

## FUNGAL DIVERSITY IN THE GRAPES-TO-WINES CHAIN WITH EMPHASIS ON *PENICILLIUM* SPECIES

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### ABSTRACT

The aim of this work was the description of surface and endogenous mycobiota colonisation of grapes, fresh grape juice, grape must, and wine primarily focused to the current spectrum of the penicillium species. One sample of white grape variety Palava and one sample of blue grape variety Dornfelder were collected in Small Carpathian wine growing region of Slovakia in the year 2017. Direct plating of grapes on agar plates was used for analysis of surface mycobiota of grapes while surface sterilised grapes were used for endogenous mycobiota analysis. Mycobiota of juice, must, and wine was analysed by plate dilution method. Overall, we isolated 148 strains belonging to 13 genera of filamentous microscopic fungi and *Mycelia sterilia* from grape variety Palava, while the most frequent was *Alternaria*. *Alternaria* was the most common genus in the surface and endogenous colonisation with an average relative density 50% and 73.6%, respectively. A total of 2 species of *Penicillium* were detected from the grapes to wine, potentially toxigenic *Penicillium expansum* and *P. chrysogenum*. A total of 39 strains belonging to 6 genera and *Mycelia sterilia* were identified from grape variety Dornfelder. The most abundant genus was also *Alternaria* (51.3%), followed by *Penicillium* (12.8%). *Alternaria* was the most common genus in the surface and endogenous colonisation and fresh grape juice with an average relative density from 20% (grape juice) to 71% (endogenous colonisation of grapes). A total of 3 species of *Penicillium* were detected from the grapes to wine, where *Penicillium expansum* were detected most commonly. In the second part of our work some selected isolates were tested to the ability to produce mycotoxins such as patulin, citrinin, and roquefortin C in *in vitro* condition by thin layer chromatography method. All tested strains of *Penicillium* species were able to produce at least one mycotoxin.

**Keywords:** wine grapes; must; wine; mycobiota; mycotoxin

### INTRODUCTION

Wine is a significant contributor to the economies of many countries. However, the commodity can become contaminated with mycotoxins produced by certain fungi. Most information on mycotoxins in wine is from Spain, Italy and France (Russell et al., 2017). Over 500 mycotoxins are currently known, and their number continues to rise. Mycotoxins are secondary metabolites produced by fungi that contaminate agricultural products as a result of fungal spoilage, and they may be produced before, during, or after harvest, or at any stage during the food chain. Some of the challenging aspects related to the pathogenesis of mycotoxins-caused illnesses are that one fungal species may generate more than one mycotoxin, and several fungal species may be concomitantly present in food products (Stein and Bulboacă, 2017). The incidence of filamentous fungi and toxin levels in grapes and wines varies depending on the variety of grapes, the wine region, agricultural practices, weather conditions, the harvest and the winemaking process (Alshannaq and Yu, 2017). The major fungi causing frequent and problematic grape rotting and spoilage are members of the fungal

genera *Penicillium*, *Aspergillus*, *Alternaria*, *Botrytis*, *Cladosporium* and *Rhizopus* (Marin et al., 2013). The genus *Penicillium* seems to be more frequent in temperate and cold climates, such as those in northern Europe whereas *Aspergillus* is more frequently associated with warmer and wetter regions (Serra et al., 2006). The mycotoxins of greatest significance include aflatoxins, citrinin, patulin, ochratoxin A (OTA) and fumonisin B<sub>2</sub> (Susca et al., 2010). Many mycotoxins are not easily eliminated during food processing because of their stability against heat, physical, and chemical treatments (Marin et al., 2013). Most mycotoxins are chemically and thermally stable during storage and food processing, including cooking, boiling, baking, frying, roasting, and pasteurization. This makes it important to avoid the conditions that lead to mycotoxin formation at all levels of production, harvesting, transport and storage, which is not always possible and not always achieved in practice. It has been demonstrated that environmental stress conditions such as insect infestation, drought, cultivar susceptibility, mechanical damage, nutritional deficiencies, and unseasonable temperature, rainfall or humidity can

promote the distribution of fungal population, including those present on grapes, thereby also affecting the presence of mycotoxins or off-flavours in wine. In fact, changes in farming practices in the past few decades may result in increasing stress on plants and therefore enhance fungal invasion and mycotoxin contamination. The careful selection and proper storage of fruits are the most important factors in quality control (**Fernández-Cruz et al., 2010**). We focused particularly on descriptions of the fungal microbiota on and in wine grapes, fresh grape juice, must and wine and species of genera *Penicillium* responsible for the production of mycotoxins and off-flavors from domestic crops in the year 2017.

### Scientific hypothesis

Growth in the must and wine habitat is limited by low pH values and high ethanol concentrations. Therefore, only acid and ethanol tolerant microbial groups can grow in grape juice, must and wine, which include yeasts and fungi.

## MATERIAL AND METHODOLOGY

### Study area and samples

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 (around 5800 ha of vineyards) spreads in the western of Slovakia. 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. The Small Carpathian wine-growing region has medium climates and abundant moisture. Grapes (*Vitis vinifera* cultivars *Palava* and *Dornfelder*) used in this study were provided by farms located in the Small Carpathians wine region, Vrbovsky subregion, village Vrbove. Two samples – 1 of white grape variety Palava and 1 of blue grape variety Dornfelder were mycologically analyzed. One sample of a wine grape variety was represented by three subsamples of wine grapes, which were sampled in left, middle and right part of the vineyard. Samples were collected at the end of September 2017, in the maturation stages corresponding to harvest. The berries from the vineyards sampled were generally in good condition without visible damage. Three kilograms of samples were taken to sterile plastic bags and transported to a mycological laboratory for immediate processing. Microfungi were monitored in fresh grape juice, must and wine. Fermentation was carried out in 15 litre tanks with yeast culture Excellence® FTH (Lamothe Abiet, France) for white wine and Excellence® TDS (Lamothe Abiet, France) for red wine. At least 50 ml of must were collected after stirring the tank content at each sampling. The fermentation lasted 3 weeks at 18 ± 2 °C.

### Mycological analysis of samples

A total of 50 berries (7 – 8 berries per bunch) from each sample were plated in Dichloran Rose Bengal Chloramphenicol agar medium (DRBC, MERCK,

Germany) and incubated at 25 ± 1 °C in the dark for one week. The detection of fungi in grape samples was also made by plating methods with surface disinfection. A total of 50 intact berries 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 DRBC and incubated at 25 ± 1 °C in the dark for 5 – 7 days. In this way was determined an endogenous mycobiota. Mycobiota of grape juice, must and wine was analysed by plate dilution method. From each sample were squeezed more than 250 g of randomly selected berries and 20 ml of the stum has been added to 180 ml of sterile peptone water containing 0.02% Tween 80. Prepared suspensions were shaken on a Stomacher easyMix®. Dilutions 10<sup>-1</sup>, 10<sup>-2</sup>, 10<sup>-3</sup> and 10<sup>-4</sup> were in the double surface inoculated in amount of 0.1 ml on DRBC agar plates. Cultivation lasted from 5 to 7 days in darkness at 25 ± 1 °C. We used conventional identification techniques, such as macroscopic and microscopic observations, with guidelines by **Pitt and Hocking (2009)** facilitating the identification of isolated microorganisms. *Penicillium* strains were isolated and cultivated in MEA (Malt extract agar, **Samson et al., 2010**), CYA (Czapek yeast agar, **Samson et al., 2010**), CREA (Creatine-Sucrose agar, **Samson et al., 2010**) and YES (Yeast Extract agar, **Samson et al., 2010**) to obtain pure cultures and identify further species. Genus *Penicillium* was identified to species level based on morphological characters according to special mycological literature of **Pitt and Hocking (2009)**, **Samson and Frisvad (2004)** and **Samson et al. (2002a, 2010)**.

### 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 (**Gautam et al., 2009**). These values were calculated according to **González et al. (1999)** as follows:

$$RD (\%) = (n_i / N_i) \times 100$$

where  $n_i$  – number of isolates of a species or genus;  $N_i$  – 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. (2002b)**, modified by **Labuda and Tančinová (2006)**. 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  $Ce(SO_4)_2 \times 4 H_2O$  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.

**RESULTS AND DISCUSSION**

The filamentous fungi identified in white grape variety Palava from surface and endogenous mycobiota of grapes, fresh grape juice, must, and wine are indicated in Table 1. A total of 148 strains belonging to 13 genera and *Mycelia sterilia* were identified. The most abundant genus was *Alternaria* (59.5%), followed *Botrytis*, *Mucor* (6.8%, each), *Rhizopus*, *Sordaria* (4%, each), *Penicillium* (2.7%), *Arthrimum*, *Fusarium*, and *Trichoderma* (2%, each) of all the fungi found. *Cladosporium* and *Talaromyces* were detected in more than 1 %, *Acremonium* and *Aspergillus* in less than 1% of all isolates.

Without surface disinfection, a total of 70 strains belonging to 11 genera and *Mycelia sterilia* were identified. The four most abundant genera found by descending order were *Alternaria*, *Mucor*, *Rhizopus*, and *Sordaria*. The occurrence of *Penicillium* spp. in our sample was generally low. *Arthrimum*, *Cladosporium*, *Fusarium* and *Penicillium expansum* were detected in more than 2% of the berries analyzed. The remaining genera were detected in less than 2% of all the isolates. *Alternaria* was one of the main fungal genera isolated also from Argentina grape berries (Prendes et al., 2015), Tunisian grapes (Fredj et al., 2009), Spanish grapes (Bau et al., 2005, 2006; García-Cela et al., 2015) or Slovakian grapes (Felšöciová, 2016). Outbreaks of *Alternaria* bunch rot on grapevines in Slovakia occurred during unusually hot summer weather in 2007 and 2008 (Kalíková et al., 2009). *Mucor* was one of the main fungal genera isolated

also from French and Tunisian grapes (Sage et al., 2002; Fredj et al., 2009). The relative density (RD) from surface mycobiota colonisation of grapes in the six wine growing regions of Slovakia in the years 2011 – 2013 was lower. *Mucor* and *Sordaria* were identified less than 1%, and *Rhizopus* higher (2.66% RD) (Felšöciová, 2016). *Penicillium expansum* can cause rot in grapes (Serra et al., 2005). In our study was isolated in low relative density (2.9%). Other studies have identified *P. expansum* as the species most frequently isolated from Portuguese (Abrunhosa et al., 2001) and French vineyards (La Guerche et al., 2004, 2005; Bejaoui et al., 2006). Felšöciová et al. (2015) from Small Carpathian winemaking region during the years 2011 and 2013 identified 13 different *Penicillium* species from the 251 *Penicillium* strains. The most abundant were *Penicillium chrysogenum* (64%), *P. crustosum* (12%) and *P. griseofulvum* (8%) of the isolates. Isolation frequency among species was maximum for *P. chrysogenum* (36%), *P. crustosum* (29%), *P. expansum* and *P. griseofulvum* (21%, each). Fungal species capable of causing rot in grapes (*Aspergillus niger*, *Botrytis cinerea*, *Penicillium expansum*, *Rhizopus*) were also common inhabitants of the berries surface from Portuguese vineyards in four winemaking regions (Serra et al., 2006). The most frequent *Penicillium* species were other than in our samples, namely *P. brevicompactum*, *P. thomii* and *P. glabrum/spinulosum* which together accounted for approximately 71 % of the strains identified in the genus.

Grape berries harbour a complex microbial community comprising yeasts, bacteria and filamentous fungi that inhabit not only the skin surface but also endosphere of the berry. A total of 72 isolates of microscopic fungi belonging to 7 genera and *Mycelia sterilia* were obtained from endogenous mycobiota. The most isolated genera by descending order were *Alternaria* (73.6%) and *Botrytis* (12.5%). *Trichoderma* and *Talaromyces purpurogenus* (previous name *Penicillium purpurogenum*) were detected

**Table 1** Fungi identified from exogenous and endogenous mycobiota of grapes, grape juice, must and wine from variety Palava

Fungal taxa	grapes exo		grapes endo		juice		must		wine	Total	RD (%)
	No	RD (%)	No	RD (%)	No	No	RD (%)	No			
<i>Acremonium</i>	-		-		-	1	16.7	-	-	1	0.7
<i>Alternaria</i>	35	50	53	73.6	-	-	-	-	-	88	59.5
<i>Arthrimum</i>	2	2.9	1	1.4	-	-	-	-	-	3	2.0
<i>Aspergillus</i>	1	1.4	-		-	-	-	-	-	1	0.7
<i>Botrytis</i>	1	1.4	9	12.5	-	-	-	-	-	10	6.8
<i>Cladosporium</i>	2	2.9	-		-	-	-	-	-	2	1.3
<i>Fusarium</i>	2	2.9	-		-	1	16.7	-	-	3	2.0
<i>Mucor</i>	8	11.4	-		-	2	33.3	-	-	10	6.8
<i>Penicillium</i>	2	2.9	-		-	2	33.3	-	-	4	2.7
<b>from it:</b>											
<i>P. expansum</i>	2		-		-	-	-	-	-	2	
<i>P. chrysogenum</i>	-		-		-	2		-	-	2	
<i>Rhizopus</i>	5	7.1	1	1.4	-	-	-	-	-	6	4.0
<i>Sordaria</i>	5	7.1	1	1.4	-	-	-	-	-	6	4.0
<i>Talaromyces</i>	-		2	2.8	-	-	-	-	-	2	1.3
<i>Trichoderma</i>	1	1.4	2	2.8	-	-	-	-	-	3	2.0
<i>Mycelia sterilia</i>	6	8.6	3	4.2	-	-	-	-	-	9	6.1
<b>Total isolates</b>	<b>70</b>		<b>72</b>		-	<b>6</b>		-	-	<b>148</b>	

Note: No - number of isolates, RD – relative density.

in more than 2%, *Arthrinium*, *Rhizopus* and *Sordaria* in less than 2% of all the fungi found. Several studies have shown that grape rot, due to the association of *B. cinerea* with order, less visible, fungi (*Penicillium* spp., *Rhizopus* spp.) frequently leads to the development of organoleptic defects in grapes and wines. These compounds have been identified as 2-methylisborneol, (-)-geosmin, 1-octen-3-one, 1-octen-3-ol, 2-octen-1-ol, and 2-heptanol (La Guerche et al., 2006). This mould also induces the production of a pathogenesis-related (PR) protein causing haziness in white wines (Girbau et al., 2004). Felšöciová (2016) described 19 genera and *Mycelia sterilia* with 1689 isolates from endogenous mycobiota of 14 wine grapes from Small Carpathians wine region, during the years 2011 and 2013. In all samples were found *Alternaria* (100%), *Cladosporium*, *Fusarium* (92.86%, each) and *Penicillium* (78.57%). Eight *Penicillium* species, namely *P. aurantiogriseum*, *P. citrinum*, *P. expansum*, *P. griseofulvum*, *P. chrysogenum*, *P. oxalicum*, *P. polonicum* and *P. thomii* were identified. *Penicillium chrysogenum* and *P. expansum* were the predominant in mycobiota, because they were the most frequent (42.9%, 35.7%, respectively) of the isolates with maximum relative density among species (63.6%, 22.6%, respectively).

In the grape juice filamentous fungi were surprisingly missed on DRBC agar medium. In grape juice and wine were identified only yeasts, but in must two isolates of *Mucor*, two isolates of *Penicillium chrysogenum* and one isolate of *Fusarium* were detected. Felšöciová (2016) described 14 genera and *Mycelia sterilia* with 2515 isolates from grape must. The highest frequency (100% FR) and relative density (91.2%) reached *Cladosporium*, followed *Alternaria* (86% FR) and *Penicillium* (64% FR). *Penicillium chrysogenum* (40.5%) and *Penicillium expansum* (29%) obtained the highest frequency from 4 *Penicillium* species. Barboráková et al. (2011) obtained the information about the mycobiota of Slovak origin wines during the production process in the year 2009. Altogether thirty three samples from the production process of 5 species white Slovak origin wines were mycologically analysed. The spectrum of isolated penicilia consisted of 21 species: *Penicillium aurantiogriseum*, *P. brevicompactum*, *P. citreonigrum*, *P. citrinum*, *P. corylophilum*, *P. crustosum*, *P. decumbens*, *P. expansum*, *P. funiculosum*, *P. glabrum*, *P. griseofulvum*, *P. implicatum*, *P. oxalicum*, *P. paneum/carneum*, *P. pinophilum*, *P. polonicum*, *P. purpurogenum*, *P. restrictum*, *P. roqueforti*, *P. rubrum* and *P. rugulosum*.

The freshly crushed must present one of the richest and most complex microbial communities, which functions as inoculum in spontaneous fermentations. The initial yeast diversity rapidly evolves in extremely stressful conditions, dominated by high sugar and low initial temperatures. In the grape juice the concentration of yeasts was  $1.10^4$  CFU.ml<sup>-1</sup>, in must  $1.7.10^8$  CFU.ml<sup>-1</sup>, and at the end of the process, only a few strains survive ( $7.4.10^3$  CFU.ml<sup>-1</sup>). As observed in other fermentations, glucose and ethanol concentrations and must pH have a significant role in shaping the microbial population, with must acidity playing the predominant role, both in selecting the initial fungal population (Charoenchai et al., 1998) and in

defining the fermentation properties of fungi (Liu et al., 2015).

The filamentous fungi identified in blue grape variety Dornfelder from surface and endogenous mycobiota of grapes, grape juice, must and wine are indicated in Table 2. A total of 39 strains belonging to 6 genera and *Mycelia sterilia* were identified. The most abundant genus was also *Alternaria* (51.3% RD), followed *Penicillium* (12.8%), *Aspergillus* (7.7%), *Cladosporium*, *Fusarium* (5.1%, each) and *Botrytis* (2.6%) of all the isolates found. Abrunhosa et al. (2001) reported that *Alternaria* and *Cladosporium* were more often isolated from blue grape varieties than white, regardless of the vineyard in Portugal, which can not be confirmed from our study. The effect of *Cladosporium* rot was reported in delayed harvests in Chile (Briceño et al., 2009). This type of rot reduced colour, aroma, and flavor in Cabernet Sauvignon and Carménère wines. Without surface disinfection, a total of 18 strains belonging to 4 genera and *Mycelia sterilia* were identified. The most abundant genera were *Alternaria* (50% RD) and *Penicillium* (22.2% RD). *Fusarium* and *Botrytis* were detected very rarely of all the fungi found. The *Penicillium* genus 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 (Rousseaux et al., 2014). Two isolates of *Penicillium expansum*, one isolate of *P. aurantiogriseum* and one isolate of *P. brevicompactum* were detected. Berries affected by *P. expansum* have an off-flavor and even a small amount of infected berries add a mouldy taste to the wine (König et al., 2009). *Penicillium brevicompactum* is a cosmopolitan species but never particularly frequent. However, Serra et al. (2006) isolated the species frequently from grape surfaces at 100% rate in some samples.

A total of 14 isolates of microscopic fungi belonging to 3 genera and *Mycelia sterilia* were obtained from endogenous mycobiota. The most isolated genus was *Alternaria* (71.4%). *Aspergillus* and *Cladosporium* were isolated only once. Medina et al. (2005) referred the diversity of filamentous fungi isolated from muscat grape varieties grown in Spain. *Cladosporium* was the most common strain isolated from two blue varieties Garnacha and Monastrell (78.2% and 92.2%, respectively) of all isolates.

In the grape juice were obtained 5 isolates of microfungi belonging to same 3 genera and *Mycelia sterilia* as from endogenous mycobiota. They were isolated only once. Filamentous fungi slowly missed. In must one isolate of *Alternaria* and one isolate of *Penicillium expansum* were detected. *Alternaria* already underwent a drastic decrease, suggesting their inability to survive, either due to the stressful environment of the fermenting must or due to competition with other species. In wine were not detected any fungi.

Yeast counts in fresh grape juice were  $2.10^5$  CFU.ml<sup>-1</sup>, in must remained nearly stable -  $5.3.10^5$  CFU.ml<sup>-1</sup> and until the end of fermentation slightly decreased on  $5.7. 10^4$  CFU.ml<sup>-1</sup>.

**Table 2** Fungi identified from exogenous and endogenous mycobiota of grapes, grape juice, must and wine from variety Dornfelder

Fungal taxa	grapes exo		grapes endo		juice		must		wine	Total	RD (%)
	No	RD (%)	No	RD (%)	No	RD (%)	No	RD (%)	No		
<i>Alternaria</i>	9	50	10	71.4	1	20	-	-	-	20	51.3
<i>Aspergillus</i>	-	-	1	7.1	1	20	1	50	-	3	7.7
<i>Botrytis</i>	1	5.5	-	-	-	-	-	-	-	1	2.6
<i>Cladosporium</i>	-	-	1	7.1	1	20	-	-	-	2	5.1
<i>Fusarium</i>	2	11.1	-	-	-	-	-	-	-	2	5.1
<i>Penicillium</i> from it:	4	22.2	-	-	-	-	1	50	-	5	12.8
<i>P. aurantiogriseum</i>	1	-	-	-	-	-	-	-	-	1	-
<i>P. brevicompactum</i>	1	-	-	-	-	-	-	-	-	1	-
<i>P. expansum</i>	2	-	-	-	-	-	1	-	-	3	-
<i>Mycelia sterilia</i>	2	11.1	2	14.3	2	40	-	-	-	6	15.4
<b>Total isolates</b>	<b>18</b>		<b>14</b>		<b>5</b>		<b>2</b>			<b>39</b>	

Note: No – number of isolates, RD – relative density.

Accurate fungal identifications and mycotoxin detection from the fungi are important. The genus *Penicillium*, in particular, has been associated with the production of secondary metabolites (including mycotoxins) in food and fruits (Pitt and Hocking, 2009). Two potentially toxigenic species were tested for their toxigenic ability (Table 3). All tested isolates of *Penicillium expansum* were able to produce roquefortin C and citrinin, but only one isolate produced patulin. The metabolite roquefortin C was also produced by *Penicillium chrysogenum*.

**Table 3** Toxinogenicity of selected *Penicillium* strains

Species	Isolated from	P	C	RC
<i>P. expansum</i>	Palava, exo	0/2	2/2	2*/2**
<i>P. expansum</i>	Dornfelder, endo	1/2	2/2	2/2
<i>P. expansum</i>	Dornfelder, must	0/1	1/1	1/1
<i>P. chrysogenum</i>	Palava, must			1/1

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

Patulin is a polyketide mycotoxin discovered in 1943. It is produced by certain species of *Penicillium*, *Aspergillus*, and *Byssoschlamys* growing on fruit and vegetables, with *P. expansum* recognized as the most fungus for its production (Drusch and Ragab, 2003). The temperature range for *P. expansum* growth and patulin production is 0 – 24 °C. Minimum  $a_w$  for patulin production is 0.99 (Fernández-Cruz et al., 2010). While it predominantly contaminates apples, apple juice, and apple products, other fruit including grapes may also be vulnerable to patulin contamination (Yang et al., 2014). Its presence in grapes has been associated with moldy berries, even if patulin is degraded to some extent during the fermentation process (Abrunhosa et al., 2001). Patulin was initially studied as a potential antibiotic, but subsequent research demonstrated human toxicities (Puel et al., 2010). The acute symptoms in animals include lung and brain oedema, liver, spleen and kidney damage and toxicity to the immune system. For humans, nausea, gastrointestinal disturbances, and vomiting have been reported. The chronic symptoms include genotoxic, neurotoxic, immunotoxic, immunosuppressive and teratogenic effects. The IARC has

classified patulin as category 3, not classifiable regarding its carcinogenicity to humans (Fernández-Cruz et al., 2010). Citrinin is a mycotoxin of moderate toxicity (Pitt and Hocking, 2009). Citrinin 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 (Pitt and Hocking, 2009). Felšöciová et al. (2015) tested 68 strains on roquefortin C from Small Carpathian winemaking region from exogenous mycobiota which all were positive, too. The metabolite citrinin, a characteristic yellow-lemon pigment, was also produced by all strains of *P. expansum* under laboratory conditions. Tančinová et al. (2015) analysed 47 samples of grapes, harvested in 2011, 2012 and 2013 from various wine-growing regions of Slovakia. The potential producers of patulin were isolated from 23 samples berries, 19 samples of surface sterilized berries and 6 samples of grape juice. Overall, the representatives of producers of patulin were detected in 32 (68.1%) samples (75 isolates). The ability to produce patulin in *in vitro* condition was detected in 82% of isolates of *Penicillium expansum*, 65 % of *Penicillium griseofulvum* and 100% of *Aspergillus clavatus*. The secondary metabolite profiles of microfungi of the genus *Penicillium* isolated from samples of grape berries collected in two different phases during two vegetative seasons in Slovakia is described by Santini et al. (2014). Three Slovak vine regions have been selected for this study, based on their climatic differences and national economic importance. The species *Penicillium brevicompactum*, *P. crustosum*, *P. chrysogenum*, *P. expansum*, *P. palitans* and *P. polonicum* were identified according to growth and morphology. The related strains were found to produce a broad spectrum of fungal metabolites, including roquefortin C, chaetoglobosin A, penitrem A, cyclopeptin, cyclophenin, viridicatin, methylviridicatin, verrucofortine, secalonic acid D, cyclopiazonic acid, fumigaclavine and mycophenolic acid. Chemotaxonomy was performed using high-performance liquid chromatography (HPLC) and mass spectrometry (MS). Considering the 52 total strains examined, the 63 % of them produced patulin. The metabolite citrinin was produced by almost all strains under laboratory conditions.



Roquefortine C, a mycotoxin produced by this species, was produced by the 68.4 % of the total extracted strains.

## CONCLUSION

Two grape varieties Palava and Dornfelder, from Small Carpathian wine growing region were analyzed by plating methods and grape juice, must and wine by plate dilution method. Samples were mycological analysed with focus on genera *Penicillium*. The most presented genera on and in grape variety Palava were *Alternaria* and *Botrytis*. The mycobiota changed with wine making process, microfungi were isolated only from grape must. The most abundant genus from grape variety Dornfelder was also *Alternaria*. A few isolates were detected also from grape juice and must. The results shown, that fermentation is a dynamic process with considerable variations in the composition of the mycobiota. In contrast with fungal species, the relative abundance of yeasts gradually increased over time. Potentially toxigenic *Penicillium* species isolated from surface colonisation of grapes Palava and must, surface sterilised grapes Dornfelder and must were tested for their toxigenic ability by thin layer chromatography method. All tested strains were able to produce at least one mycotoxin. In the research, ochratoxigenic *Penicillium* species were not found in grape samples.

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