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MYCOTOXIN-PRODUCING *PENICILLIUM* SPP. AND OTHER FUNGI ISOLATED FROM GRAPES FOR WINE PRODUCTION IN SMALL CARPATHIANS WINE REGION

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ABSTRACT

The diversity of mycobiota associated with grapevine in Vrbove, Slovakia at the harvest time in 2018 was evaluated. Fourteen samples of grapes were analyzed by plating methods and by plating methods with surface disinfection. The identification of fungi was performed using the morphological and microscopical characteristics. From the 1001 strains detected and identified from exogenous mycobiota, the most frequent genera were *Alternaria, Rhizopus* and *Sordaria*. Their relative density was low, except *Alternaria*. The most frequently encountered moulds and with the highest relative density from endogenous mycobiota were *Alternaria, Cladosporium* and *Penicillium*. Most of all genera had relative density less than 1%. *Penicillium* contributed small proportion in both sources. *Penicillium citrinum* was the most dominant species in exogenous and endogenous mycobiota. *Penicillium expansum* and *P. glabrum* were recorded in exogenous source and *P. hordei*, *P. chrysogenum* and *P. griseofulvum* in endogenous. Potentially toxigenic *Penicillium* species were tested for their toxigenic ability by thin layer chromatography method. Out of 15 tested isolates representing five potentially toxigenic species 11 produced at least one mycotoxin. Positive toxinogenity was detected in all tested strains of *Penicillim citrinum* (9/9).

Keywords: grapes; *Penicillium*; microfungi; mycotoxins; TLC

INTRODUCTION

Fresh grapes are prone to fungal contamination in the fields, during harvesting, transporting, marketing and during storage under domestic conditions. If the spoiling fungi are toxigenic or pathogenic, they could pose a health risk for the consumer. Grape fruit contain high levels of sugars and other nutrients, and they possess and ideal water activity for microbial growth, their low pH makes them particularly susceptible to fungal spoilage **(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)**. The grape mycrobiota may change in response to various factors such as: the climate, grape variety and geographical region **(Raspor et al., 2006)**.

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 **(Visagie et al., 2014)**. Species of *Penicillium* are economically, ecologically and medically important microorganisms playing vital roles in natural ecosystems, agriculture and biotechnology **(Visagie, 2008)**. Many species of the *Penicillium* are among the common postharvest pathogens on a wide range of fruits and vegetables. *Penicillium expansum*, the main cause of green (blue) mold of apple, pear and fruits of other deciduous trees, is an example of destructive pathogens that causes much of post-harvest economic losses on food during storage and marketing stages **(Barkai-Golan, 2008)**. Two main genera are responsible for mycotoxin production in grapes: *Aspergillus* and *Penicillium*. The mycotoxin production is characteristic of the species and therefore by identifying the species one can predict potential mycotoxin hazards **(Serra et al., 2006)**. Green mould produces mycotoxins for example patuline which is however degraded during fermentation and by sulphurization. Berries affected by green mould have an off-flavour and even a small amount of infected berries add a mouldy taste to the wine **(Kassemeyer and Berkelmann-Löhnertz, 2009)**. Ochratoxin A (OTA) producing fungi are members of the genera *Aspergillus* and *Penicillium*. Natural occurrence of OTA has been reported from temperate to tropical climates mainly on cereals and their products, beverages such as bread, beer, coffee, dried fruits, grape juice and wine among others. Only some OTA producing species are known to be a potential source of OTA contamination of these commodities, including *A. niger* aggregate, *A. carbonarius, A. ochraceus, A, westerdijkiae,*

A. steynii, Penicillium verrucosum and *P. nordicum* **(Cabañes et al., 2010)**.

In our work the goal was to assess the fungi present on the surface and inside of healthy grapes destined for commercial winemaking at harvest time. Emphasis was given to *Penicillium* species due to their relevance for mycotoxin production.

Scientific hypothesis

During maturation of grapes, the spoilage agents, *Aspergillus, Penicillium* and *Rhizopus*, increase their incidence. When the temperature is higher then 37 °C, species in *Aspergillus* section *Nigri* become predominant **(Valero et al., 2007)**. At harvest time, the conditions are optimal for fungal invasion, especially if physical damage had occurred on grape. Certainly the *Aspergillus* species are present worldwide, in all the grape products and under all environmental conditions.

MATERIAL AND METHODOLOGY

Study area

The grapes (*Vitis vinifera*) used in this study originated from a small family winery founded in 2002. Their goal is to produce quality wines mainly from grapes from their own production. The farm is situated in Vrbovsky subregion, in the village of Vrbove, which is part of the Small Carpathians wine region. In the years 2014 – 2016, 10 ha of their own vineyards were planted in Vrbove. Young vineyards are great advantage because they had the opportunity to choose many varieties according to the climate, soil type, but also their own experience so that they could obtain a high-quality raw material for the production of unique wines. They planted old varieties that were grown in Vrbove in the past, for example Green Veltliner, Müller Thurgau, Sauvignon, but also modern varieties such as Pálava, Cabernet Sauvignon and Alibernet.

The Small Carpathians wine region has a medium climate and abundant moisture. The spring of 2018 was an extremely abnormal temperature in Slovakia. April and May were two recordable warm months in Hurbanovo. The average monthly air temperature reached 16.2 °C in April and 19.7 °C in May. Summer was extremely hot with the absence of longer, cooler days. Intense rainfall occurred at the beginning of September **(Siman, 2019)**.

Grape samples

Wine company provided the grapes grown for commercial winemaking. A total of 14 samples were taken: ten from white varieties (Green Veltliner, Seteasca Regala, Chardonnay, Rheinriesling, Welschriesling, Sauvignon, Pálava, Pinot Blanc, Irsai Oliver, Müller Thurgau and the remaining 4 from red varieties: Dornfelder, Blaufränkisch, Alibernet, Cabernet Sauvignon, during the period from the end of August to the beginning of September 2018, in the maturation stages corresponding to harvest. The samples comprised 10 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.

Isolation and identification of fungi

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. The detection of fungi in grape samples was also made by plating methods with surface disinfection. The whole 50 grapes were surface-disinfected in 1% NaClO for 1 min according methods of **Magnoli et al. (2003)**. After thorough washing with sterile distilled water (3 times, total amount 1L), and drying were inserted on the agar surface DRBC as a whole fruit and incubated at 25 ±1 °C in the dark for 5 – 7 days.

The identification of fungal taxa based on macroscopic and microscopic features, with guidelines by **Pitt and Hocking (2009)**.

Media used for isolation of fungi were:

a) Dichloran Rose Bengal Chloramphenicol agar medium (DRBC, MERCK, Germany) to which rose bengal (25 $\mu g.mL^{-1}$) was used to inhibit rapidly growing fungi and chloramphenicol (100 $\mu g.mL^{-1}$) was used as bacteriostatic agents.

Penicillium strains were isolated and cultivated on these medias:

b) Malt extract agar (MEA),
c) Czapek yeast agar (CYA),
d) Creatine-Sucrose agar (CREA),
e) Yeast Extract agar (YES) **(Samson et al., 2010)**.

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)**.

Mycotoxins analysis

A total of 15 different isolates of filamentous fungi from different samples belonging to *Penicillium* spp. were examined for their ability on mycotoxins production by thin layer chromatography (TLC) according to **Samson et al. (2002b)**, modified by **Labuda and Tančinová (2006)**. Extracellular metabolites – citrinin, griseofulvin and patulin were carried out on YES agar and intracellular roquefortine 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, – Carlsbad, CA, USA).

Mycotoxins detection

Mycotoxins extracted from fungal isolates cultures were determined by thin layer chromatographic technique on pre-coated silica gel plate (Alugram ® SIL G, Macherey – Nagel, Germany). The volume 30 µL of liquid phase of extracts along with 10 µL standards (Sigma, Germany) was applied on TLC plate. 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. Mycotoxins were identified by comparison with appropriate reference standards of mycotoxins.

Table 1 Fungi identified in Slovak wine grapes from 2018 by the direct plating method.

Fungal taxa	No.	Fr (%)	RD (%)
Alternaria	868	100	87
Aspergillus	5	14	<1
Aureobasidium	1	7	<1
Botrytis	18	43	2
Cladosporium	4	21	<1
Epicoccum	7	36	<1
Mucor	3	14	<1
Penicillium	5	21	<1
from this			
P. citrinum	3	7	
P. expansum	1	7	
P. glabrum	1	7	
Rhizopus	49	93	5
Sordaria	13	71	1
Trichoderma	1	7	<1
Mycelia sterilia	19	71	2
Total isolates	**1001**		

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

Table 2 Fungi identified in Slovak wine grapes from 2018 by the direct plating method with surface disinfection.

Fungal taxa	No.	Fr (%)	RD (%)
Alternaria	414	100	81
Aspergillus	1	7	<1
Botrytis	1	7	<1
Cladosporium	49	79	10
Epicoccum	3	21	<1
Fusarium	2	14	<1
Penicillium	14	43	3
from this			
P. citrinum	6	21	
P. griseofulvum	1	7	
P. hordei	4	7	
P. chrysogenum	3	21	
Rhizopus	7	29	1
Trichoderma	1	7	<1
Mycelia sterilia	17	64	3
Total isolates	**509**		

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

Table 3 Toxinogenity of selected *Penicillium* strains, isolated from exogenous and endogenous mycobiota of wine grapes.

Species/Exo	C	G	P	CPA	RC
P. citrinum	3*/3**				
P. expansum	1/1		0/1		0/1
Species/Endo					
P. citrinum	6/6				
P. griseofulvum		1/1	1/1	0/1	0/1
P. hordei					0/1
P. chrysogenum					0/3

Note: * - number of isolates with ability to produce mycotoxin, ** - number of tested isolates, C – citrinin, G – griseofulvin, P – patulin, CPA – cyclopiazonic acid, RC – roquefortin C.

Roquefortine 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. 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 were visualized citrinin as a yellow-green-tailed spot and griseofulvin as a blue spot.

Statisic analysis

The obtained results were evaluated and expressed according to relative density (RD) and isolation frequency (Fr). The relative density (%) is defined as the percentage of isolates of the species or genus, occurring in the analyzed sample **(Guatam et al., 2009)**. These values were calculated according to **González et al. (1999)** as follows:

$$RD\ (\%) = (ni / Ni) \times 100$$

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

The isolation frequency (%) is defined as the percentage of samples within which the species or genus occurred at least once. These values were calculated according to **González et al. (1999)** as follows:

$$Fr\ (\%) = (ns / N) \times 100$$

where: ns – number of samples with a species or genus, N – total number of samples.

RESULTS AND DISCUSSION

A survey study was conducted on 14 samples of fresh grape which collected from Vrbove, Slovakia. Isolation of fungi contaminated fresh grape resulted in collecting of 1001 fungal isolates. Data in Table 1 show that, eleven fungal genera namely *Alternaria, Aspergillus, Aureobasidium, Botrytis, Cladosporium, Epicoccum, Mucor, Penicillium, Rhizopus, Sordaria, Trichoderma* and *Mycelia sterilia* (unidentified fungus without creation fruiting bodies) were identified from fresh grape samples.

Data in the same Table 1 showed that, *Alternaria* was the most frequently occurring genus (100 %) which record 868 isolates, with the highest relative density 87%. *Rhizopus* was the second predominant genus which recorded frequency of 93%, but low relative density (5%), followed by *Sordaria* (71% Fr, 1% RD). *Botrytis, Epicoccum, Penicillium, Cladosporium, Aspergillus, Mucor Aureobasidium* and *Trichoderma* were less fungal frequency occurred in all grape samples.

Khashaba et al. (2018) 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%. In our study *Aspergillus* and *Penicillium* were less fungal frequency occurred in all grape samples. Similar results that *Aspergillus* is the main fungal genus were obtained by several studies **(Alisa et al., 2007; Fredj et al., 2007)**. *Alternaria, Botrytis* and *Cladosporium* were three of the most frequent genera in all four winemaking regions in Portuguese **(Serra et al., 2006)**, representing 16, 17 and 24% of the total identified strains, respectively.

Figure 1 *Penicillium expansum.*

Figure 2 *Penicillium chrysogenum.*

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. *Aspergillus* was more frequent than *Penicillium* in almost all regions. In our samples *Botrytis* represented 2% of the total identified strains and *Cladosporium* less than 1%, but *Alternaria* was the most frequent genus, too. **Serra et al. (2006)** described that the most frequent *Penicillium* species were *P. brevicompactum, P. thomii* and *P. glabrum* which together accounted for approximately 71% of the strains identified in the genus. Five species of *Penicillium* including *P. brevicompactum, P. citrinum, P. echinulatum, P. expansum* and *P. solitum* reported from grapes in Korea **(Won et al., 2007)**. **Serra and Peterson (2007)** described two new species of *Penicillium*, namely, *P. astrolabium* and *P. neocrassum* from contaminated grapes in Portugal. The reports on *Penicillium* species occurring on grape and raisin from Iran are very rare **(Rahmani et al., 2012; Maulani et al., 2012)**, mainly *P. expansum, P. brevicompactum* and *P. glabrum* **(Houbraken et al., 2014)**. Five species represent new records for the mycobiota of Iran are *Penicillium crocicola, P. olsonii, P. sumatrense, Talaromyces atroroseus* and *T. minioluteus* **(Khodaei et al., 2016)**. During our survey, five isolates belonging to three *Penicillium* species (*P. citrinum, P. expansum* and *P. glabrum*) were isolated and identified from exogenous colonisation (Table 1). Their occurrence was very sporadically. On the other hand, *Penicillium* were between the most common species identified in grape from Small Carpathian area during harvesting 2011 to 2013 **(Felšöciová et al., 2015)**. From 13 different *Penicillium* species with high frequency (93%) were 4 main species: *P. chrysogenum, P. crustosum, P. expansum* and *P. griseofulvum*. *Penicillium citrinum* was less common species identified in grape samples.

By the endogenous (surface-disinfected) plating method were identified nine different genera from the 509 fungal strains (Table 2): *Alternaria, Aspergillus, Botrytis, Cladosporium, Epicoccum, Fusarium, Penicillium, Rhizopus, Trichoderma* and *Mycelia sterilia*. The three most abundant genera found by descending order and with the highest relative density were *Alternaria, Cladosporium* and *Penicillium*. Most of all genera had relative density less than 1%.

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 **(Serra et al., 2006)**. Fungal species capable of causing rot in grapes (e.g. *Aspergillus niger, Botrytis cinerea, Rhizopus*) were not common inhabitants of the berry surface.

Four *Penicillium* species were found: *Penicillium citrinum, P. griseofulvum, P. hordei* and *P. chrysogenum* (Table 2). According **Felšöciová et al. (2013)** some similar species but in lower number of abundances were isolated from the Nitra wine growing region in 10 analysed samples, namely *P. citrinum, P. corylophilum, P. crustosum, P. decumbens, P. expansum, P. chrysogenum* and *Penicillium* spp. The occurrence of most was also very sporadically, except *P. crustosum, P. chrysogenum* and *P. expansum*. Nine *Penicillium* species (*P. canescens, P. citrinum, P. crustosum, P. expansum, P. funiculosum, P. glabrum, P. griseofulvum, P. chrysogenum* and *P. variabile*) were found from grapes grown in the Central Slovak wine region, but there occurrence was also very sporadically except two *P. expansum* and *P. chrysogenum* **(Felšöciová et al., 2015)**.

As shown in Table 3, thin layer chromatographic analysis of 15 tested isolates representing 5 potentially toxigenic species showed that 11/15 (73%) produced at least one mycotoxin. Positive toxigenity was detected in *P. citrinum* (9 out of 9 strains screened). *Penicillium expansum* (Figure 1) produced only citrinin, did not produce patulin and roquefortin C, *Penicillium griseofulvum* produced griseofulvin and patulin, the production of cyclopiazonic acid and roquefortin C was not confirmed. Negative toxigenity were detected in *Penicillium hordei* and *P. chrysogenum* (Figure 2) on roquefortin C.

Penicillium spp. in our samples was generally low (3% RD). On the other hand, *Penicillium* was a common component of the grapes mycobiota from 2011 to 2013 in the Small Carpathian area **(Felšöciová et al., 2015)**. Ninety three percent of samples were colonies by the genus *Penicillium*. During the survey, 251 isolates belonged to 14 *Penicillium* species. Out of 124 strains, 84% produced at least one mycotoxin by TLC method. The most frequent was *P. chrysogenum*. Interesting was, that almost all tested strains on roquefortin C were producted (100 out of 102). No mycotoxin (RC) was formed by the examined four species (*P. expansum*, *P. griseofulvum*, *P. hordei* and *P. chrysogenum*) isolated from our grape samples.

CONCLUSION

The present work indicated that the examined grape fruits were contaminated with several fungi especially members of *Alternaria*. Members of *Aspergillus* and *Penicillium*, fungi capable of producing mycotoxins such as aflatoxins and ochratoxin A, were very rare. These findings indicate that strict hygiene microbiological must be applied during different stages of harvest, transport, storage and handling to avoid the harmful effects on human health.

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