Mycobiota in Traditional Grapes and Grape for Ice Wine Production Cultivated in Slovakia

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Abstract

From Small Carpathian wine-growing region were collected 3 samples of wine grapes (Alibernet, Blue Portugal and Rheinriesling) during harvesting 2016 and 2017 except one variety Alibernet, which was collected for production of ice wine at the beginning of December in 2016. The objectives of this study were to gain more knowledge about mycobiota on grapes originating from Slovakia, with a focus on genus *Penicillium* and its ability to produce mycotoxins in *in vitro* conditions by thin layer chromatography method. Direct plating of grapes on agar plates was used for analysis of surface mycobiota while surface sterilized grapes were used for endogenous mycobiota analysis. Of these samples were isolated 762 strains belonging to 14 genera of filamentous microscopic fungi and unidentified genus *Mycelia sterilia*. From variety Alibernet for ice wine production was isolated the most varied mycobiota (11 genera and *Mycelia sterilia*) in 2016. The most abundant genera were *Cladosporium* and *Epicoccum. Penicillium* and *Botrytis* were only found in this sample. *Alternaria* and *Cladosporium* were the most common genera in the surface and endogenous colonisation. *Penicillium* was the most frequently encountered moulds with the highest relative density from both mycobiota in 2017. During the survey only two *Penicillium* species were isolated: *P. expansum* and *P. glabrum*. Potentially toxigenic *Penicillium expansum* were tested for their toxigenic ability on patulin, citrinin and roquefortin C by thin layer chromatography method. Out of 67 tested isolates 60 produced at least one mycotoxin as revealed by the method used here.

Keywords: mycotoxin, *Penicillium*, TLC method, wine grapes

1. Introduction

Grapes and wine musts harbor a complex microbiome, which plays a crucial role in wine fermentation as it impacts on wine flavour and, consequently, on its final quality and value [1]. The grape microbial ecosystem is composed of highly diverse microorganisms, including yeasts, bacteria and fungi with different physiological and metabolic characteristics [2]. Molds are ubiquitous with various genera commonly found on grapes [3]. The major fungi causing frequent and problematic grape rotting and spoilage are members of the fungal genera *Penicillium, Aspergillus, Alternaria, Botrytis, Cladosporium* and *Rhizopus* [4]. Among the contaminating microorganisms, there is greater concern about the mycotoxin-producing fungi, *Aspergillus, Penicillium,* and *Alternaria*, the main producer genera in grapes (*Vitis vinifera* L.) [5,6]. *Penicillium* species are one of the most common fungi occurring in a diverse range of habitats, from soil to vegetation, air, indoor environments and various food products [7]. Many species of the *Penicillium* are among the common postharvest pathogens on a wide range of fruits and vegetables [8]. The species of *Penicillium* have gained attention as grapevine pathogens that cause blue mold decay at harvest [9]. The genus *Penicillium* seems to be more frequent in temperate and cold climates, such as those in northern Europe,

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whereas *Aspergillus* is more frequently associated with warmer and wetter regions [6]. Among the mycotoxins, ochratoxin A (OTA) is the main contaminant of grapes and wines [10, 11]. The incidence of these fungi and toxin levels vary depending on the grape variety, wine region, agricultural practices, weather conditions, physicochemical characteristics of the grapes, harvest and the winemaking process [12].

2. Materials and methods

Study area

Grape samples were collected in moderate region of Slovakia, as it can be common in the Middle Europe areas, from Terra Parna winery, Suchá nad Parnou, Small Carpathian wine region. Slovak republic has 6 distinct wine-growing zones (the Small Carpathians, the Southern Slovak, the Nitra, the Central Slovak, the Eastern Slovak and the Tokaj wine regions). They spread from the west to the east of the country along its southern and south-western borders. The largest in size and the most important over the centuries has been the Small Carpathian area (4260 ha of vineyards) spreads in the western of Slovakia [13]. The Small Carpathian wine region is divided to 12 subregions. The subregion is the area with the same soil and climate conditions. Wine-growing zones are defined as geographic regions with distinct climatic conditions for grape cultivation [14]. The Small Carpathian wine-growing region has medium climates and abundant moisture.

Sampling

Two samples of red grapes (Alibernet, Blue Portugal) and one sample of white grape (Rheinriesling) were collected in the final stages of maturation of the berries (harvest season), in the September/October 2016 and 2017 harvest, except Alibernet in 2016, which was collected at the beginning of December, 2016 near – 7 °C for production of ice wine. The sample comprised 3 bunches of grapes collected across two diagonal transects. Grape samples were put directly each into a sterile plastic bag. Samples were brought into the laboratory and kept at 5 °C till fungal analysis.

Mycological analysis of grapes

A total of 50 berries (7 - 8 berries per bunch) from each sample were plated in Dichloran Rose Bengal Chloramphenicol agar medium (DRBC) and incubated at 25±1 °C in the dark for one week. In this way was determined an exogenous mycobiota. Fifty another grapes were surface-disinfected in 1% NaClO for 1 min according methods of Magnoli et al. [15] and 3 times rinsed by submersion in sterile distilled water (total amount 1L) to remove incidental surface contaminants, dried, plated in the same medium and incubated at 25 °C in the dark for 7 days. This way was determined an endogenous mycobiota.

The identification of fungal taxa based on macroscopic and microscopic features, with guidelines by Pitt and Hocking [16]. *Penicillium* strains were isolated and cultivated on MEA (Malt extract agar) [17], CYA (Czapek yeast agar) [17], Creatine-Sucrose agar (CREA) [17] and Yeast Extract agar (YES) [17]. From the pure cultures, genus *Penicillium* was identified to species level based on macroscopic and microscopic characteristics according to the manuals of Pitt and Hocking [18], Samson and Frisvad [19] and Samson et al. [20,17].

Results evaluation

The obtained results were evaluated and expressed according to relative density (RD). The relative density (%) is defined as the percentage of isolates of the species or genus, occurring in the analyzed sample [21]. These values were calculated according to González et al. [22] as follows:

RD (%) = (ni / Ni) x 100

where ni – number of isolates of a species or genus; Ni – total number of isolated fungi.

Toxinogenity analysis

Toxinogenity of selected isolates was screened in *in vitro* conditions by means of thin layer chromatography (TLC) according to Samson et al. [23], modified by Labuda and Tančinová [24]**.** Extracellular metabolites – citrinin and patulin were carried out on YES agar and intracellular roquefortin C 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 TLC plate (Alugram ®

SIL G, Macherey – Nagel, Germany). The plate was put into TEF solvent (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 metabolite standards. Roquefortin C was visible after spraying with $Ce(SO_4)_2$ x 4 H_2O as an orange 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.

3. Results and discussion

The filamentous fungi identified in white grape variety Rheinriesling and red grape varieties Alibernet and Blue Portugal from surface and endogenous mycobiota from two years 2016 and 2017 are indicated in table 1. Samples belonging to red varieties showed different total isolates from surface colonization in the year 2016 - Alibernet 95 and Blue Portugal 26 (Table 1).
Variety Alibernet was harvested for production of ice wine at the beginning of December, so the presence of microscopic fungi were the most varied (11 and *Mycelia sterilia*). The highest number of isolates was detected in *Cladosporium* (32) and *Epicoccum* (27), followed *Alternaria* (11) and *Penicillium* (8), on the other hand from variety Blue Portugal was isolated only three genera *Alternaria, Cladosporium* and *Epicoccum*. From variety Rheinriesling were isolated the highest number of isolates (115), mainly genus *Alternaria* was dominated (75). All tested samples were colonized by *Alternaria, Cladosporium* and *Epicoccum.* The colonization by *Penicillium* and *Aspergillus* was not often only 8 isolates of *Penicillium expansum* and one isolate of *A. flavus* belong to the sample Alibernet. A total of 236 strains belonging to 12 genera and *Mycelia sterilia* were identified. The relative density was the highest in genera *Alternaria* (45%), *Cladosporium* and *Epicoccum* (19%, each) from surface (exogenous) mycobiota colonisation of grapes.

Table 1. Fungi identified in Slovak wine grapes from exogenous mycobiota from 2016 to 2017 by the direct plating method

Harvest year	**2016**					**2017**				
Fungal taxa	**AL**	**RR**	**BP**	**No**	**RD (%)**	**AL**	**RR**	**BP**	**No**	**RD (%)**
Alternaria	11	75	20	106	45	36	16	7	59	20
Arthrinium	-	-	-	-	-	3	-	2	5	2
Aspergillus	1	-	-	1	<1	3	4	1	8	3
A. flavus	1	-	-	1	<1	1	3	-	4	1
A.section nigri	-	-	-	-	-	2	1	1	4	1
Botrytis	4	-	-	4	2	2	6	-	8	3
Cladosporium	32	11	2	45	19	19	13	31	63	21
Epicoccum	27	13	4	44	19	8	12	20	40	14
Eurothium	-	-	-	-	-	6	-	-	6	2
Fusarium	1	-	-	1	<1	6	-	-	6	2
Mucor	-	1	-	1	<1	-	3	-	3	1
Mycelia sterilia	5	4	-	9	4	-	-	-	-	-
Phoma	3	1	-	4	2	-	-	-	-	-
Rhizopus	1	5	-	6	3	-	4	7	11	4
Penicillium	8	-	-	8	3	19	29	29	77	26
P. expansum	8	-	-	8		19	29	29	77	
Sordaria	1	3	-	4	2	-	2	4	6	2
Trichoderma	1	2	-	3	1	1	-	-	1	<1
Total isolates	95	115	26	236		103	89	101	293	

AL – Alibernet, RR – Rheinriesling, BP - Blue Portugal, No. – number of isolates, RD – relative density

Other genera were detected in less than 5% of all isolates. Most of the fungi found are ubiquitously distributed, such as the field fungi *Alternaria, Cladosporium* and *Epicoccum*, which occur commonly in the air, plant surfaces, debris and soil [6]. Our results corroborate the findings of Felšöciová et al. [25] in which 42% of isolates from grapes from Small Carpathian wine-growing region were *Alternaria* and 33% *Cladosporium.* The difference demonstrates *Epicoccum* which was detected in more than 1% and *Penicillium* 9% of the berries analyzed. Occurrence of *Aspergillus* was detected in 1% of the berries what was similar results as our. Felšöciová [26] monitored the mycobiota in Devín grape variety from Nitra wine growing region at 3 maturation stages (pea berry, early veraison and ripe berry) in the year 2014. The most abundant genera found by descending order were *Alternaria* (42.8%), *Cladosporium* (24.2%) and *Epicoccum* (10.0%). *Penicillium expansum* was isolated from all 3 maturation stages but in low average relative density (1.2%). Khashaba et al. [27] reported that prevalence of *Alternaria* was moderate fungal frequency (50%) and *Aspergillus* was the most frequently occurring genus (100%) in Egypt. *Penicillium* was the second predominant genus which recorded frequency of 92.5%. Similar results that *Aspergillus* is the main fungal genus were obtained by several studies [28,29].

Of the 102 identified fungi from endogenous mycobiota in 2016, 61 belong to the variety Alibernet, 36 to Rheinriesling and only 5 belong to the variety Blue Portugal (Table 2).

Table 2. Fungi identified in Slovak wine grapes from endogenous mycobiota from 2016 to 2017 by the direct plating method

Harvest year	**2016**					**2017**				
Fungal taxa	**AL**	**RR**	**BP**	**No**	**RD (%)**	**AL**	**RR**	**BP**	**No**	**RD (%)**
Alternaria	14	31	5	50	49	7	15	8	30	24
Arthrinium	-	-	-	-	-	6	2	3	11	9
Aspergillus	2	-	-	2	2	-	-	-	-	-
A. clavatus	1	-	-	1	<1					
A.section nigri	1	-	-	1	<1					
Botrytis	16	-	-	16	16	-	1	2	3	2
Cladosporium	15	5	-	20	20	9	11	18	38	30
Epicoccum	8	-	-	8	8	3	1	-	4	3
Fusarium	2	-	-	2	2	-	1	-	1	<1
Mycelia sterilia	-	-	-	-	-	-	5	-	5	4
Penicillium	3	-	-	3	3	10	2	25	37	29
P. expansum	2	-	-	2	2	10	2	25	37	29
P. glabrum	1	-	-	1	<1	-	-	-	-	-
Sordaria	1	-	-	1	<1	1	1	-	2	2
Total isolates	61	36	5	102		36	39	56	131	

AL – Alibernet, RR – Rheinriesling, BP - Blue Portugal, No. – number of isolates, RD – relative density

The most abundant genus was again *Alternaria* (49%), which was presented in all three varieties, followed *Cladosporium* (20%) and *Botrytis* (16%) of all eight the fungi found. *Penicillium* spp. in our sample was generally low. There was no occurrence of *Penicillium* in the Rheinriesling and Blue Portugal varieties. From variety Alibernet species as *P. expansum* and *P. glabrum* were isolated. *Aspergillus* species were also found in the wine grape Alibernet - *A. clavatus* and *A. section nigri*, but the most abundant genera were *Botrytis, Cladosporium, Alternaria* and *Epicoccum*. *Alternaria* was the most common genus in the surface and endogenous colonisation from white grape variety Palava from Small Carpathian wine growing region in the year 2017 with an average relative density 50% and 73.6%, respectively [30]. *Alternaria, Botrytis* and *Cladosporium* were three of the most frequent genera in all four winemaking regions in Portuguese [6], representing 16, 17 and 24 % of the total identified strains, respectively. According to the region considered, other frequent fungi were *Aureobasidium pullulans, Aspergillus niger, Epicoccum nigrum, Penicillium brevicompactum, P. thomii* and *Rhizopus*. *Aspergillus* and

Penicillium were also an important part of the mycobiota representing 15 and 24 %, respectively, of all the fungi found in the regions.

Samples without surface disinfection belonging to variety Blue Portugal showed higher colonization of fungi in 2017 than in 2016 (Table 1), which demonstrates that despite the sample the same variety, other factors contribute to contamination. All three varieties were harvested at the end of September, differences between number of isolates were not observed. *Alternaria*, *Cladosporium* and *Epicoccum* were found in all tested samples as in the year 2016. The most commonly microscopic fungi found were *Aspergillus* and *Penicillium expansum* in this year, too. The identified species of the genus *Aspergillus* were *A. flavus* and *A. section nigri*. A total of 293 strains belonging to 13 genera were identified. The most abundant genus was *Penicillium* (26%), followed *Cladosporium* (21%), *Alternaria* (20%) and *Epicoccum* (14%) of all the fungi found. Other genera were detected in less than 5% of all isolates.

Our results corroborate the findings of Felšöciová [26] where from Frankovka modra (Frankish) grape variety from Nitra wine growing region the most abundant genera found by descending order were *Alternaria* (30.6%), *Cladosporium* (21.3%) and *Penicillium* (20.3%). Felšöciová et al. [25] also reported that from the 251 *Penicillium* strains identified from Small Carpathian wine-growing region, the most frequent were *Penicillium chrysogenum* (36%), *P. crustosum* (29%), *P. expansum* and *P. griseofulvum* (21%, each) of the isolated species. These species except *P. expansum* were not reported in our study, which demonstrates the difference in occurrence in different years. The species of *Aspergillus* section *Nigri* were the predominant in mycobiota (64%) of *Aspergillus* spp. The species of *A. clavatus* and *A. flavus* were the other most important species recorded with high isolation frequency (21%, each). On the other hand the relative densities were low (1%) as in our case (3%). The contamination by *Aspergillus* was higher than by *Penicillium* in most samples of grapes (9/11) except Muscat Canelli and Ruby Cabernet that showed the higher incidence of *Penicillium* in semi-arid tropical region of Brazil [12]. Kizis et al. [31] evaluated the colonization of grapes by filamentous fungi from cultivation areas in Greece found *Aspergillus* as the most frequently isolated genus in all regions except in Macedonia where the *Alternaria* genus was the most commonly found. However, Serra et al. [6] found a higher contamination by *Penicillium* when evaluating grapes from Portugal, which indicates the influence of geographical location on the incidence of fungi.

A total of 131 strains belonging to 8 genera and *Mycelia sterilia* were obtained from endogenous mycobiota in 2017 (Table 2). The four most abundant genera found by descending order were *Cladosporium* (30%), *Penicillium* (29%), *Alternaria* (24%) and *Arthrinium* (9%). The remaining genera were detected in less than 5% of all the isolates. The relative density from endogenous mycobiota colonisation of grapes in *Alternaria* was lower in the year 2017 than in 2016. The occurrence of *Penicillium expansum* was confirmed again, but in higher relative density (29%) than in 2016. A total of 3 species of *Penicillium* were detected from the grapes to wine, where *Penicillium expansum* were detected most commonly [30], too. The genus *Penicillium* has long been known to grow on grapes and to be the causal agent of green mold, a secondary disease on mature berries resulting in a loss of must color and a decrease in sugar concentration. This genus is less frequently isolated from warmer and wetter vineyards than from cooler and drier vineyards [5]. Berries affected by *P. expansum* have an off-flavor and even a small amount of infected berries add a mouldy taste to the wine [32].

Only one potentially toxigenic *Penicillium* species were isolated from the exogenous and endogenous mycobiota of wine grapes (Table 3).

Table 3. Toxinogenity of selected *Penicillium* strains

Species	**P**	**C**	**RC**
Toxinogenity from exogenous mycobiota			
P. expansum	40*/44**	41/44	41/44
Toxinogenity from endogenous mycobiota			
P. expansum	20/23	23/23	23/23

* - number of isolates with ability to produce mycotoxin, ** - number of tested isolates, P – patulin, C – citrinin, RC – roquefortin C

In total 67 isolates representing *Penicillium expansum* were tested for their toxigenic ability by thin layer chromatography method. From the exogenous mycobiota 44 strains were tested. *Penicillium expansum* produced patulin (40 out of 44 strains screened), citrinin and roquefortin C (41

out of 44 strains screened, each). From the endogenous mycobiota 23 strains were tested. Positive toxigenity was detected for citrinin and roquefortin C (100%, each) and three strains did not produce patulin. Out of 67 strains, 89% produced at least one mycotoxin as revealed by the method used here.

Patulin contaminates apples and apple derivates [33]. However, patulin has also been reported in grapes [34], processed grape juice and fermenting wine [35]. Patulin is produced by several species of the genera *Aspergillus* and *Penicillium*, including *P. expansum* in particular [36,37]. Patulin inhibits the fermenting yeast *Saccharomyces cerevisiae* in the must. It is partially degraded by the addition of sulfur dioxide and completely degraded during alcoholic fermentation [37]. It is therefore unlikely to be present in wine. Patulin, produced primarily by *P. expansum* is a thermal resistant, causes gastrointestinal problems, skin rashes, and is known to be mutagenic, immunologic, and neurotoxic mycotoxin [38].

Citrinin ($C_{13}H_{14}O_5$) is produced principally by species of the genera *Penicillium* and *Aspergillus* (*A. terreus, A. niveus*). *Penicillium citrinum*, the main producer of citrinin, has been isolated from grapes, *P. expansum* could produce this mycotoxin, too. Citrinin, a hepato-nephrotoxic compound, is not degraded during alcoholic fermentation and may be present in very small amounts in wine. However, wine contamination is unlikely, due to the low abundance of citrinin producing species on grapes [5]. Roquefortin C is a mycotoxin produced by various fungi, particularly species from the *Penicillium* genus. Roquefortin C is an important neurotoxic (paralytic) secondary metabolite.

A 50% of *P. expansum* from Frankovka modrá (Frankish) were positive for citrinin, a higher percentage (80%) of isolates were positive for patulin and all were positive for roquefortin C from grapes came from Nitra wine growing region [26]. In total 102 *Penicillium* isolates representing 7 potentially toxigenic species isolated from exogenous mycobiota from grapes were tested for their toxigenic ability from 2011 to 2013 by Felšöciová et al. [25]**.** *Penicillium expansum* produced roquefortin C (RC), patulin (3 out of 5) and citrinin (2 out of 5). A small percentage (20%) of *P. expansum* isolates were positive for patulin on YES agar, whereas they were all positive for citrinin, reported Abrunhosa et al. [38] from the wine-producing regions of Portugal.

4. Conclusions

From 3 samples of wine grapes from exogenous and endogenous mycobiota were isolated 762 strains belonging to 14 genera and unidentified genus *Mycelia sterilia*. From variety Alibernet for ice wine production was isolated the most varied mycobiota (11 genera and *Mycelia sterilia*) in 2016. The most abundant genera were *Cladosporium* and *Epicoccum. Penicillium* and *Botrytis* were only found in this sample. *Alternaria* and *Cladosporium* were the most common genera in the surface and endogenous colonisation. During the survey only two *Penicillium* species were isolated: *P. expansum* and *P. glabrum*. Of the 125 fungi of the *Penicillium* genus isolated from grapes, 124 (99%) were *P. expansum*, which shows a higher risk of the toxin in grapes and their derivatives from the region. Out of 67 tested isolates 89% produced at least one mycotoxin as revealed by the method used here.

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