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MYCOCENOSIS OF GRAPE BERRIES CULTIVATED IN THE CENTRAL SLOVAK WINE REGION

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ABSTRACT

Eleven samples of grapes from the Central Slovak wine-growing region were examined at the time of harvest in 2021 using the plating method and the plating method with surface disinfection. Plates were incubated aerobically at 25±1 °C in the dark for one week. Identification of fungi was based on morphological and microscopic characteristics. Of the 574 strains detected and identified in the exogenous mycobiota, the most common genus was *Alternaria* (90.9%), followed by *Aspergillus* (72.7%), *Cladosporium*, *Penicillium*, and *Rhizopus* (63.6%, each). The most abundant genera found by descending order were *Alternaria* (53.3%), *Rhizopus* (9.4%), *Aspergillus* (5.5%) and only in minor percentage by *Penicillium* (3%), among others. Of the 512 strains detected and identified in the endogenous mycobiota, the most common genus was *Alternaria* (100%), followed by *Rhizopus* (81.8%), *Penicillium* (72.7%), *Aspergillus* (63.6%), *Cladosporium* (45.5%), and *Epicoccum* (27.3%). The most abundant genera were *Alternaria* (61.1%), *Cladosporium* (13.1%), *Aspergillus* (11.7%), and *Rhizopus* (9%). The major fungal species isolated from the genera *Aspergillus* and *Penicillium* were *Aspergillus* section *Nigri* and *Penicillium glabrum*. Potentially toxigenic *Aspergillus* and *Penicillium* species were evaluated for toxinogenicity by thin-layer chromatography. Positive toxinogenity was found for all strains of *Aspergillus clavatus* tested and for almost all strains of *Penicillium expansum* and *P. crustosum*. Out of the two *Aspergillus flavus* isolates tested, none produced AFB_1, AFG_1, and CPA. Ochratoxigenic microfungi did not produce OTA.

Keywords: wine grapes, microscopic filamentous fungi, *Aspergillus* spp., *Penicillium* spp., mycotoxins, thin layer chromatography

INTRODUCTION

The grape berry surface hosts a microbiota of filamentous fungi, yeast, and bacteria that can have an impact on the quantity of the yield as well as the quality of must and wine (**Fleet, 2003; Ribéreau-Gayon *et al.*, 2006**). Grape fruit is rich in sugars and other nutrients and has ideal water activity for the growth of microorganisms, its low pH makes it particularly susceptible to spoilage by fungi (**Tournas and Katsoudas, 2005**). The most frequent filamentous fungi found in grapes were species of *Cladosporium*, *Penicillium*, *Botrytis*, *Alternaria*, and *Aspergillus* (**Serra *et al.*, 2005; Fredj *et al.*, 2007**). Microbiota of grapes may change depending on various factors such as climate, grape variety and geographical region (**Raspor *et al.*, 2006**). Associated microorganisms found in or on grape berries are known to affect the taste of grapes and wines by producing volatile organic compounds (**Verginer *et al.*, 2010**). *Botrytis cinerea* is considered one of the most destructive fungi in cool-climate viticulture, causing botrytis grape rot or grey mould and dramatically altering the physiochemical state of grape berries (**Dean *et al.*, 2010**). Several other microorganisms are involved in the development of grape rot, including fungi from the genera *Penicillium*, *Trichothecium*, *Aspergillus*, *Alternaria*, *Cladosporium*, and *Rhizopus* (**Barata *et al.*, 2012; Rousseaux *et al.*, 2014**). The most important mycotoxigenic fungal species belonging to the genera *Aspergillus*, *Penicillium* and *Fusarium* can contaminate staple crops and widely consumed foods, such as also fruit, dried fruit, wine, and therefore pose a risk to a large number of people (**Altomare *et al.*, 2021**). An important food safety issue in viticulture is ochratoxin A (OTA), a potent nephrotoxic, nephrocarcinogenic, teratogenic and immunosuppressive metabolite (**Battaglia *et al.*, 1996**). This mycotoxin is recognized worldwide as the main contaminant in grape, wine and fruit wines, as well as in other grape products such as grape juice or grape must (**Zimmerli and Dick, 1996; Cabañes *et al.*, 2010**). The most important and the most predominant producers of OTA are the "black aspergilli" (*Aspergillus* section *Nigri*) *A. carbonarius* and *A. niger*, commonly found in grapes, dried grape fruit, wine and coffee. *Aspergillus ochraceus* and related species, which produce this toxin in coffee and sometimes in stored grains are less common producers (**Altomare *et al.*, 2021**). They are considered the main causative agent of this toxin in grapes and their derivatives in the postharvest phase, and *A. carbonarius* is considered as the strongest producer of OTA (**Battilani *et al.*, 2004, 2006; Perrone *et al.*, 2006; Tjamos *et al.*, 2006**). The only *Penicillium* species that produce OTA are *P. verrucosum*, and the closely related *P. nordicum*. *Penicillium verrucosum* commonly occurs in cereals in cool temperate climates, whereas *P. nordicum* has been isolated, uncommonly, from processed meats. In Mediterranean regions, the incidence of *Penicillium* spp. can be very high, but the frequency of OTA-producing strains in grapes is extremely low (**García-Cela *et al.*, 2015; Rousseaux *et al.*, 2014; Battilani *et al.*, 2004**). Patulin (PAT) is a toxin produced primarily by *Penicillium expansum*, and secondarily by numerous other *Penicillium* and *Aspergillus* species that affect fruits. PAT -producing fungi have been isolated from various moldy fruits and vegetables. PAT has been found primarily in apples and apple-based foods, occasionally in other fruits such as pears, plums, peaches, figs, oranges, and grapes. Toxicological studies at PAT have shown that ingestion causes convulsions, agitation, ulcers, edema, intestinal inflammation, vomiting, and DNA damage in the brain, liver, and kidneys of experimental animals. Chronic exposure also has neurotoxic, immunotoxic, genotoxic and teratogenic effects. PAT is destroyed by the fermentation process and is therefore not included in fermented beverages (**Smith *et al.*, 2016**).

The objective of our study was to identify the fungi present on the surface and inside healthy grapes for commercial winemaking at the time of harvest. The focus was on *Aspergillus* and *Penicillium* species because of their importance in mycotoxin production.

MATERIAL AND METHODS

Study area

Viticulture and winemaking in Slovakia belong among the important traditional industries. With its light wines, Slovakia is making an unexpected breakthrough in the very exclusive world of wine. These wines are unique. They are delicious. They express the territory from which they come. 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 southwestern borders. During 2021 vintage, grape clusters from the vineyard of the four wineries from two subregions Vinický and Modrokamenský in the Central Slovak wine region were collected (Table 1). The wine-growing region of Central Slovakia is divided into 7 sub-regions. The sub-region is the area with the same soil and climatic conditions. The wine-growing zones are defined as geographical regions with different climatic conditions for viticulture (**ÚKSÚP, 2022**). The Central Slovak wine region covers the wines produced in the lower central part of the country. This wine regiónu continues just across the border in Hungary. About 80% of the wines grown in this region are white, while 20% are reds. This region produces varietal wines with a strong bouquet.

Table 1 Wine grape varieties from the Central Slovak wine-growing region used in the study

Village	Subregion	Winery	Grape variety	Date of harvest
1. Seľany	Vinický	Selvino	Irshay Oliver	20.09.2021
2. Seľany	Vinický	Selvino	Pinot gris	20.09.2021
3. Seľany	Vinický	Selvino	Cabernet Sauvignon	20.09.2021
4. Čebovce	Modrokamenský	Movino	Chardonnay	05.10.2021
5. Čebovce	Modrokamenský	Movino	Blaufränkisch	05.10.2021
6. Čebovce	Modrokamenský	Movino	Cabernet Sauvignon	05.10.2021
7. Opatovská Nová Ves	Modrokamenský	Historic Wine Cellar	Pinot noir	11.10.2021
8. Opatovská Nová Ves	Modrokamenský	Historic Wine Cellar	Welschriesling	11.10.2021
9. Opatovská Nová Ves	Modrokamenský	Historic Wine Cellar	Alibernet	11.10.2021
10. Selešťany	Modrokamenský	Chateau Selešťany	Alibernet	19.10.2021
11. Selešťany	Modrokamenský	Chateau Selešťany	Devin	19.10.2021

Sampling of grape berries

Eleven grape samples were taken in the 4 Slovak wineries: Selvino (n = 3), Movino (n = 3), Historic Wine Cellar (n = 3) and Chateau Selešťany (n = 2) (Table 1). Four samples belonged to white varieties - Chardonnay, Devin, Irshay Oliver, Welschriesling and 7 to red - Alibernet 2x, Cabernet Sauvignon 2x, Blaufränkisch, Pinot Gris, and Pinot Noir. Samples were placed directly into a sterile plastic bag, each without damaged grapes or infected signals. The grapes were mycologically examined within 24 hours after harvest.

Mycological analysis of grape berry samples

For the isolation of microscopic filamentous fungi from the surface of the grapes, the method of direct plating was used. Seven – eight grape berries per bunch were randomly selected (total 50), plated onto DRBC (Dichloran Rose Bengal Chloramphenicol agar, Merck, Germany), and incubated at 25 ± 1 °C, for 5–7 days in the dark. The endogenous mycobiota was determined so that 50 another grape berries were suface-disinfected in 1% NaCIO for 1 min according methods of **Magnoli *et al.* (2003**) 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 ± 1 °C, for 5–7 days in the dark. Conventional macroscopic and microscopic observation was used, with guidelines by **Pitt and Hocking** (**2009**). Dense conidia suspensions of *Aspergillus* spp. were inoculated on CYA (Czapek-yeast agar) (**Samson *et al.,* 2010**), MEA (Malt extract agar) (**Samson *et al.,* 2010**), and CY20S (Czapek yeast extract with 20% sucrose) (**Pitt and Hocking, 2009**) and *Penicillium* strains on CYA, MEA, CREA (Creatine Sucrose agar) (**Samson *et al.,* 2010**), and YES (Yeast extract agar) (**Samson *et al.,* 2010**) in 9 cm plastic Petri plates and incubated in the dark at 25 ± 1 °C. Phenotypic identification of isolated *Aspergillus* and *Penicillium* species was performed by studying their morphology. The *Aspergillus* colonies were identified to species level after 7 days, according to the relevant special mycological literature (**Klich, 2002; Pitt and Hocking, 2009**) and *Penicillium* strains using the keys of **Pitt and Hocking** (**2009**), **Samson and Frisvad** (**2004**), and **Samson *et al.*** (**2002a, 2010**).

Data analysis

The obtained results were evaluated and expressed in terms of relative density and isolation frequency. The relative density (RD%) is defined as the percentage of isolates of the species or genus present in the analyzed sample (**Guatam *et al.,* 2009**). These values were calculated following **González *et al.*** (**1999**):
RD (%) = (ni / Ni) x 100
where: ni - number of isolates of one species or genus; Ni - total number of isolated fungi.
Isolation frequency (IF%) is defined as the percentage of samples in which the species or genus occurred at least once. These values were calculated following **González *et al.*** (**1999**):
IF (%) = (ns / N) x 100
where: ns - number of samples with one species or genus, N - total number of samples.

Toxinogenity analysis

The toxinogenicity of selected isolates was studied under *in vitro* conditions by 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 determined on YES agar and intracellular roquefortin C, penitrem A, and cyclopiazonic acid on CYA agar. From the colonies (after 14 days of incubation), some mycelial pieces of about 5 x 5 mm size were cut and placed in an Eppendorf tube containing 500 µl 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). Mycotoxins extracted from cultures of fungal isolates were determined by thin-layer chromatographic technique on a precoated silica gel plate (Alugram ® SIL G, Macherey - Nagel, Germany). 30 µL of the liquid phase of the extracts was applied to the TLC plate together with 10 µL of the standards (Sigma, Germany). The plate was placed in the solvent TEF (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. Mycotoxins were identified by comparison with appropriate reference standards for mycotoxins. Cyclopiazonic acid was directly visible as a purple-tailed spot after spraying with Ehrlich reagent in daylight. Penitrem A was visible as a dark blue spot after spraying with 20% $AlCl_3$ in 60% ethanol and heating at 130 °C for 8 min. Roquefortine C was visible as an orange spot after spraying with $Ce(SO_4)_2$ x 4 H_2O. Patulin was detected by spraying with 0.5% methylbenzothiazolone hydrochloride (MBTH) (Merck, Germany) in methanol and heating at 130 °C for 8 min and was then visible as a yellow-orange spot. Directly under UV light with a wavelength of 365 nm, citrinin was visualized 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

Exogenous mycobiota from 11 grape varieties

The diversity of grapevine-associated surface mycobiota in the Central Slovak wine growing region at the time of grape harvest in 2021 was assessed (Table 2). A total of 574 strains belonging to 12 fungal genera and *Mycelia sterilia* (unidentified fungi without fruiting body formation) were identified. The highest number of isolates (82) and the highest number of genera (7 + *Mycelia sterilia*) were found in Welschriesling grape variety (8) from Opatovská Nová Ves village. The lowest number of isolates (2) was isolated from the white variety Devin (11) from Selešťany village. This wine grape was colonized mainly by yeasts. Almost all samples were of the genus *Alternaria* (90.9% IF), followed by *Aspergillus* (72.7% IF), *Cladosporium*, *Penicillium*, and *Rhizopus* (63.6% IF, each). The most abundant genus was *Alternaria* (53.3% RD) of all the fungi found, *Rhizopus* was the second one (9.4% RD). From the previous study by **Felšöciová and Kačániová (2019**), *Alternaria* was the most genus (100%), also with the highest relative density (87%) in the Vrbovský subregion (Small Carpathians wine-growing region, Slovakia) at harvest time 2018. *Rhizopus* was the second most abundant genus of 93% but a low relative density (5%). The most frequently occurring genera in grapes in the same vineyard, but one year later, were again *Alternaria* and *Rhizopus* (92%, each), followed by *Cladosporium* (85%), *Penicillium* (77%), *Botrytis*, and *Epicoccum* (54%, each) (**Felšöciová *et al.,* 2020**).
In our study *Aspergillus* strains were present on grapes in higher numbers (72.7% IF) but their relative density was lower (5.5%). Fungi of genus *Aspergillus* were found in 8 grape varieties except 3 - Pinot gris (2) and Cabernet Sauvignon (3) from Seľany and Chardonnay (4) from Čebovce villages. Three *Aspergillus* species were found: *A. clavatus, A, flavus* and *A.* section *Nigri*. *Aspergillus* section *Nigri* were the most frequent and abundant isolates (58.3% IF, 77% RD). The most colonized grape variety of these species was Cabernet Sauvignon from Čebovce village. Thirty of the 32 *Aspergillus* isolates were identified as *A.* section *Nigri* also in the study by **Felšöciová *et al.* (2020**).
From exogenous mycobiota 23 strains of 6 *Penicillium* species, namely *P. brevicompactum, P. crustosum, P. expansum, P. glabrum, P. oxalicum*, and *P. thomii* were isolated. Seven samples were colonised by *Penicillium* species. Grape varieties Cabernet Sauvignon (3), Chardonnay (4), Blaufränkisch (5) and Welschriesling (8) were free from penicillia. From the 574 strains detected and identified, *Penicillium* was represented in 3%. According to **Pitt and Hocking** (**2009**), *Penicillium* species do not infect grapes before harvest, but are widespread in stored grapes, where *P. expansum* is the most common contaminating species. *Penicillium expansum* is common in certain wine regions such as the bordering regions in northern Portugal and Galiza (Spain) (**Serra *et al.,* 2006**). *Penicillium expansum* dominated in the Müller Thurgau variety, Blaufränkisch, Seteasca Regala, Rheinriesling, and Palava from the area of the Small Carpathians, Slovakia (**Felšöciová *et al.,* 2020**). In our case, *Penicillium expansum* occurred only once. The most frequent isolate was *Penicillium glabrum* (27.3% IF) with the highest relative density from all penicillium isolates (26.1% RD). A total of 10 wine grapes

varieties were collected from the Central Slovak region in 2011 and 2012, which involved 7 vineyards (**Felšöciová *et al.*, 2014**). In general representatives of *Penicillium* genus were isolated with higher frequency from the surface of the berries than from their interior. Overall, two species were dominant: *P. chrysogenum* and *P. expansum*. Colonization of *P. glabrum* was not detected from exogenous mycobiota, one isolate was found only from endogenous mycobiota. Occurrence of *Penicillium* was very sporadically at the harvest time in 2018 from the Small Carpathian wine-growing region (**Felšöciová and Kačániová, 2019**). *Penicillium citrinum* was the dominant species in the exogenous mycobiota, *P. expansum* and *P. glabrum* were less isolated. On the other hand, *Penicillium* spp. were among the most common species identified in grapes from the Small Carpathians area during the 2011 to 2013 harvests (**Felšöciová *et al.*, 2015**). The major species responsible for the ochratoxin production in foods from temperate climate, *P. verrucosum* (**Pitt and Hocking, 2009**), was not isolated from our grape samples.

Table 2 Fungi identified in Slovak grapes, isolation frequency and relative density from exogenous mycobiota from 2021 by the method of direct plating

Fungal taxa	**1.**	**2.**	**3.**	**4.**	**5.**	**6.**	**7.**	**8.**	**9.**	**10.**	**11.**	**No**	**IF (%)**	**RD (%)**
Alternaria	24	48	62	66	43	35	11	45	32	14	-	380	90.9	53.3
Aspergillus	3	-	-	-	2	15	5	2	4	7	1	39	72.7	5.5
A. clavatus	-	-	-	-	-	-	1	-	1	1	-	3	27.3	7.7
A. flavus	-	-	-	-	-	-	2	-	-	3	1	6	27.3	15.4
A. section *Nigri*	3	-	-	-	2	15	2	2	3	3	-	30	58.3	77
Bipolarys	-	-	-	-	-	-	1	-	-	-	-	1	9	6.6
Botrytis	2	-	-	-	-	-	-	-	-	-	-	2	9	<1
Cladosporium	-	-	4	6	2	3	3	24	5	-	-	47	63.6	<1
Epicoccum	-	-	-	-	1	-	-	-	-	-	-	1	9	<1
Fusarium	-	-	-	-	-	-	-	1	-	-	-	1	9	<1
Mucor	-	-	-	-	-	-	-	1	-	-	-	1	9	<1
Mycelia sterilia	-	-	-	-	-	-	2	-	3	-	-	5	18.2	<1
Paecilomyces	-	-	-	-	-	-	-	-	3	-	-	3	9	<1
Penicillium	1	13	-	-	-	1	1	-	3	3	1	23	63.6	3
P. brevicompactum	-	2	-	-	-	-	-	-	-	-	-	2	9	8.7
P. crustosum	1	-	-	-	-	-	-	-	-	2	-	3	18.2	13
P. expansum	-	1	-	-	-	-	-	-	-	-	-	1	9	4.3
P. glabrum	-	4	-	-	-	1	-	-	-	-	1	6	27.3	26.1
P. oxalicum	-	-	-	-	-	-	-	-	-	1	-	1	9	4.3
P. thomii	-	-	-	-	-	-	-	-	1	-	-	1	9	4.3
P. sp.	-	6	-	-	-	-	1	-	2	-	-	9	27.3	39.1
Rhizopus	23	10	9	-	-	-	4	9	12	2	-	69	63.6	9.4
Trichoderma	-	-	-	-	-	1	1	-	-	-	-	2	18.2	<1
Total	53	71	75	72	48	55	28	82	62	26	2	574		

Legend: 1 – Irshay Oliver, 2 – Pinot gris, 3, 6 – Cabernet Sauvignon, 4 – Chardonnay, 5 – Blaufränkisch, 7 – Pinot noir, 8 – Welschriesling, 9, 10 – Alibernet, 11- Devin, No – number of isolated micromycetes, IF – isolation frequency, RD – relative density

Endogenous mycobiota from 11 grape varieties

In the endogenous mycobiota of grapes, a total of 512 strains belonging to 8 fungal genera and *Mycelia sterilia* were identified (Table 3). The highest number of isolates (91) was found in Alibernet grape variety (10) from Selešťany village. The highest number of genera (6 + *Mycelia sterilia*) was found in Alibernet grape variety (9) from Opatovská Nová Ves village. The lowest quantitative representation of micromycetes (3) was isolated from the blue variety Blaufränkisch (5) from Čebovce village. *Alternaria* spp. represent the most common species occurring on grape berry skin (**Kačániová *et al.*, 2019**) in particular during the pea berry and early veraison stages, while at harvest the frequencies of these species drastically decrease (**Serra *et al.*, 2005**) what did not correspond with our results. All samples were colonized by genus *Alternaria* (100% IF), whereas in most samples, *Alternaria* reached the highest numbers except for two - Alibernet (10) from Selešťany, where the genus *Cladosporium* dominated, and Cabernet Sauvignon (6) from Čebovce, where the species *Aspergillus* section *Nigri* dominated. Some of *Alternaria* secondary metabolites, such as alternariol and alternariol monomethyl ether, have been detected in wines and grape juice (**Asam *et al.*, 2009**). However, to date, maximum and recommended levels have not been established for any *Alternaria* mycotoxin in foodstuffs by the EU (**Tini *et al.*, 2020**).

The most frequent genera of micromycetes in the present work were *Rhizopus* (81.8% IF), *Penicillium* (72.7%), *Aspergillus* (63.6%), and *Cladosporium* (45.5%). The most abundant genus was *Alternaria* (61.1% RD) of the total mycobiota, followed by *Cladosporium* (13.1%), *Aspergillus* (11.7%), and *Rhizopus* (9%). The genus *Botrytis*, which is considered the most damaging pathogen of on grapes, responsible for gray rot or noble rot disease, was found only in a small proportion (< 1% RD) of the total mycobiota and only in grapes Cabernet Sauvignon (3) from Seľany and Alibernet (9) from Opatovská Nová Ves. Must from botrytized grapes is more susceptible to oxidation due to the polyphenol oxidizing activity of the laccase of *B. cinerea* and is not suitable for winemaking (**Morales *et al.*, 2013**).

Fungi of genus *Aspergillus* were found in 7 grape varieties except 3 - Pinot gris (2) and Cabernet Sauvignon (3) from Seľany village, Chardonnay (4) from Čebovce and Alibernet (9) from Opatovská Nová Ves. Two *Aspergillus* species were found: *A. clavatus,* and *A.* section *Nigri*. *Aspergillus* section *Nigri* were the most frequent and abundant isolates (54.5% IF, 98.3% RD) from all aspergillus isolates.

Table 3 Fungi identified in Slovak grapes, isolation frequency and relative density from endogenous mycobiota from 2021 by the method of direct plating with surface disinfection

Fungal taxa	**1.**	**2.**	**3.**	**4.**	**5.**	**6.**	**7.**	**8.**	**9.**	**10.**	**11.**	**No**	**IF (%)**	**RD (%)**
Alternaria	32	30	26	35	1	1	21	36	48	36	47	313	100	61.1
Aspergillus	1	-	-	-	1	51	2	3	-	1	1	60	63.6	11.7
A. clavatus		-	-	-	-	-	-	-	-	-	1	1	9	1.7
A. section *Nigri*	1	-	-	-	1	51	2	3	-	1	-	59	54.5	98.3
Botrytis		-	1	-	-	-	-	-	3	-	-	4	18.2	<1
Cladosporium	3	-	-	-	-	-	5	11	4	44	-	67	45.5	13.1
Epicoccum	-	-	-	-	-	-	2	-	1	1	-	4	27.3	<1
Mycelia sterilia	-	-	-	-	-	-	-	-	2	-	-	2	9	<1
Penicillium	2	4	2	4	-	-	1	-	1	-	1	15	72.7	3
P. brevicompactum	-	-	-	-	-	-	1	-	1	-	-	2	18.2	13.3
P. expansum	-	3	2	-	-	-	-	-	-	-	-	5	18.2	33.3
P. glabrum	-	1	-	4	-	-	-	-	-	-	-	5	18.2	33.3
P. sp.	2	-	-	-	-	-	-	-	-	-	1	3	18.2	20
Rhizopus	8	1	-	15	-	2	3	3	2	9	3	46	81.8	9
Trichoderma	-	-	-	-	1	-	-	-	-	-	-	1	9	<1
Total	46	35	29	54	3	54	34	53	61	91	52	512		

Legend: 1 – Irshay Oliver, 2 – Pinot gris, 3, 6 – Cabernet Sauvignon, 4 – Chardonnay, 5 – Blaufränkisch, 7 – Pinot noir, 8 – Welschriesling, 9, 10 – Alibernet, 11- Devin, No – number of isolated micromycetes, IF – isolation frequency, RD – relative density

Fifteen strains of 3 *Penicillium* species, namely *P. brevicompactum, P. expansum,* and *P. glabrum* were isolated from endogenous mycobiota in this study. Seven samples were colonised by *Penicillium* species but at a low frequency of occurrence. Grape varieties Blaufränkisch (5), Cabernet Sauvignon (6), Welschriesling (8) and Alibernet (10) were free from penicillia. From the 512 strains detected and identified, *Penicillium* was represented in 3% as in exogenous mycobiota. Fourteen samples of grapes were analyzed by plating methods with surface disinfection by **Felšöciová and Kačániová (2019)**. *Penicillium citrinum, P. hordei, P. chrysogenum,* and *P. griseofulvum* were recorded in endogenous source. *Penicillium citrinum* was the most dominant species.

Toxinogenity of *Aspergillus* and *Penicillium* isolates from selected grape varieties

The toxicogenic profile of the 35 *Aspergillus* isolates and 4 *Penicillium* isolates representing *Aspergillus clavatus, A. flavus*, *A.* section *Nigri, P. crustosum* and *P. expansum* from the exogenous mycobiota of Slovak wine grapes is shown in Table 4. Positive toxinogenity was detected for PAT by *Aspergillus clavatus*, for penitrem A by *P. crustosum* (2 strains out of 3), for roquefortin C by *P. crustosum* and *P. expansum* (100%, each), and for citrinin by *P. expansum*. All tested strains of *Aspergillus* section *Nigri* were negative for OTA and two strains of *Aspergillus flavus* negative for aflatoxin B_1, G_1, and cyclopiazonic acid. These results differ from other reports demonstrating OTA contamination in *Vitis vinifera* grapes (**Battilani *et al.*, 2006; Chiotta *et al.*, 2013; García-Cela *et al.*, 2015; Mikušová *et al.*, 2014; Mogensen *et al.*, 2010a,b; Qi *et al.*, 2016, Somma *et al.*, 2012; Soto *et al.*, 2014**). *Aspergillus* species belonging to *A.* section *Nigri* were found in the majority of grape samples collected in the Brazilian states of Paraná, Rio Grande do Sul, Pernambuco, and São Paulo. Regarding mycotoxin production ability, 3.2% of the total isolates of *A.* section *Nigri* (2042) were positive for OTA production (**de Souza Ferranti *et al.*, 2018**). Aflatoxins are potent carcinogens, produced by *Aspergillus flavus* and *A. parasiticus* (**Pitt, 2000**). Both species were isolated from grapes from the Small Carpathian area by **Felšöciová *et al.* (2015)**. The strain of *A. parasiticus* was able to produce aflatoxin B_1 and G_1 *in vitro*, *A. flavus* produced AFG_1 (2 out of 5) but not AFB_1.

Table 4 Toxinogenity of selected *Aspergillus* and *Penicillium* strains, isolated from the exogenous mycobiota of grapes

***Aspergillus* species**	**ochratoxin A**	**patulin**	**citrinin**	**roquefortin C**	**penitrem A**	**AFB_1**	**AFG_1**	**CPA**
Toxinogenity of exogenous mycobiota								
***Aspergillus** clavatus*	-	3/3	-	-	-	-	-	-
Aspergillus flavus	-	-	-	-	-	0/2	0/2	0/2
Aspergillus section *Nigri*	0*/30**	-	-	-	-	-	-	-
***Penicillium** crustosum*	-	-	-	3/3	2/3	-	-	-
P. expansum	-	0/1	1/1	1/1	-	-	-	-
Toxinogenity of endogenous mycobiota								
***Aspergillus** clavatus*	-	1/1	-	-	-	-	-	-
Aspergillus section *Nigri*	0/55	-	-	-	-	-	-	-
***Penicillium** expansum*	-	1/1	1/1	1/1	-	-	-	-

Legend: * - number of isolates with ability to produce mycotoxins, ** - number of tested isolates, AF – aflatoxin, CPA – cyclopiazonic acid

Data in Table 4 also show that, 57 isolates belonging to 2 *Aspergillus* species (*A. clavatus* and *A.* section *Nigri*) and *Penicillium expansum* from endogenous mycobiota of wine grapes were screened for their toxinogenity by TLC method. One strain of *Aspergillus clavatus* was positive for patulin, one strain of *Penicillium expansum* was positive for patulin, citrinin and roquefortin C. Negative toxinogenity was detected for ochratoxin A by *Aspergillus* section *Nigri*. *Penicillium expansum* is the most widespread fungus in soil and air with good adaptability to environmental conditions. However, growth occurs at an optimal temperature of 25 °C and 90% relative humidity. Essentially, it grows on decaying plant material (**Li *et al.*, 2020**). However, the fungus can produce PAT at a temperature range of 1-20 °C (**Garcia *et al.*, 2011**), with the optimal temperature being 25 °C (**Tannous *et al.*, 2016**). Processes such as clarification, filtration, and enzymatic treatment during processing of juices and fermentation in winemaking significantly reduce the content of PAT. Washing, sorting and grading, as well as removing spoiled parts of the fruit, can also reduce the PAT content (**Cunha *et al.*, 2014**). *Penicillium* spp. in our samples was generally low (3% RD). On the other hand, *Penicillium* was a common component on the grapes from the Small Carpathian area from 2011 to 2013 (**Felšöciová *et al.*, 2015**). Ninety three percent of samples (IF) were colonized by the genus *Penicillium*. *Penicillium expansum* produced roquefortin C (5 out of 5), patulin (3 out of 5) and citrinin (2 out of 5). Positive toxinogenity was detected in *P. crustosum* on penitrem A and roquefortin C (14/14, 100%). Potential producer of OTA *Aspergillus* section *Nigri* was the most frequent mycotoxigenic species isolated from grapes. However, the production of OTA was not confirmed, as in our study. The ability to produce patulin under *in vitro* conditions was demonstrated in 100% of the isolates of *A. clavatus* (5/5), which is consistent with our results.

CONCLUSION

Grape samples were grown in the Central Slovak wine region, in the Modrokamenský and Vinický subregions in 2021. All grape samples analyzed (n = 11) showed fungal contamination. The most common filamentous fungal genera found on the surface of the grapes were *Alternaria* (90.9%), *Aspergillus* (72.7%), *Cladosporium, Penicillium*, and *Rhizopus* (63.6%, each). The *Botrytis* genus, which is regarded as the main spoilage cause in wine grapes, was isolated in this study but in low frequency (9%). The most abundant genera found were *Alternaria* (53.3%) and *Rhizopus* (9.4%). Potencional producers of mycotoxins *Aspergillus* (5.5% RD) and *Penicillium* (3% RD) contributed a small proportion from mycobiota associated with grapevine in the Central Slovak wine region. The most frequent genera from endogenous mycobiota were *Alternaria* (100%), *Rhizopus* (81.8%), *Penicillium* (72.7%), *Aspergillus* (63.6%), *Cladosporium* (45.5%), and *Epicoccum* (27.3%). The most abundant genera were *Alternaria* (61.1%), *Cladosporium* (13.1%), *Aspergillus* (11.7%), and *Rhizopus* (9%). *Penicillium* was one less abundant genus (3% of all fungi found). Mycotoxin production is characteristic of each species, and therefore species identification can predict potential mycotoxin hazards. From *Aspergillus* species we isolated *A. clavatus, A. flavus*, *A.* section *Nigri* and from *Penicillium* species *P. brevicompactum*, *P. crustosum, P. expansum, P. glabrum, P. oxalicum*, and *P. thomii*. Ochratoxin A, patulin, citrinin, roquefortin C, penitrem A, aflatoxin B_1, aflatoxin G_1, and cyclopiazonic acid production was tested by thin layer chromatography method in *in vitro* condition. Although several grape samples analyzed were contaminated with *A.* section *Nigri*, none of the 88 isolates produced this mycotoxin *in vitro*.

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