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REGULAR ARTICLE

OCCURRENCE OF *PENICILLIUM* SPECIES IN GRAPES FROM NITRA WINE REGION

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ABSTRACT

A study was carried out to investigate *Penicillium* species present on grapes grown in Slovakia. The survey involved Nitra wine region, located on southern Slovakia. In 2011 bunches of grapes (10) were collected from all 9 vineyards. A total of 50 berries (5 berries per bunch) from each sample were placed in Dichloran Rose Bengal Chloramphenicol agar medium. In this way was determined by using plate dilution method an exogenous mycobiota. The endogenous mycobiota was determined by the method of direct placing of superficially sterilized 50 wine grapes on the same medium. Cultivation lasted at 25 ± 1 °C in the dark from 5 to 7 days. During the survey, 170 isolates belonging to 6 *Penicillium* species were collected: *P. citrinum, P. corylophilum, P. crustosum, P. decumbens, P. expansum* and *P. chrysogenum.* The number of colonized samples was from 10 to 60 % and relative density from 0.6 to 28 %. *P. chrysogenum* was the most frequent, isolated in 40 % of the samples. The highest relative density belongs to *P. crustosum* (28 %) and *P. chrysogenum* (27 %). Toxinogenity of selected isolates was analysed by means of thin layer chromatography. Three potentially toxigenic species isolated from endogenous mycobiota were tested for their toxigenic ability. Out of 8 strains, 50 % produced at least one mycotoxin. Of all 15 potencially toxigenic strains from exogenous mycobiota all of them were positive on screening mycotoxins.

Keywords: *Penicillium*, wine grapes, vineyard

INTRODUCTION

Grape is a fruit appreciated by consumer as fresh (table grapes), dried (raisins), or as processed products, such as grape juice and wine (**Aydogdu and Gucer, 2009**).
Contamination of grapes by different moulds occurs during pre-harvesting, harvesting and grape processing. The fungal growth begins in grapes if temperature and humidity are suitable (**Magnoli *et al.*, 2003; Valero *et al.*, 2005**). The concern about filamentous fungi in the vineyard has been traditionally linked to spoilage of grapes due to fungal growth (**Serra *et al.*, 2005**). Rotting and spoilage of grape berries before harvest can be caused by a variety of fungal species such as *Alternaria* spp., *Aspergillus* spp., *Botrytis cinerea*, *Cladosporium* spp., *Eurotium* spp., *Penicillium* spp. and *Rhizopus* spp. These genera are regarded as the main natural contaminants of grapes or mycotoxin production (**Magnoli *et al.*, 2003; Valero *et al.*, 2005**). However, these fungi do not have the ability to grow in wines and their effect on wine quality is due to grape damage (**Barata *et al.*, 2012**). The mycotoxins of greatest significance in grapes and grape products are produced by *Aspergillus* and *Penicillium* spp. include ochratoxin A, aflatoxins, patulin and citrinin (**Battilani *et al.*, 2003; Magnoli *et al.*, 2003**). Mycotoxins such as patulin, aflatoxin and citrinin are less common that ochratoxin A in grapes before storage (**Serra *et al.*, 2003**). The main concern from the viewpoint of mycotoxin contamination is the black Aspergilli, *Aspergillus carbonarius*, *A. niger* and *Penicillium verrucosum*. These fungi are capable of producing ochratoxin A (OTA) which may contaminate grapes and grape products such as wine, grape juice and dried vine fruit. In the vineyard, careful management of cultivation, irrigation and pruning can assist in minimizing the levels of black Aspergilli in the soil, which in turn, can minimize contamination of grapes by these fungi. Minimising damage to grapes on the wine by the use of open wine canopies, grape varieties with resistance to rain damage and by the management of insect pests and fungal diseases can reduce the incidence of *Aspergillus* rot in mature berries. The risk of OTA in table grapes can be minimized by careful visual inspection to avoid damaged and discoloured berries. In wine, harvesting grapes with minimal damage, rapid processing and good sanitation practices in the winery assist in minimizing OTA (**Hocking *et al.*, 2007**).

The aim of this study was to evaluate the occurrence of mycobiota in wine grape samples collected from Nitra wine region, Slovakia with focus on genera *Penicillium*. The potentially toxigenic *Penicillium* species were tested for the ability to produce selected mycotoxins.

MATERIAL AND METHODS

Study area

Slovak republic has 6 distinct wine-growing zones (Malokarpatská, Južnoslovenská, Nitrianska, Stredoslovenská, Východoslovenská and Tokaj) which are defined as geographic regions with distinct climatic conditions for grape cultivation. Nine vineyards were studied in 2011 in Nitra wine-growing region: Šintavský, Nitrianský, Radošinský, Zlatomoravecký, Vrábeľský, Žitavský, Želiezovský, Tekovský and Pukanecký (Table 1).

Table 1 Wine grape varieties used in the study from Nitra wine region

Village	Vineyard	Grape variety	Date of harvest	Date of analyses
1. Báb	Šintavský	Chardonnay	18.09.2011	19.09.2011
2. Nitra	Nitrianský	Mixture	18.09.2011	19.09.2011
3. Oponice	Radošinský	Chardonnay	18.09.2011	19.09.2011
4. Beladice	Zlatomoravecký	Rhine Riesling	26.09.2011	27.09.2011
5. Vinodol	Vrábeľský	Chardonnay	26.09.2011	27.09.2011
6. Komjatice	Žitavský	Rhine Riesling	26.09.2011	27.09.2011
7. Čaka	Želiezovský	Welschriesling	2.10.2011	3.10.2011
8. Nová Dedina	Tekovský	Green Veltliner	2.10.2011	3.10.2011
9. Brhlovce	Pukanecký	Pinot blanc	2.10.2011	3.10.2011
10. Báb	Šintavský	Traminer	27.10.2011	27.10.2011

Mycological analysis of grapes

Ten wine grape varieties were collected from the middle of September to the beginning of October 2011 in the harvest time (Table 1). The berries from the vineyards sampled were generally in good condition without visible damage. A total of 50 berries (5 healthy berries

per bunch) from each sample were plated in a Petri dishes containing Dichloran Rose Bengal Chloramphenicol agar medium (DRBC), (MERCK, Germany) and incubated at 25 °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.* (2003)** and 3 times rinsed by submersion in sterile distilled water (total amount 1L), dried, plated in the same medium and incubated at 25 °C in the dark for 7 days (endogenous mycobiota).

Penicillium strains were isolated and cultivated in MEA (Malt extract agar, **Samson *et al.*, 2010**) and CYA (Czapek yeast agar, **Samson *et al.*, 2010**). Additional agar media: Creatine-Sucrose agar (CREA, **Samson *et al.*, 2010**) and Yeast Extract agar (YES, **Samson *et al.*, 2010**) were used for some species in the terverticillate *Penicillium* group. Genus *Penicillium* was identified to species level based on morphological characters according to the manuals of **Pitt and Hocking (1997), Samson and Frisvad (2004), Samson *et al.* (2002a, 2010).**

Results evaluation

The obtained results were evaluated and expressed according to isolation frequency (Fr) and relative density (RD). The isolation frequency (%) is defined as the percentage of samples within which the species or genus occurred at least once. 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.* (1996)** as follows:

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

ns – number of samples with a species or genus; N – total number of samples; 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.* (2002b)**, modified by **Labuda and Tančinová (2006).** Extracellular metabolites – citrinin and patulin was carried out on YES and intracellural roquefortin C on CYA. 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 and drying, the visualization passed: roquefortin C after spraying with $Ce(SO_4)_2$ x 4 H_2O was visible as an orange spot. Patulin by spraying with 0,5 % methylbenzothiazolone hydrochloride (MBTH), (Merck, Germany) in methanol, heated 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 (yellow-green-tailed spot).

RESULTS AND DISCUSSION

Fungi of genus *Penicillium* were found in 8 types of grapes except two - Chardonnay from Radošinský (3) and Vrábeľský (5) vineyards. Six *Penicillium* species were found in 10 tested samples from 10 wine-growing regions. There occurrence were very sporadically (Table 2). According to **Pitt and Hocking** (**1997**) *Penicillium* species do not attack grapes before harvest, but are prevalent in stored grapes where *P. expansum* is the most common contaminant species (**Snowdon, 1990**) what almost corresponded with our results.

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

Penicillium species	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.
P. citrinum									1	
P. corylophilum	1									
P. crustosum								13		
P. decumbens						1				
P. expansum				1			6			
P. chrysogenum		1								8
Penicillium. sp.									5	

Legend: 1-10 wine growing regions; 1- Šintavský, 2 – Nitrianský, 3 – Radošinský, 4 – Zlatomoravecký, 5 – Vrábeľský, 6 – Žitavský, 7 – Želiezovský, 8 – Tekovský, 9 – Pukanecký, 10 - Šintavský

Penicillium species were found less frequently. That is the reason why the percentages of a colonized samples (isolation frequency) was low (Table 3). *Penicillium expansum* and *Penicillium chrysogenum* grew on 20 % from 10 tested samples. Other samples were contaminated only once (10 %). *P. crustosum* was present in 35 % of all tested isolates. The

second most frequent isolate was *P. chrysogenum* (24 %), follow by *P. expansum* (19 %). *P. citrinum, P. corylophilum* and *P. decumbens* were present only in 3 %.

Table 3 Isolation frequency and relative density of endogenous strains from genus *Penicillium*, isolated of grapes (n=10) harvested in Nitra wine region

Penicillium species	No of isolates	Isolation frequency (%)	Relative density (%)
P. citrinum	1	10	3
P. corylophilum	1	10	3
P. crustosum	13	10	35
P. decumbens	1	10	3
P. expansum	7	20	19
P. chrysogenum	9	20	24
Penicillium. sp.	5	10	13

Legend: n = number of samples

Penicillium species in exogenous contamination were present also in 8 types of berries tested from various vineyards: 2 x Šintavský, Nitriansky, Radošinský, Zlatomoravecký, Želiezovský, Tekovský and Pukanecký. Vineyards Vrábeľský and Žitavský were free from penicillia. *Penicillium* profiles of the various grape varieties are summarized in Table 4. *Penicillium* strains were present on grapes in higher numbers. Most samples (5) were colonised by *Penicillium* sp. However these isolates could not be taxonomically identified because of contamination.

Table 4 The overall exogenous *Penicillium* contamination of tested various types of grape wine samples

Penicillium species	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.
P. citrinum							15	9		
P. crustosum							9	25		
P. expansum				11						
P. chrysogenum		1								36
Penicillium. sp.	8	2	2				6		9	

Legend: 1-10 wine growing regions; 1- Šintavský, 2 – Nitrianský, 3 – Radošinský, 4 – Zlatomoravecký, 5 – Vrábeľský, 6 – Žitavský, 7 – Želiezovský, 8 – Tekovský, 9 – Pukanecký, 10 - Šintavský

With regard to species representation, the most frequently encountered *Penicillium* were *Penicillium sp*. (50 %, Table 5). *P. citrinum, P. crustosum* and *P. chrysogenum* were isolated less often. Colonization of *P. expansum* was established once. The largest number of

isolates belongs to *P. chrysogenum* (37) and *P. crustosum* (34). From this point of view, the relative density of both species is the highest (28 %) and (26 %) respectively.

Table 5 Isolation frequency and relative density of exogenous strains from genus *Penicillium*, isolated of grapes (n=10) harvested in Nitra wine region

Penicillium species	No of isolates	Isolation frequency (%)	Relative density (%)
P. citrinum	24	20	18
P. crustosum	34	20	26
P. expansum	11	10	8
P. chrysogenum	37	20	28
Penicillium sp.	27	50	20

Legend: n = number of samples

From endogenous and exogenous mycobiota were isolated 170 strains of 6 *Penicillium* species, namely *P. citrinum, P. corylophilum, P. crustosum, P. decumbens, P. expansum* and *P. chrysogenum* (Table 6). *Penicillium* species in both cases were present in 9 types of wine berries except one: Chardonnay from Vrábeľský vineyard (5). The most colonized vineyards by *Penicillium* were Želiezovský (7) with Welschriesling grape variety and Šintavský (1) with Chardonnay grape variety. According **Barkai-Golan (1980)** and **Benkhemmar *et al.* (1993**) some similar species but in higher number of abundance are isolated from stored grapes: *P. aurantiogriseum, P. brevicompactum, P. chrysogenum, P. citrinum, P. decumbens* and *P. glabrum*. More of them were also found in our wine grape samples.

The major species responsible for the ochratoxin production in foods from temperate climate, *P. verrucosum* (**Pitt and Hocking, 1997**), has not been isolated from these wine grape samples.

Table 6 The overall endogenous and exogenous *Penicillium* contamination of tested various types of grape wine samples

Penicillium species	1	2	3	4	5	6	7	8	9	10
P. citrinum							15	9	1	
P. corylophilum	1									
P. crustosum							9	38		
P. decumbens						1				
P. expansum				12			6			
P. chrysogenum	2									44
Penicillium. sp.	8	2	2				6		14	
Σ	11	2	2	12	-	1	36	47	15	44

Legend: 1-10 wine growing regions; 1- Šintavský, 2 – Nitrianský, 3 – Radošinský, 4 – Zlatomoravecký, 5 – Vrábeľský, 6 – Žitavský, 7 – Želiezovský, 8 – Tekovský, 9 – Pukanecký, 10 - Šintavský

From a total of 10 wine grapes samples of endogenous and exogenous *Penicillium* mycobiota (Table 7), the most frequently encountered *Penicillium* were *Penicillium sp*. (60 %). The second isolation frequency belongs to *P. chrysogenum* (40 %). The highest relative density from all samples belongs to *P. crustosum* (28 %) and *P. chrysogenum* (27 %). Another species were isolated less often - under 20 %. The number of there isolates were 47 and 46 respectively. **Magnoli *et al.*,** (**2003**) reported some similar results. Fifty samples of wine grapes from a winery of Mendoza province, Argentina were to evaluate the mycoflora. *Penicillium* genus was frequent in 30 % of the samples. *P. chrysogenum* was the most frequent, isolated in 22 % of the samples according to our study. *P. glabrum* and *P. crustosum* were isolated in smaller frequency. In the study of **Tournas** and **Katsoudas** (**2005**) various types of grapes (red seedless, red seeded, green seedless, black seedless and black seeded) were purchased from local supermarkets in Washington, DC area and prone to fungal contamination. *Penicillium* grew only on 10 % of green seedless grape samples. Very low contamination level of black grapes *Penicillium* can probably be explained by the fact that these varieties possess very hard, difficult to break skin, whereas the higher sensitivity of the green seedless grapes to fungal contamination could be due to their thinner, easier to invade epidermis. Additionally, grapes are sprayed with fungicides very near the harvest time, such pesticide residues remaining on the fruit during marketing protect against fungal spoilage. Grapes from 4 Portuguese wine-growing regions were analyzed by plating methods between 2001 and 2003. From the 10 602 strains detected and identified, *Penicillium* was represented in 9 %. From 885 *Penicillium* strains identified, the most frequent were *P. brevicompactum, P. thomii* which represented 29 % of the isolates and *P. glabrum/spinulosum* 14 % (**Serra *et al.*, 2005**). In our study we didn't isolated any of them.

Table 7 Isolation frequency and relative density of endogenous and exogenous strains from genus *Penicillium*, isolated of grapes (n=10) harvested in Nitra wine region

Penicillium species	No of isolates	Isolation frequency (%)	Relative density (%)
P. citrinum	25	30	15
P. corylophilum	1	10	0,6
P. crustosum	47	30	28
P. decumbens	1	10	0,6
P. expansum	18	30	11
P. chrysogenum	46	40	27
Penicillium. sp.	32	60	19

Legend: n = number of samples

In total 8 endogenous strains representing 3 potentially toxigenic species were tested for their toxigenic ability, namely *P. citrinum*, *P. expansum* and *P. chrysogenum* (Table 8). Out of 8 strains, 50 % produced at least one mycotoxin as revealed by the method used here. *P chrysogenum* produced roquofortin C (2 out of 4 strains screened). This species may produce a very wide range of toxic compounds: roquefortine C, meleagrin and penicillin. These metabolites could be considered as a potential hazard to human health (**Samson *et al.*, 2002a**). Positive toxigenity was detected in *P. expansum* (2 out of 3), patulin produced 1 out of 3 strains and citrinin any of 3 tested strains. On the other hand citrinin was produced by *P. citrinum*. The citrinin has been known as nephrotoxic, hepatotoxic and carcinogenic to humans and animals (**Li *et al.*, 2012**).

Table 8 Toxinogenity of selected *Penicillium* strains, isolated from endogenous mycobiota of wine grapes

Species	C	P	RC
P. citrinum	1/1		
P. expansum	0/3	1/3	2/3
P. chrysogenum			2/4

Legend: C – citrinin; P – patulin; RC – roquofortin C

Of all 15 potencially toxigenic strains from exogenous mycobiota all of them were positive (Table 9).

Table 9 Toxinogenity of selected *Penicillium* strains, isolated from exogenous mycobiota of wine grapes

Species	C	P	RC
P. citrinum	4/4		
P. crustosum		6/6	6/6
P. chrysogenum			5/5

Legend: C – citrinin; P – patulin; RC – roquofortin C

The highest toxinogenity (100 %) was observed in 15 strains of exogenous mycobiota and out of 8 strains of endogenous mycobiota 50 % produced at least one mycotoxin.

Ochratoxin A is a mycotoxin with nephrotoxic, nephrocarcinogenic, teratogenic and immunosuppressive properties, which recently has received a great deal of interest from the scientific community (**Battaglia *et al.*, 1996, Walker, 1999**). It has been detected in foods and beverages, including grape juice and wine, where it was reported for the first time by **Zimmerli** and **Dick** (**1995**). Since then, fungi responsible for OTA production have been

studied in foods and beverages. *Penicillium verrucosum* is the only species of *Penicillium* capable of OTA production. During the 1997-98 harvests, 50 grape samples were collected from Malbec and Chardonnay varieties in Argentina and Brazil. Penicillia were isolated in both countries, but *P. verrucosum* was not identified (**Da Rocha *et al.*, 2002**). Similar results were obtained in France, where 11 samples of grape and must used in red table wine making were investigated. Several penicillia were identified, but *P. verrucosum* was absent (**Sage *et al.*, 2002**). During 1999 and 2000, nine vineyards in Italy were sampled. Five hundred and eight fungal isolates were collected, 31 belonging to *Penicillium* spp. *P. verrucosum* was not find (**Battilani *et al.*, 2003**).

CONCLUSION

In the 2011 harvest, 10 grape samples were collected from 3 x Chardonnay varieties, 2 x Rhine Riesling, Mixture, Welschriesling, Green Veltliner, Pinot blanc and Traminer in Slovak Republic. *Penicillia* were isolated in both mycobiota – endogenous and exogenous. In southern Slovakia, *Penicillium* contamination in endogenous mycobiota at harvesting was lower than in exogenous. Mycological endogenous and exogenous survey of the 10 samples of wine grapes indicated the presence of 6 species of the *Penicillium* genus: *P. citrinum, P. corylophilum, P. crustosum, P. decumbens, P. expansum* and *P. chrysogenum*. Among them *Penicillium* sp. was the most frequent mould of the mycobiota that occurred in 60 % of samples, followed by *P. chrysogenum* in 40 %. The species isolated in smaller frequency were *P. citrinum, P. crustosum, P. expansum* (30 %) and *P. corylophilum* with *P. decumbens* in 10 %. The largest number of isolates belongs to *P. crustosum* (47) and *P. chrysogenum* (46). From this point of view, the relative density of both species is the highest (28 %) and (27 %). The results on fungal dynamics are important, both because these genera is usually considered post-harvest moulds and because they were all isolated from berries without visible symptoms. Some vineyards were free of *Penicillium* – Radošinský and Vrábeľský from endogenous mycobiota and Vrábeľský and Žitavský from exogenous mycobiota. Three potentially toxigenic species isolated from endogenous mycobiota were tested for their toxigenic ability. Out of 8 strains, 50 % produced at least one mycotoxin. Of all 15 potencially toxigenic strains from exogenous mycobiota all of them were positive on screening mycotoxin.

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