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MICROFUNGI AND MYCOTOXINS OF GRAPES FROM EASTERN SLOVAK WINE REGION

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ABSTRACT

The current study investigated an endogenous mycobiota of grapes in Eastern wine region, Slovakia and detection a potentially pathogenic isolates to produce selected mycotoxins. Intact berries from four wine grape cultivars were tested. Seven/eight berries superficially sterilized from each samples were placed on a Dichloran Rose Bengal Chloramphenicol agar in a total of 50 and incubated at 25 °C, 5 - 7 days. A total of 582 isolates were obtained that belonged to ten genera: *Alternaria, Aspergillus, Botrytis, Cladosporium, Epicoccum, Fusarium, Mucor, Penicillium, Rhizopus, Trichoderma* and one unidentified genus *Mycelium sterillium* without creation fruiting bodies. The most frequent were genera *Alternaria, Aspergillus, Botrytis* and *Penicillium* with 100 % frequention. The most frequent *Aspergillus* species was *Aspergillus* section *Nigri* (100 %) and *Penicillium* species was *Penicillium chrysogenum* (50 %). The largest number of isolates belonged to *Alternaria* (275 isolates), *Cladosporium* (114 isolates) and *Penicillium* (92 isolates). For that reason the relative density of both genera were the highest 42 %, 19.6 % and 15.8 %, respectively. The selected isolates – *Aspergillus flavus*, *Aspergillus* section *Nigri*, *Penicillium citrinum, Penicillium expansum* and *Penicillium chrysogenum* were tested for patulin, citrinin, penitrem A, roquefortin C, ochratoxin A, aflatoxin B_1, G_1 and cyclopiazonic acid producing ability. Out of 11 strains 54 % produced at least one mycotoxin. In our research ochratoxigenic microfungi (some species of *Aspergillus* section *Nigri*) were found in grape samples but without production of OTA

Keywords: Wine grapes, Slovakia, toxigenic fungi, mycotoxins

INTRODUCTION

Most vineyards in Eastern wine region lies on gentle slopes of Vihorlat and hilly outskirts of the Eastern lowlands. Under Vihorlat are different types of soils from the heavy clay-loam to light sandy soils on the four wine areas. The vineyards extend up to an altitude of 180 meters above sea level and are in most windiness. Wine with a fine aroma may, in these northern and harsher conditions, also create well, but it must be ensured that the grapes on shrubs in late fall is a well matured. The climate is continuous, stable, warm and slightly moist, slightly dry up, with colder winters. Rainfall during the growing season reaches the average of 373 mm and average temperatures are around 16.6 °C (**Novotný, 2007**).
The concern about filamentous fungi in the vineyard has traditionally been linked to spoilage of grapes due to fungal growth. Two main genera are responsible for mycotoxin production in grapes: *Aspergillus* and *Penicillium*, but *Aspergillus* are important in hot and dry climates, where black *Aspergillus*, namely *A. carbonarius*, can lead to achratoxin A production in grapes (**Serra *et al.*, 2006**). The mycotoxin production is characteristic of the species and therefore by identifying the species one can predict potential mycotoxin hazards. The treat to wines by mycotoxins, in particular ochratoxin A (OTA), instigated detailed studies of the grape mycobiota. Surveys of the fungi to which grapes are exposed in the vineyard were conducted in the main wine-producing countries in Europe such as France (**Sage *et al.*, 2004**), Greece (**Tjamos *et al.*, 2004**), Italy (**Battilani *et al.*, 2003**), Portugal (**Abrunhosa *et al.*, 2001, Serra *et al.*, 2003**) and Spain (**Bau *et al.*, 2005**), and also in countries such as Australia (**Leong *et al.*, 2004**), Argentina and Brazil (**Rocha Rosa *et al.*, 2002**). Black aspergilli are good representative of the mycobiota associated with grapes, and include a high percentage of ochratoxin-producing isolates particularly within the *Nigri* section (**Cabanes *et al.*, 2002, Battilani *et al.*, 2003**). The occurrence of OTA has been reported frequently in wine and grape juice (**Zimmerli and Dick, 1996, Visconti *et al.*, 1999, Pietri *et al.*, 2001**). It is almost impossible to eliminate the mycotoxin from the grape, mainly because it is highly stable and the producing fungi are an integral part of the microbiota associated with grape (**Bleve *et al.*, 2006**).
In our work the goal was to assess the endogenous fungi of healthy grapes destined for commercial winemaking at harvest time and to analyse recognized mycotoxin-producing fungi for ability to produce some selected mycotoxins.

MATERIAL AND METHODS

Grapes

Four cultivars of wine grapes were collected from the Eastern Slovak wine region, which is divided to 4 subregions. The subregion is the area with the same soil and climate conditions. The grape cultivars, locations of the vineyards, and the sampling times are shown in Table 1. Climatic conditions in Slovakia are relatively moderate during the critical stages of maturation and harvesting of the grapes, as it can be common in the Middle Europe areas. From sampling grape berries, ten intact bunches (no damage and no visible signs of fungal growth) of each grape variety were randomly collected along a cross-section in the vineyard. Each bunch was manually picked by hand using sterilized gloves and placed into sterilized paper bags to prevent cross-contamination. The picked grapes were transported to the laboratory in cooled boxes, and analysed in the shortest time possible, within 24 h of collection.

Table 1 Grape varieties used in the study from Eastern Slovak region

Village	Subregion	Grape variety	Types	Date of harvest	Date of analyses
1. Orechová	Sobranecký	Pinot gris	Wine grape (white)	23.10.2012	24.10.2012
2. Vinné	Michalovský	Green Veltliner	Wine grape (white)	23.10.2012	24.10.2012
3. Streda nad Bodrogom	Kráľovskochlmecký	Traminer	Wine grape (white)	7.11.2012	8.11.2012
4. Hrušov	Moldavský	Alibernet	Wine grape (red)	7.11.2012	8.11.2012

Mycological analysis

The mycobiota of grapes was determined by direct placing of superficially sterilized grains on agar plates. A total of 50 intact berries (7 – 8 healthy berries per bunch) were surface-disinfected in 1 % NaClO for 1 min according methods of **Magnoli *et al.* (2003)** and 3 times rinsed by submersion in sterile distilled water (total amount 1L), dried, plated onto Dichloran Rose Bengal Chloramphenicol agar medium (DRBC) (MERCK, Germany) and incubated at 25 °C in the dark for 5 - 7 days. In this way was determined an endogenous mycobiota. Identification of the obtained isolates was carried out according to macroscopic and microscopic morphological criteria. Different media were used for the taxonomic identification of obtained fungi according to that used for standard strains. Specifically, *Penicillium* and *Aspergillus* strains were identified down to the species level first using Malt extract agar (MEA, **Pitt and Hocking, 1997**), Czapek yeast extract agar (CYA, **Samson *et al.*, 2002a**), Czapek yeast extract with 20 % sucrose agar (CY20S, **Pitt and Hocking, 1997**), Yeast Extract agar (YES, **Samson *et al.*, 2010**), CREA (Creatine-Sucrose agar, **Samson *et al.*, 2010**) and identified to species level according to the manuals of **Klich (2002), Samson *et al.* (2002a), Pitt and Hocking (1997).** Other fungi were identified down to the genus level based on morphological characters as previously described **Pitt and Hocking,** (**1997**).

Data analysis

Occurrence of micromycetes was expressed according to isolation frequency (Fr) and relative density (RD), which are calculated as followed (**González *et al.*, 1999**):

Fr (%) = (ns/N) x 100; RD (%) = (ni/Ni) x 100

Legend: ns – number of samples in which the genus or species is detected; N – total number of samples; ni – number of isolates of a species or genus, Ni – total number of isolated fungi.

Toxinogenity analysis

For the determination of toxinogenity was used thin layer chromatography (TLC) according to **Samson *et al.* (2002b)**, modified by **Labuda and Tančinová (2006).** Extracellular metabolites – ochratoxin A, aflatoxin B_1, aflatoxin G_1, citrinin and patulin were carried out on YES agar and intracellural penitrem A, roquefortin C and cyclopiazonic acid on CYA agar. A few pieces of mycelium with approximate size 5 x 5 mm were cut from colonies and placed in an Eppendorf tube with 500 µL of chloroform : methanol – 2 : 1 (Reachem, Slovak Republic). The content of the tubes was stirred for 5 min. by Vortex Genie ® 2 (MO BIO Laboratories, Inc. – Carlsbad, CA, USA). The volume 30 µL of liquid phase of extracts along with 10 µL standards (Sigma, Germany) was applied on chromatographic plate (Alugram ® SIL G, Macherey – Nagel, Germany). The plate was put into developing solution (toluene : ethyl acetate : formic acid – 5 : 4 : 1, toluene – Mikrochem, Slovak Republic; ethyl acetate and formic acid – Slavus, Slovak Republic). After elution the plate was air-dried. Identification of the metabolites was done by comparison with standards (Merck, Germany). Roquefortin C was visible after spraying with $Ce(SO_4)_2$ x 4 H_2O as an orange spot. Cyclopiazonic acid was visible directly in daylight after spraying with the Ehrlich reagent as a violet-tailed spot. Penitrem A after spraying with 20 % $AlCl_3$ in 60 % ethanol and heating at 130 °C for 8 min as a dark blue spot. Patulin by spraying with 0.5 % methylbenzothiazolone hydrochloride (MBTH), (Merck, Germany) in methanol and heating at 130 °C for 8 min and then detectable as a yellow-orange spot. Directly under UV light with a wavelength of 365 nm was visualized citrinin as a yellow-green-tailed spot, ochratoxin A as a blue-green spot, aflatoxin B_1 as a blue spot and aflatoxin G_1 as a green-blue spot.

RESULTS AND DISCUSSION

Endogenous fungal contamination

Ten contaminating fungal genera were identified: *Alternaria, Aspergillus, Botrytis, Cladosporium, Epicoccum, Fusarium, Mucor, Penicillium, Rhizopus, Trichoderma* and one unidentified genus *Mycelium sterillium.* Table 2 shows the distribution of each contaminating fungal genus found in different grape cultivars. *Alternaria* spp., *Aspergillus* spp., *Botrytis* spp. and *Penicillium* spp. genera were found in all tested subregions with the highest frequency 100 %. The genera isolated in smaller frequency were *Cladosporium* spp., *Fusarium* spp., *Rhizopus* spp. and *Mycelium sterillium* in 75 %, among others. The moulds belonging to the species *Aspergillus niger, Penicillium expansum* and *Penicillium crustosum* are common grape pathogens (**Tournas and Karsoudas, 2005; Diguta *et al.*, 2011**). *Aspergillus flavus and Aspergillus* section *Nigri* were indicated from *Aspergillus* genus and also eight species of *Penicillium* (*Penicillium aurantiogriseum, Penicillium brevicompactum, Penicillium citrinum, Penicillium corylophilum, Penicillium expansum, Penicillium funiculosum, Penicillium chrysogenum* and *Penicillium solitum*). Between them the mention fungi were isolated. The *Aspergillus* section *Nigri*, "black aspergillosis", was isolated with a high frequency (100 %). Black aspergilli were reported as the predominant mycobiota of grapes by many authors (**Cabañes *et al.*, 2002, Da Rocha Rosa *et al.*, 2002, Sage *et al.*, 2002, Battilani *et al.*, 2003**). The predominant from *Penicillium* genus was *Penicillium chrysogenum* (50 %). *Penicillium aurantiogriseum, Penicillium brevicompactum, Penicillium citrinum, Penicillium corylophilum, Penicillium expansum, Penicillium funiculosum* and *Penicillium solitum* were in smaller frequency, isolated in 25 % of the samples. *Penicillium expansum*, represented 29.7 % of the *Penicillium* genus, together with *Penicillium verrucosum, Penicillium glabrum, Penicillium citrinum* and *Penicillium crustosum* from grape berries were collected in three most important Slovakia winemaking areas in the harvest year 2008 (**Mikušová *et al.*, 2010**). **Serra *et al.* (2003)** evaluated the incidence of fungi in Portuguese wine grapes. Penicillia and aspergilli were isolated from 17 % and 22 % of the total sampled grapes, respectively. Predominant mycobiota in Spanish wine grapes according to **Bau *et al.* (2005)** belonged to *Alternaria* spp., *Cladosporium* spp. and *Aspergillus* spp. These three genera were isolated in 75.6 %, 22.5 % and 17.3 % of plated berries, respectively. *Penicillium* spp. were only isolated from 2.3 % of grapes. Mucorales were isolated from 8.3 % of berries and other genera such as *Trichoderma*, *Monilia*, *Acremonium* and *Botrytis* among others, were isolated at frequencies of less than 0.8 %. No fungal growth was obtained from the seeds removed from berries. This leads to the assumption that the contamination by OTA-producing species comes from the surface of the berries and not from the inner fruit. Sixteen *Penicillium* species were identified, but *P. verrucosum*, the OTA-producing species of the genus was not isolated. The infection of grape by *Botrytis cinerea*, often occurs at bloom time, followed by a period of latency without causing disease symptoms, generally until grape berries begin to ripen. After veraison, *Botrytis cinerea* are distributed throughout the pulp and on steams (**Keller *et al.*, 2003**).

The relative density is defined as the percentage of isolates of the species or genus occurring in the analyzed sample (**Guatam *et al.*, 2009**). The most presented genera of all isolates (582) were *Alternaria* spp. (47 %), *Cladosporium* (19.6 %) *and Penicillium* (15.8 %). The most presented species of the 92 *Penicillium* strains isolated were *Penicillium aurantiogriseum* (34 %), *Penicillium corylophilum* (17 %) and *Penicillium brevicompactum* (13 %). **Mikušová *et al.* (2012)** obsereved the occurrence of spoilage fungi in Slovakia. The genera *Penicillium* (present in the range 27 – 54 %) was predominant in harvest time, and it was represented by *P. brevicompactum, P. chrysogenum, P. crustosum, P. expansum, P. palitans, P. polonicum, P. verrucosum, P. citrinum and P. glabrum*. The most important mycotoxin-producing fungi were *Aspergillus* (*A. carbonarius, A. niger, A. citrinum, A. flavus*) contributing for about 33 %, while *Fusarium* fungi constituted up to the 19 % of all the toxigenic fungi. *Alternaria* accounted by itself for the 21 %. Other potential toxigenic species, e.g. *Trichoderma, Cladosporium, Epicoccum, Rhizopus, Ulocladium, Trichothecium* were present, but with a minor contribution. From Portuguese wine grapes were isolated the main *Penicillium* species: *Penicillium aurantiogriseum, Penicillium brevicompactum, Penicillium citrinum, Penicillium crustosum, Penicillium expansum, Penicillium simplicissimum, Penicillium spinulosum* and *Penicillium thomii*, constituting 86 % of the total *Penicillium* strains isolated. None of the 301 penicillia strains were ochratoxigenic species what correspond with our results. Black aspergilli (*Aspergillus carbonarius, Aspergillus japonicus, Aspergillus niger* aggregate) constituted 90 % of the 370 *Aspergillus* strains isolated (**Serra *et al.* 2003**). In Spanish wine grapes *Penicillium citrinum, P. glabrum* and *P. chrysogenum* constituted 18.5 %, 16.9 % and 13.8 %, respectively, of the 65 *Penicillium* strains recovered. Black aspergilli (mainly *A. niger* aggregate and *A. carbonarius*) constituted 92 % of the total *Aspergillus* strains isolated. *Aspergillus niger* aggregate were isolated from 16.9 % of plated berries, and *A. carbonarius* from 3.6 %. The occurrence of the remaining *Aspergillus* spp. ranged from 0.04 % to 0.5 % (**Bau *et al.*, 2005**). In our research the occurrence of black aspergilli were presented in 55 % of all *Aspergillus* strains isolated.

Table 2 The overall endogenous *Penicillium* contamination of tested various types of grape

Microfungi	1.	2.	3.	4.	Total	Frequency (%)	Density (%)
Alternaria	86	16	94	79	275	100	47.2
Aspergillus	4	2	2	1	9	100	1.5
A. flavus	3	1			4	50	0.7*/44**
A. section Nigri	1	1	2	1	5	100	0.9/55
Botrytis	16	18	7	1	42	100	7.2
Cladosporium	4		36	74	114	75	19.6
Epicoccum			1	2	3	50	0.5
Fusarium		1	11	3	15	75	2.6
Mucor			2		2	25	0.3
Penicillium	5	3	61	23	92	100	15.8
P. aurantiogriseum			31		31	25	5.3/34
P. brevicompactum			12		12	25	2.1/13
P. citrinum	1				1	25	0.2/1
P. corylophilum				16	16	25	2.7/17
P. expansum			2		2	25	0.3/2
P. funiculosum				1	1	25	0.2/1
P. chrysogenum	4	3			7	50	1.2/8
P. solitum			2		2	25	0.3/2
P. sp.			14	6	20	50	3.4/22
Rhizopus	1		5	6	12	75	2.1
Trichoderma	5			2	7	50	1.2
Mycelia sterilia		2	5	4	11	75	1.9

Legend: * the percentage of isolates of the species from the total of genera; ** the percentage of isolates of the species from the total of genus; 1. Pinot gris variety, 2. Green Veltliner, 3. Traminer, 4. Alibernet

In total 11 endogenous strains representing 5 potentially toxigenic species were tested for their toxigenic ability, namely *Aspergillus flavus*, *Aspergillus* section *Nigri*, *Penicillium citrinum*, *Penicillium expansum* and *Penicillium chrysogenum* (Tab. 3).

Two isolates of *Aspergillus flavus* did not produce aflatoxin G_1, cyclopiazonic acid but one isolate produced aflatoxin B_1. *Penicillium citrinum* produced citrinin. From two *Penicillium expansum* species all produced patulin, roquefortin C and one citrinin. Two *Penicillium chrysogenum* tested isolates produced roquefortin C. Ochratoxin A production was tested in 4 strains belonging to *Aspergillus* section *Nigri*. Among them, the production of ochratoxin A was not confirmed. In the European diet, wine (especially red wine) has been identified as the second major source of human exposure to ochratoxin A (OTA), following cereals. Ochratoxin A is a mycotoxin with nephrotoxic, nephrocarcinogenic, teratogenic and immunosuppressive properties, which has received growing interest from the scientific community and food committees in the last few years (**Battaglia *et al*., 1996; Walker, 1999**). It has been detected in different kinds of foods and beverages, including grape juice and wine, where it was reported for the first time by **Zimmerli and Dick** (**1995**). The only reported species capable of producing OTA belong to the genera *Aspergillus* and *Penicillium*. Some species of black aspergilli - *Aspergillus niger* group (**Raper and Fennell, 1965**), *Aspergillus* section *Nigri* (**Gams *et al*., 1985**) have been described as capable of producing OTA (**Abarca *et al*., 1994; Téren *et al*., 1996)**. These species are commonly present in the vineyards and have the ability to cause rot in berries, known as *Aspergillus* rot (**Snowdon, 1990**). Among the species of this group, *Aspergillus carbonarius* shows the highest ochratoxigenic potential, with most of the isolates having the ability to produce OTA in media (**Heenan *et al.*, 1998**). *Aspergillus carbonarius* is the fungus responsible for OTA production in grapes (**Pitt, 2000; Cabañes *et al*., 2002**). Spain is a world class producer of wines, both in quality and in quantity. The wines of Spain can be divided into more than 50 recognized wine regions, each of which is very different. It appears that some wines from southern wine-growing regions of Europe and northern Africa, mainly in the Mediterranean basin, have higher OTA contents than those from the northern areas (**Zimmerli and Dick, 1996**). Black aspergilli, mainly *Aspergillus carbonarius* and members of the *Aspergillus niger* aggregate, have been described as a main possible sources of OTA contamination in grapes from Argentina and Brazil (**Da Rocha Rosa *et al.*, 2002**), France (**Sage *et al.*, 2002**) and Italy (**Battilani *et al*., 2003**).

Table 3 Toxinogenity of selected strains, isolated from endogenous mycobiota of wine grapes

Species	Patulin	Citrinin	Penitrem A	Roquefortin C	Ochratoxin A	AFB_1	AFG_1	CPA
Aspergillus flavus						1*/2**	0/2	0/2
Aspergillus section *Nigri*					0/4			
Penicillium citrinum		1/1						
Penicillium expansum	2/2	1/2		2/2				
Penicillium chrysogenum				2/2				

Legend: * positive isolates; ** number of tested isolates, AF – aflatoxin, CPA – cyclopiazonic acid

CONCLUSION

Four grape samples were collected from Pinot gris, Green Veltliner, Traminer and Alibernet grape varieties in the Eastern wine growing region. Mycological survey indicated the presence of ten genera: *Alternaria, Aspergillus, Botrytis, Cladosporium, Epicoccum, Fusarium, Mucor, Penicillium, Rhizopus, Trichoderma* and one unidentified genus *Mycelium sterillium*. *Alternaria, Aspergillus, Botrytis* and *Penicillium* were the most frequent isolated in 100 % of the samples, followed by *Cladosporium*, *Fusarium*, *Rhizopus* and *Mycelium sterillium* in 75 % frequency. The species isolated in higher frequency were *Aspergillus* section *Nigri* (100 %) and *Penicillium chrysogenum* (50 %). The largest number of isolates belongs to *Alternaria* (275 isolates), *Cladosporium* (114 isolates) and *Penicillium* (92 isolates). From this point of view, the relative density of three genera was the highest 47 %, 20 % and 16 %, respectively. Five potentially toxigenic species isolated from endogenous mycobiota were tested for their toxigenic ability by thin layer chromatography, namely *Aspergillus flavus, Aspergillus* section *Nigri*, *Penicillium citrinum, Penicillium expansum* and *Penicillium chrysogenum*. Out of 11 strains 54 % produced at least one mycotoxin. The most important mycotoxin-producing species found was *Aspergillus* section *Nigri*, which is an ochratoxin A producer. In our research OTA was not detected. In line with the results on OTA content of Slovak grapes, it appears that the mycotoxin does not present a significant hazard to consumers.

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