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Contamination by moulds of Grape Berries in Slovakia

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Abstract

This work describes the first map, albeit partial, of toxigenic fungi re-isolated from grape berries collected in three out of the six most important Slovakia wine making areas in two different periods of the harvest year 2008. Low temperatures and high relative humidity during July 2008 favoured the development of grape fungal diseases that cause rots such as *Plasmopara*, *Uncinula*, *Botrytis*, *Metasphaeria*, *Elsinoë*, and *Saccharomycetes*. In the analysed samples, the following genera of toxigenic fungi were identified in the range 1-4%: *Aspergillus*, *Alternaria*, *Cladosporium*, *Epicoccum*, *Fusarium*, *Penicillium*, *Rhizopus*, *Ulocladium*, and *Trichoderma*. *Trichothecium* while the genera *Aspergillus*, *Alternaria*, *Fusarium*, and *Penicillium* were in the range 11-29%. *A. niger*, *A. carbonarius*, some strains of *A. carbonarius* - with “crystals” and strains of *A. uvarum*-uniserial were identified; these species are considered ochratoxigenic (able to produce variable amounts of toxins). In addition a non-ochratoxigenic strain of *A. ibericus* and a *Fusarium* strain able to biosynthesize small amount of fumonisins, beauvericin, and enniatins was identified. *P. expansum*, able to produce citrinin, represents 29.7%, of the *Penicillium* genus together with *P. verrucosum*, *P. glabrum*, *P. citrinum*, and *P. crustosum*. An analysis for the identification and quantification of main toxins was done: ochratoxin A, fumonisins, beauvericin, enniatins, and fusaproliferin was performed on grape samples; it is consistent with the results of the mycological analysis.

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3 Toxigenic fungi should be checked throughout the years and their occurrence compared with
4 all environmental factors to avoid health risks.
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11 **Keywords:** vineyard, toxigenic and non toxigenic fungi, ecophysiological conditions, toxins,
12 microscopy scan.
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23 24 25 26 **Introduction**

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28 Slovakia is a country with a rich and consolidated wine and vine cultural history. Under the
29 European territorial segmentation, Slovak vineyards are classified as Zone B and are
30 represented by six different wine regions for a total of 22000 acres. Cooler temperatures and
31 high relative humidity and/or moisture are suitable conditions for the development of fungal
32 diseases that affect the leaves, shoots, stems, and fruit. This situation represents a serious
33 phytopathological problem especially for the operators in the wine sector for whom the
34 economic losses are very relevant (Šrobárova and Kakalíková 2007) since the presence of
35 pathogenic fungi disvalue the plant and the berries. The presence of pathogenic fungi can lead
36 to the development of opportunistic microfungi such as *Aspergillus* section *Nigra* that is an
37 important producer of ochratoxin A (OTA) that can cause the contamination of grapes and
38 wine products. The number of micro organisms and the virulence of the mycoflora of grapes
39 is important to determine because this is a relevant indicator that can predict the presence of
40 mycotoxins on the grapes. The potential mycotoxigenic fungi grown on/in grapes or grape
41 musts used for grape juices or wine production and the related exposure risks have to be
42 evaluated (Belli et al. 2002).
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56 *Penicillium expansum* is known as a patulin producer but can also produce citrinin another
57 mycotoxin that has been frequently identified in grape juice (Vinas et al. 1993), but after the
58 first detection of OTA in wine, several studies particularly in Europe (see Galvano et al. 2005
59 for a review) were conducted, and a clear relationship between geographical position and the
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3 accumulation of OTA in red wines has been demonstrated in Germany (Otteneder and
4 Majerus 2000), in Italy (Pietri et al. 2001), and in Greece (Stefanaki et al. 2003). Several
5 factors, e.g. agronomic and genetic, influence the qualitative and quantitative variability of the
6 mycotoxins present in the grapes, but the climatic conditions are considered to be the deciding
7 factor. The weather conditions of humidity and temperature determine the growth of
8 particular moulds (Pitt and Hocking 1997); this observation also explains the correlation
9 between the geographic site and the presence of OTA. The natural occurrence of OTA and of
10 the produced moulds in grapes manufactured in Slovakia is relatively moderate because of the
11 climatic conditions during the critical stages of maturation and harvesting of the grapes (mild
12 in the regions of the Middle Europe). The OTA can contaminate not only the wine but also
13 other grape products such as dried grapes (currants, raisins, and sultanas), which when eaten
14 can become an important food source for OTA for the population. For children, considered
15 strong consumers of these products, the risk may be high. Taking into account these
16 observations, the Commission of European Communities (2005) set the limit of OTA to 10 μg
17 kg^{-1} in dehydrated grapes.
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32 This paper presents for the first time the native mycoflora of the year 2008 in the Slovak
33 vineyards. This research during two months has considered eco-physiological factors
34 identified as important during the growing season. The source of meteorological data was the
35 Hydro Meteorological Data (HMD) for each of the three regions where the samples were
36 collected. The aim of our work was to verify if toxigenic moulds as producer of dangerous
37 toxins are present in Slovakia and if its representative's strain can produce them. OTA, one
38 of the most important mycotoxins potentially present in wine grapes, and other relevant
39 mycotoxins besides fusariotoxins present in the grapes sampled have also been identified and
40 quantified.
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50 **Material and methods**

51 *Study area*

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53 Three winemaking regions were chosen for this study based on their climatic differences and
54 national economical importance: Little Carpathian: Modra, Šenkvice, Pezinok (HMD-
55 Slovenský Grob); Nitria: Malanta, Hrnčiarovce, Nitra (HMD in Nitra-Janíkovce); and the
56 South Slovakian region: Radošina, Galanta, Kralova (HMD Žiharec). A total of 9 localities
57 and 18 vineyards were investigated.
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Sample collection

Grapes were collected twice during the year: in July and before harvest at the end of September 2008. These collection periods represent two important developmental stages of the berries: green berry at early maturation and ripe berry (harvest time). At each sampling time and in each vineyard, the samples were taken to the laboratory in closed paper bags, transported in cooled boxes, and analyzed within 24 hours of collection.

Mycological analysis of the grapes

From each bunch, 10 berries were randomly selected, cut in half, and aseptically plated on Czapek Dox Agar medium, CDA, (Biomark Laboratories Pune, India). For each sampling period, a total of 50 berries were collected per vineyard, and, as referenced above, a mixed berry sample was pressed against a plate of the same medium (CDA) in order to compare the fungal species isolated in the two sample collection periods. The plates were incubated for 7 days at 25°C (Pitt and Hocking 1997). From the second day of incubation, the plates were monitored in the stereomicroscope for the presence of toxigenic moulds and other species. In days when the moulds were present in each grape, they were re-isolated on CDA and cultivated for seven days for *Aspergillum* and *Penicillium*, while for the other moulds species they were cultivated for 10 to 14 days at 25°C.

The staining and microscopic identification of fungi

For a first observation, cotton blue with lactophenol was used and fungi identified according to the spore, measure of the spore and shape and colour of the culture; in case of difficulties in the form of the spore scanning Electron Microscopy (SEM) was used. SEM was used for some species of the genus *Aspergillus* and *Penicillium* with gold coated samples and micrographs that consisted of 3 averaged fast scans in a Jeol-JXA-840A (Japan) electron probe microanalyzer. The identification of fungi was made according to Nelson, Toussoun and Marasas (1983), Summerbell (1996), Samson and Frisvard (2004).

Preservation of the isolated strains

All the fungi and representative strains of the toxigenic moulds found were preserved as a suspension of spores in a 10 % glycerol solution and maintained at a constant temperature of - 8°C.

Mycotoxins analysis

Ochratoxin analysis. Agar medium (1 g) was cut from each plate and were added 5 mL of CH₃OH. After 1 h stirring, the extracted solution was filtered through Whatman no. 4 filter paper and dried using a centrifuge evaporator (Savant Instruments Inc., Farmingdale, NY, USA). The residue was dissolved in 1 mL of methanol centrifuged at 13000 rpm for 5 min and used for HPLC analysis. For HPLC analysis a Onyx column with a flow of 1 mL min⁻¹ was used; the mobile phases were: A) H₂O in 1% CH₃COOH and B) CH₃CN in 1% CH₃COOH in isocratic conditions with 35% of B, total time 20 min. The revealing wavelength were λ_{ex} : 333 nm and λ_{em} : 460 nm. The linearity range was between 0.5 and 50 $\mu\text{g kg}^{-1}$. The limit of detection (LOD) was 0.1 $\mu\text{g kg}^{-1}$, and the limit of quantification (LOQ) was 1 $\mu\text{g kg}^{-1}$, and calculated as 3 and 10 times the value of the noise level, respectively.

For recovery studies, 1g of agar was spiked with a standard mixture dissolved in methanol, at three different concentration levels (1 ng/g, 10 ng/g and 50 ng/g), placed at room temperatures overnight and then extracted as above described. OTA recoveries in spiked samples averaged 89% .

Fumonisin analysis. Sample (1 g) was collected from the plates by cutting and extracted with 5 mL of a CH₃OH /H₂O (70:30, v/v) solution. Samples were placed for 50' in an ultrasonic bath, then were filtered using RC 0.2 μm filters (Phenomenex, U.S.A.). The extracted were dried using a centrifuge evaporator, dissolved in 5 mL of the extracting solution, centrifuged at 13000 rpm for 3' and used for LC/MS/MS investigations.

Chromatographic separation was performed using an HPLC apparatus equipped with two micropumps Series 200 (Perkin Elmer, Canada, USA) and a Gemini 5u C18 110 Å column (150 x 2 mm) (Phenomenex, CA, USA). The eluents were: A water 0.1% formic acid; B CH₃CN/MeOH (80:20 v/v), 0.1% formic acid. The gradient program was as follows: 30-100 % B (8 min), 100 % B (3 min), 100-30% B (3 min), at a constant flow of 0.2 mL/min. Injection volume was 20 μL .

MS/MS analyses of fumonisins were performed on an API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Canada) equipped with a TurboIonSpray source. The declustering potential (DP) and the collision energy (CE) were optimized for each compound infusing directly into the mass spectrometer standard solutions (10 $\mu\text{g/mL}$) at a constant flow rate of 6 $\mu\text{L/min}$ using a model 11 syringe pump (Harvard Apparatus, Holliston, MA, USA). Drying gas (air) was heated to 350°C and the capillary voltage (IS) was set to + 5000 V. Analysis were performed in the positive ion mode in MRM (multiple reaction monitoring).

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3 For recovery studies, 1g of agar was spiked with a standard mixture dissolved in methanol, at
4 three different concentrations (1 ng/g, 10 ng/g and 50 ng/g), placed at room temperatures
5 overnight and then extracted as above described. FB₁ and FB₂ recoveries in spiked samples
6 averaged 81% and 72%, respectively. The detection limits (LOD) for FB₁ and FB₂ were 0.5
7 and 1 µg kg⁻¹ respectively, quantification limits (LOQ) were 1 µg kg⁻¹ for FB₁ and 2 µg kg⁻¹
8 for FB₂. Calibration curve showed good linearity in the range 10-1000 ng/mL. Table 3 reports
9 the LC/MS/MS conditions for FB₁ and FB₂.
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17 **Beauvericin, fusaproliferin and enniatins analysis.** Sample (1 g) was collected from the
18 plates and extracted with 5 mL of methanol in a ultrasonic bath for 50 min, then was
19 centrifuged at 3000 rpm for 5 min and filtered using a RC 0.20 µm Phenomenex filter. The
20 extracted was dried using a centrifuge evaporator, dissolved in 200 µL of CH₃OH, centrifuged
21 at 13000 rpm for 5 min and used for HPLC analysis. HPLC analysis as previously described
22 (Ritieni et al. 1995, Srobarova et al. 2002) was performed using LC-10AD pumps and a diode
23 array UV/VIS detector SPD-M10A (Shimadzu, Japan). A Gemini 5 µm C₁₈ 110 300A,
24 4.6x250 mm (Phenomenex, USA), column was used. HPLC conditions were set using a
25 constant flow at 1.0 mL/min and CH₃CN/H₂O (65:35 v/v) as starting eluent system. The
26 starting ratio was kept constant for 5 min, and then linearly modified to 70% CH₃CN in 10
27 min. After 1 min at 70% CH₃CN, the mobile phase was taken back to the starting conditions
28 in four minutes. Fusaproliferin was detected at 261 nm, beauvericin and enniatins were
29 detected at 205 nm. Detection limit (LOD) and quantification limit (LOQ) were 0.1 and 1 mg
30 kg⁻¹ for fusaproliferin, 10 and 20 mg kg⁻¹ for beauvericin, 3.5 and 8 mg kg⁻¹ for enniatin B₁,
31 respectively. For recovery studies, 1g of agar was spiked with a standard mixture dissolved in
32 methanol, at three different concentrations (0.5 mg/kg, 10 mg/kg and 50 mg/kg), placed at
33 room temperatures overnight and then extracted as above described. Recovery averaged 68%
34 for fusaproliferin, 76% for beauvericin and 63% for enniatins. All the mycotoxins analyses
35 were run in triplicate. Calculated standard deviation was always lower than 5%.
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52 53 **Results and discussion**

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56 Table 1 reports the fungi identified in the grape samples and Table 2 reports the mycotoxins
57 identified and their quantity expressed as µg kg⁻¹. They were analyzed from more than seven
58 hundred and thirteen strains of fungi isolated from vineyards examined in Slovakia during the
59 2008 season. Two hundred eighty strains were not toxigenic while four hundred thirtyseven
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3 strains of fungi were potential producers of mycotoxins and belonged to the genera
4 *Aspergillus* (A), *Penicillium* (P), *Fusarium* (F). The considered mycotoxins have been OTA,
5 fumonisins, enniatins, fusaproliferin, and beauvericin.
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10 The 2008 season, see Figure 1, was very rainy from May to late July when the samples of
11 early veraison berries were collected, and from the end of August until mid-September days
12 of hot sun high temperatures were prevalent. During the second part of September the
13 weather conditions were again rainy until the end of September it was harvest time when the
14 samples were collected and examined. Figure 1 shows the ecological factors in the three
15 regions where the samples were taken. The data present daily average temperature,
16 precipitation and relative humidity during the vegetative phase of vine and are from Hydro
17 meteorological station (HMD): Little Carpatien, HMD from Slovensky Grob (A); Nitria,
18 HMD from Nitria-Velké Janíkovice (B), South Slovakia, HMD from Žiharec (C),
19 respectively.
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30 An high relative humidity was measured directly in the vineyards during the rainy days and in
31 this period a number of not toxigenic fungi were identified, such as *Guignardia Bidwell*
32 (Ellis), *Viala and Ravaz* (anamorph: *Phyllosticta ampelicida* (Englem.) van der Aa), black rot,
33 white rot *Metasphaeria diplodiella* (Viala et Ravaz) Berl. anamorph: *Coniella diplodiella*
34 (Speg.), and Pet. and Syd. and *Elsinoe* (see Figure: 1 A, B, C). On the contrary, *Botrytis*
35 *bunch rot* - *B. fuckeliana* (de bary Whetzel) was observed throughout the season and in the
36 second part of September, as a sclerotiorum group.
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44 Among the fungi considered dangerous because they were potential producers of mycotoxins
45 and collected on grape berries, some species of the genera *Aspergillus*, *Penicillium*, *Fusarium*
46 sp. and *Alternaria* (Table 1) have been identified. Approximately 25% of total toxigenic
47 fungi were present before the collection of samples in September, during harvest, while the
48 non-toxigenic fungi, that represent about 50%, were identified in early maturation phase of
49 berries collected in July. At the time of the collection, samples in early maturation phase, high
50 precipitation and humidity were observed but lower temperature values were namely
51 significant. Toxigenic fungi of the genera *Cladosporium*, *Epicoccum*, *Rhizopus*,
52 *Trichothecium*, *Trichoderma* and *Ulocladium*, were also identified but with a very low
53 frequency. On the contrary, the genera *Aspergillus* (11.4%), *Fusarium* (11.4%), *Penicillium*
54 (29.7%), and *Alternaria alternata* (14.8%) are considered to be predominant among the
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toxigenic fungi. In particular, the *Aspergillus* section *Nigri*, "black aspergillosis", was isolated with a relatively high frequency. The *A. niger* (Figure 4) is the most common species and representative of isolates producers of OTA (Table 2). *A. carbonarius* was common too, and the screened strains were ochratoxigenic. OTA was not produced by *A. carbonarius* with "crystals", and by strains of *A. ibericus*, as observed by Serra et al. (2006) for the conidia. *A. ibericus* produces conidia with spiny appearance (Samson et al. 2007). These strains, however, have characters that allow morphological distinction from the other species in the section, particularly the conidia size (5-7 μm), that allows separation of the species from the two most common biserial species in section *Nigri*: *A. carbonarius* (7-9 μm) and *A. niger* and its aggregate species (3-5 μm).

Other toxigenic fungi such as *Aspergillus flavus* and aflatoxin producer *A. citrinum*, producer of citrinin, were also identified. Table 2 shows the results of the analysis of mycotoxins for the different fungal strains isolated from grapes of Slovakian vineyards. The results show an in vitro potentially toxigenic activity for the genera: *Aspergillus* (A), *Penicillium* (P), *Fusarium* (F). These strains are considered potential producers of OTA, Fumonisin (FUM), Enniatins (ENN), beauvericin (BEA), and fusaproliferin (FUP).

The *Aspergillus* section *Niger* is the most frequently reported in the literature and it is also used in some processes for the biotechnological production of enzymes. However, recognition of this section is complex and sometimes the term of *A. niger* has been used for each member of the section. Taxonomic studies based on molecular methods allow the subdivision of *A. niger* in the two species *A. niger* and *A. tubingensis* (Abarca et al. (2004).

Black aspergilli is one of the most difficult groups to classify and identify and most isolates of *black Aspergillus* strains belong to the species *A. niger*. This classification is based on view of their spores and of their culture. These strains, dusty or powder on Czapek Dox Agar and the colonies consist of a compact white or yellow basal felt covered by a dense layer of dark-brown to black conidial heads. The strain of *A. niger* can produce small quantities of OTA in a liquid medium (0.83 $\mu\text{g mg}^{-1}$) differently from *A. ibericus* that is characterized by spores that are larger than about 5-7 μm .

The formation of sclerotium is not necessarily correlated with the production of OTA; Wicklow et al. (1996) have correlated the production of OTA with the formation of

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3 sclerotium of *A. carbonarius*. The observation of our samples showed that the production of
4 OTA, $1.03 \mu\text{g mg}^{-1}$, derived from non sclerotial strain of *A. carbonarius*, is not associated
5 with the formation of sclerotia, but to a non compact culture with radial growth.
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10 This type of strain is frequently observed in Slovakia, and the geological and climatic
11 conditions are quite similar to those existing in the Czech Republic where strains of
12 *Aspergillus* were not identified in vine berries (Ostrý et al. 2007). According to the
13 classification of the black *Aspergillus* (according to SCAN) we have identified *Aspergillus*
14 *uvarum* sp. nov., an uniseriate one. The observation of the morphology of the spores (Figure
15 3) and the shape of the culture, color and size of conidia indicate that they belong to the black
16 *Aspergillus*. The conidia are dark brown to black and the spore dimension range from 3 to 4
17 μm . Perrone et al. 2008 observed that this fungus do not produce OTA but it does produce
18 asterric acid, geodin, erdin, and secalonic acid D and F (Perrone et al. 2008).
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28 The strain of *A. flavus* isolates were able to produce *in vitro* small amounts of aflatoxin, up to
29 $1.2 \mu\text{g mg}^{-1}$; in the literature, an aflatoxigenic strain of *A. flavus* has been isolated from grapes
30 in Lebanon (El Khoury et al. 2006), and five strains of *A. citrinum* are occurred in Slovakia,
31 one of them shown to produce up to $0.59 \mu\text{g mg}^{-1}$ of citrinin.
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37 The largest source of contamination of wine grapes originates in the grapes themselves. In
38 fact, the OTA-producing fungi can easily colonize the damaged grapes before harvest and
39 easily at the end of the vegetative session and then produce and accumulate OTA (Battilani et
40 al. 2001).
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46 The concentrations of OTA in the 2005 harvest were well below the proposed European limit
47 of $2 \mu\text{g l}^{-1}$ (Belajová and Rauová 2007). According to these authors the minimal changes in
48 the concentration of OTA were found and recorded by the authors during the 2006
49 winemaking season in Slovakia. This was observed in years when the season was very rainy,
50 but there is a need to monitor the levels every year since they may differ from the previous
51 one.
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58 In the 2008 season small differences were observed between the Nitrian and Little Carpatien
59 regions, and, as expected, in regions with warmer climates during the month of September at
60 the time of harvesting, the colonization by *Penicillium* was most common after the drying of

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3 the grapes in August and collected at the end of September (see Figure 1). For example, *P.*
4 *expansum*, observed in this study as a citrinin producer (see Table 2), is a common fungus in
5 all samples and was found in 29.7% of the samples before the harvest.
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8 *P. glabrum*, *P. citrinum* and *P. verrucosum* (OTA manufacturer) are present in samples
9 collected on rainy days during July, but *P. expansum* is also present in a smaller amount like
10 *P. citrinum* strains.
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15 In a season like 2008, with very high humidity and days of rain after a long period of sunny
16 days before the harvest, the weather can be a source of danger because *Fusarium* isolates may
17 be present in grape berries (Table 1) with the production of fumonisin, beauvericin and, in
18 some small amounts, also enniatin (Table 2). These toxins are very dangerous to human
19 health and with *Alternaria* isolates are capable of producing a range of secondary metabolites
20 harmful to animals and humans (Drusch and Ragab 2003).
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28 Conclusions

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31 The analysis of representative strains for the identification and quantification of OTA, FUM,
32 ENN, FUP and BEA summarized in Table 2 are consistent with the results of mycological
33 analysis reported in Table 1. The presence of OTA is related to the presence of the producing
34 strains of the genera *Aspergillus* and *Penicillium*. The small amounts of FUM are associated
35 with the presence of *F. verticillioides* and *F. proliferatum*. Also the presence of BEA and FUP
36 is compatible with the discovery of *F. subglutinans*. These chemotaxonomic results confirm
37 the morphological data and allow speculation as to the existence and frequency of fungal
38 genera and strains present in the various areas of wine production and in the two phases of
39 sampling.
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49 The presence of OTA and moulds that colonize grapes is highly dependent on climatic
50 conditions during the ripening and harvesting of grapes. These weather conditions are
51 generally moderate in the Middle European countries like Slovakia. Most of the black
52 *Aspergillus* strains isolated were found to belong to the Niger section *Aspergillus* species and
53 their strain *in vitro* produced small amounts of OTA. Other fungi including potentially
54 toxigenic strains of *Penicillium*, *Fusarium*, and *Alternaria* were also isolated, and dangerous
55 toxin strains of them have been identified. We can concluded that further studies are needed
56 to clarify the origin of OTA and other toxin contamination the berries grapes from Slovakia,
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3 including that produced by *Alternaria*. However, the quantity of toxins produced by these
4 isolates is very low even in a medium which induces the production of toxins, so it does not
5 represent a serious health hazard for consumers of fruit juice and wine produced from
6 Slovakian grapes .
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12 **Acknowledgement:** The work was done by Project VEGA No 2/0002/09 and APVV.
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5 **Figure captions.**
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9 **Figure 1.** Summary of ecological factors (daily average temperature, precipitation and
10 relative humidity during the vegetative phase of vine) in three regions from which samples
11 were analyzed: **a)** Little Carpatien; HMD from Slovenský Grob; **b)** Nitria; HMD from Nitra-
12 Velké Janíkovice; **c)** South Slovakia; HMD from Žiharec, respectively. Data is from the
13 Hydro Meteorological Station (HMD).
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19 **Figure 2.** Scan microscopy of *Aspergillus ibericus* (*A. carbonarius*) = 6 - 9µm.
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23 **Figure 3.** Scan microscopy of *Aspergillus uvarum* < 4µm.
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27 **Figure 4.** Scan microscopy of *Aspergillus niger* < 5µm.
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31 **Figure 5.** Scan microscopy of *Penicillium expansum*.
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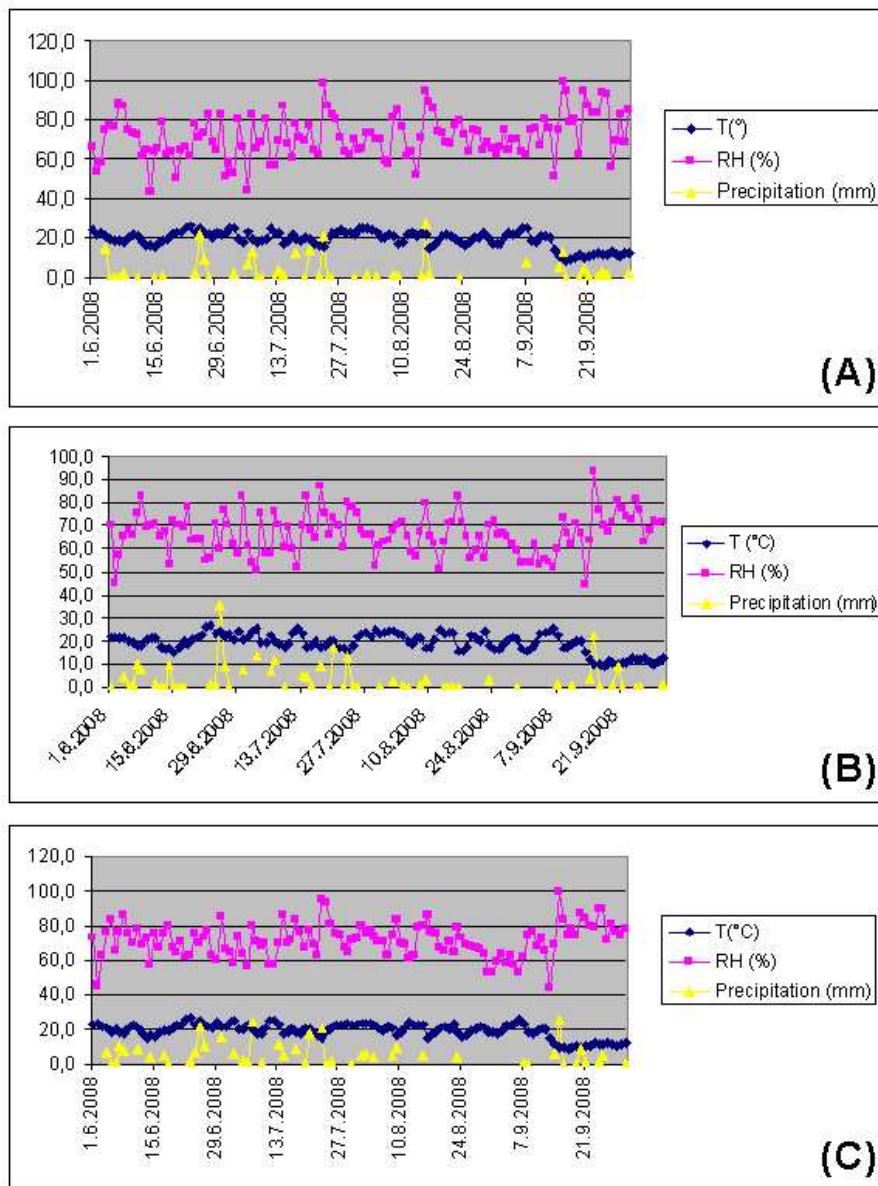


Figure 1. Characterization of ecological factors in three regions from were samples were analyzed: a) Little Carpatien; HMD from Slovenský Grob; b) Nitria; HMD from Nitra-Velké Janíkovice; c) South Slovakia; HMD from Žiharec, respectively. Data are from Hydro Meteorological Station (HMD).

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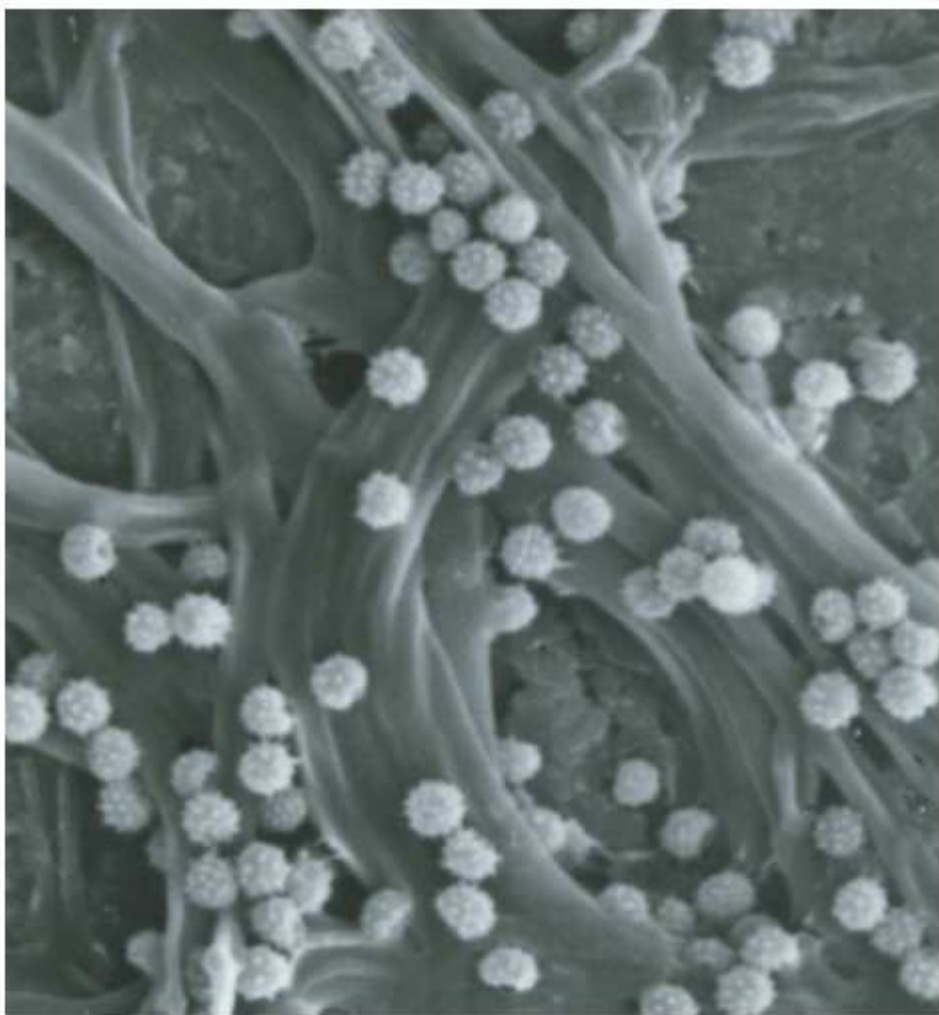


Figure 2. Scan microscopy of *Aspergillus ibericus* (*A. carbonarius*) = 6 - 9µm.

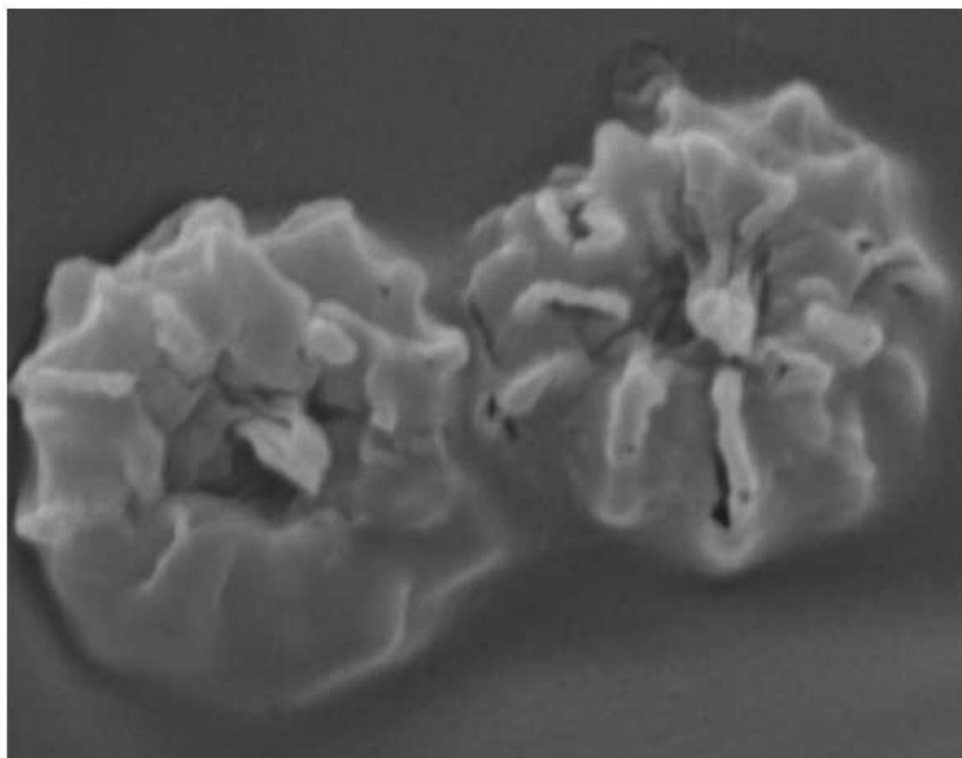


Figure 3. Scan microscopy of *Aspergillus uvarum* < 4 μ m.

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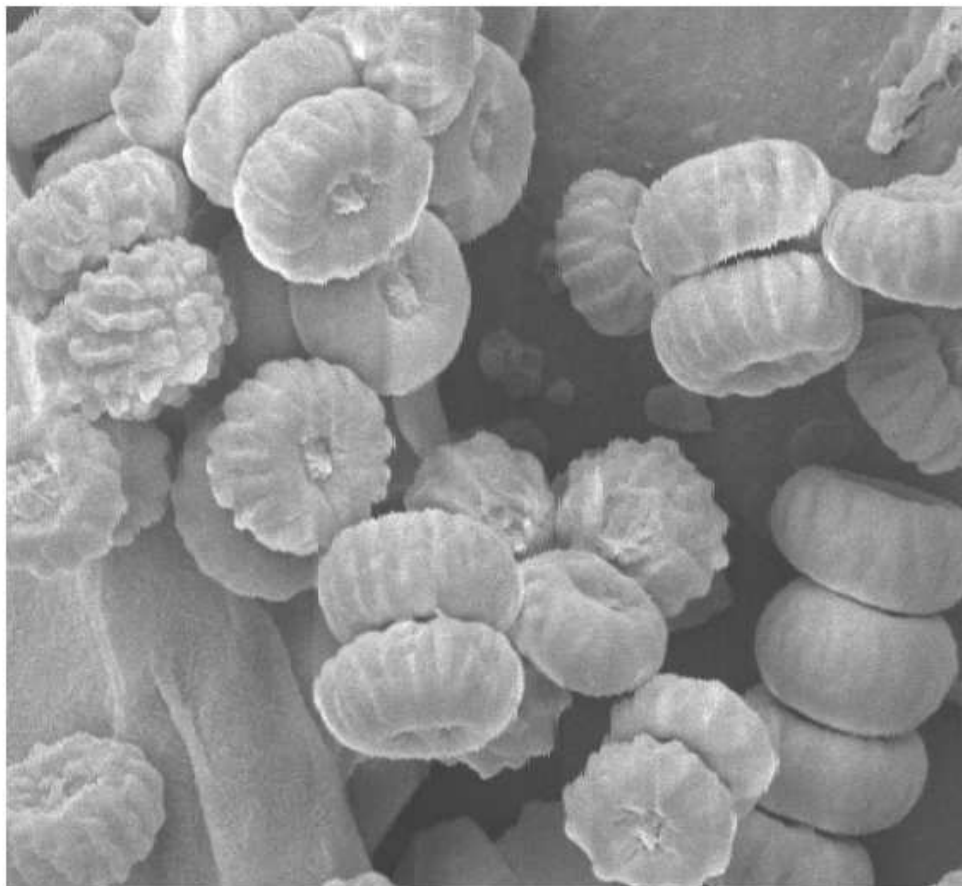


Figure 4. Scan microscopy of *Aspergillus niger* < 5µm.

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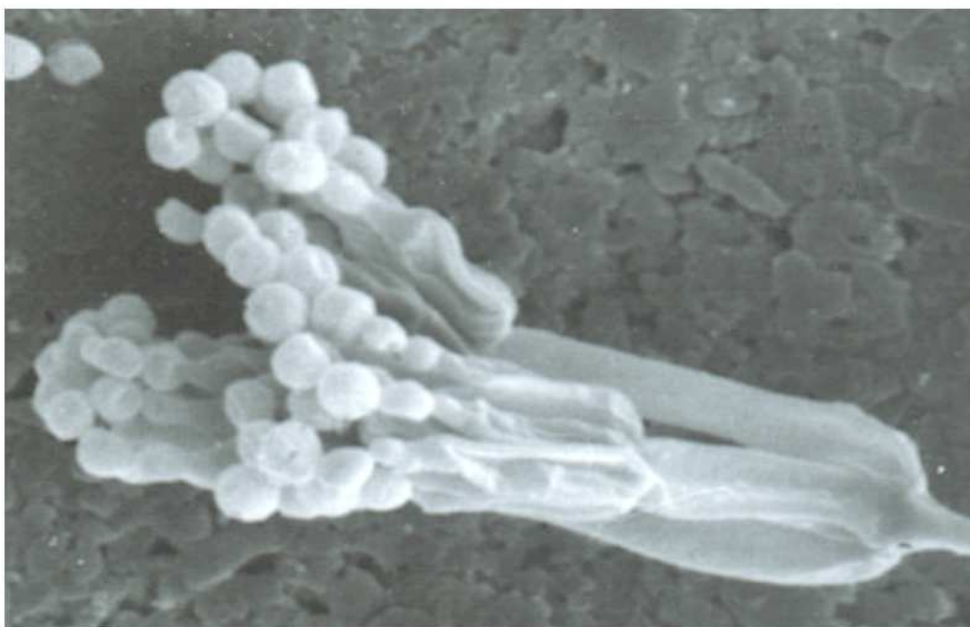


Figure 5. Scan microscopy of *Penicillium expansum*.

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Table 1. Fungi in samples of grape in the important of the Slovak vineyard regions, during the vegetative phase of the year 2008. Samples were taken in July and before harvest in September.

Fungi species	Little Carpathian		Nitrian		South Slovakian		Total		Total	%
	July	September	July	September	July	September	July	September		
<i>Aspergillus carbonarius</i>	0	5	20	5	10	5	30	15	10.3	
<i>Aspergillus niger</i>	0	5	15	5	10	5	25	15	9.15	
<i>Aspergillus uvarum</i>	0	5	10	0	10	0	20	5	5.7	
<i>Aspergillus citrinum</i>	0	0	5	0	0	0	5	0	1.14	
<i>Aspergillus flavus</i>	0	0	0	5	0	0	0	5	1.14	
<i>Alternaria alternata</i>	10	20	15	5	5	10	30	35	14.87	
<i>Alternaria tenuissima</i>	5	5	0	0	0	0	5	5	2.29	
<i>Cladosporium cladosporioides</i>	0	5	0	5	0	0	0	10	2.29	
<i>Epicoccum nigrum</i>	0	5	0	0	0	0	0	5	1.14	
<i>Fusarium oxysporum</i>	0	5	0	0	0	0	0	5	1.14	
<i>Fusarium proliferatum</i>	0	5	0	0	0	0	0	5	1.14	
<i>Fusarium semitectum</i>	5	0	5	5	0	0	10	5	3.43	
<i>Fusarium solani</i>	0	0	0	0	0	5	0	5	1.14	
<i>Fusarium subglutinans</i>	5	0	5	0	0	0	10	0	2.29	
<i>Fusarium verticillioides</i>	0	5	5	0	0	0	5	5	2.29	
<i>Penicillium citrinum</i>	0	5	5	5	0	0	5	10	3.43	
<i>Penicillium crustosum</i>	0	0	0	5	0	0	0	5	1.14	
<i>Penicillium expansum</i>	0	60	0	30	0	10	0	100	22.88	
<i>Penicillium glabrum</i>	0	0	5	0	0	0	5	0	1.14	
<i>Penicillium verrucosum</i>	0	5	0	0	0	0	0	5	1.14	
<i>Rhizopus stolonifer</i>	15	0	0	0	0	0	15	0	3.43	
<i>Ulocladium chartarum</i>	0	2	2	1	2	0	4	3	1.6	
<i>Trichoderma viride</i>	10	0	10	0	0	0	20	0	4.58	
<i>Trichothecium roseum</i>	0	5	0	0	0	0	0	5	1.14	
Total	50	142	102	71	37	35	189	248	437	
Non toxigenic							Total	Total	% species	
<i>Uncinula sp.</i>	20	0	20	0	15	0	55	0	20	
<i>Perenospora sp.</i>	20	0	25	0	15	0	60	0	21	
<i>Metasphaeria</i>	10	5	15	5	10	5	35	15	18	
<i>Botrytis</i>	10	25	20	10	0	10	30	45	27	
<i>Elsinoe</i>	5	0	5	0	0	0	10	5	5	
<i>Saccharomycetes</i>	0	10	5	5	0	5	5	20	9	
Total	65	40	90	20	40	20	195	85	280	

Table 2. The results of the main toxins are reported as $\mu\text{g kg}^{-1}$ for one representative strain of vineyards in Slovakia *in vitro* for potentially toxigenic Genus: *Aspergillus* (A), *Penicillium* (P), *Fusarium* (F), Ochratoxin A (OTA), Fumonisin (FUM), Enniatin (ENN), Fusaproliferin (FUP), Beauvericin (BEA).

Species	OTA	FUM	ENN	FUP	BEA
1. <i>A. niger</i>	0.83	n.a.	n.a.	n.a.	n.a.
2. <i>A. citrinum</i>	0.59	n.a.	n.a.	n.a.	n.a.
3. <i>A. ibericus</i>	n.d.	n.a.	n.a.	n.a.	n.a.
4. <i>A. carbonarius</i>	1.03	n.a.	n.a.	n.a.	n.a.
5. <i>P. glabrum</i>	n.d.	n.a.	n.a.	n.a.	n.a.
6. <i>P. citrinum</i>	0.52	n.a.	n.a.	n.a.	n.a.
7. <i>F. verticillioides</i>	n.d.	FB ₁ - 118.01 FB ₂ - 232.75	n.a.	n.a.	n.a.
8. <i>P. verrucosum</i>	0.85	n.a.	n.a.	n.a.	n.a.
9. <i>P. expansum</i>	0.93	n.a.	n.a.	n.a.	n.a.
10. <i>F. oxysporum</i>	n.d.	n.a.	n.d.	n.a.	49.41
11. <i>F. proliferatum</i>	n.d.	FB ₁ - 35.51 FB ₂ - 18.55	n.a.	n.d.	n.d.
12. <i>F. subglutinans</i>		n.a.	n.a.	0.73	60.34
13. <i>A. carbonarius</i> *	n.d.	n.a.	n.a.	n.a.	n.a.
14. <i>A. flavus</i>	n.d.	n.a.	n.a.	n.a.	n.a.
15. <i>F. semitectum</i>	n.d.	n.a.	n.a.	n.a.	31.71

* with crystals; n.d.= not determined (below detection limit); n.a.= not analyzed. The limit of detection (LOD) and the limit of quantification (LOQ) were $0.1 \mu\text{g Kg}^{-1}$ and $1 \mu\text{g Kg}^{-1}$ for OTA, respectively. LOD for FB₁ and FB₂ were 0,5 and $1 \mu\text{g kg}^{-1}$ respectively, the LOQ were $1 \mu\text{g kg}^{-1}$ for FB₁ and $2 \mu\text{g kg}^{-1}$ for FB₂. LOD and LOQ were 0.1 and 1 mg kg^{-1} for fusaproliferin, 10 and 20 mg kg^{-1} for beauvericin, 3.5 and 8 mg kg^{-1} for enniatin B₁, respectively.

Table 3. LC/MS/MS parameters for fumonisin B₁ and fumonisin B₂.

Compound	Retention time (min)	Precursor ion [M+H] ⁺ <i>m/z</i>	Product ions <i>m/z</i>	DP (V)	CE (V)
Fumonisin B ₁	3.8	722.4	352.4 528.1	70	50 42
Fumonisin B ₂	4.4	706.4	336.2 512.2	70	51 42