is, however, the OTA, and our observations confirm low production of this metabolite and its absence in dry grape berries. This indicates that there is low risk of OTA contamination of Slovak wine. This conclusion disagrees with reported survey data, where OTA has been detected in more than 50% of analysed wine samples (Belajová and Rauová, 2007). Ochratoxigenic microfungi have been isolated from analysed samples and they have potential toxicity, but they were not found in the grape berries in a similar way to reports from grapes in the Czech Republic, a Slovak neighbouring country (Ostrý *et al*., 2007).

Conclusions

The presence of *Aspergillus* spp. in grapes represents a serious potential risk due to the possible formation of secondary toxic metabolites. *Aspergillus flavus, A. japonicus, A. niger, A. carbonarius and A. ibericus* representative strains were found to produce a broad spectrum of fungine secondary metabolites *in vitro*. *Aspergillus niger* and *A. carbonarius*, in particular, produced OTA and fumonisin B_2. No traces of OTA and fumonisins were found on dry berries, however, while emodin, 3-nitropropionic acid, kojic acid and malformin C were detected. These results confirm low contamination of Slovak grape berries and, consequently, low likelihood of contamination of wine and grape berry products from this country. More strains of toxigenic fungi were observed at the veraison and ripening berry growth stages (temperature range from 17 to 22.5° C, humidity from 58 to 80%, and precipitations from 0.7 to 8.4 mm). Further research is required to fully assess the influence and the relationships between the climatic conditions (temperature, humidity and precipitation in the course of a season) and the presence of microfungus contaminants of grape berries.

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