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COLONIZATION OF GRAPE BERRIES BY THE GENUS *FUSARIUM* AND TOXIGENITY OF THE MOST COMMON REPRESENTATIVES

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ABSTRACT

The aim of the study was to assess mycotoxin-producing fungi, especially from *Fusarium* genus, in grapes destined for wine production and to test the ability of selected *Fusarium* strains to produce mycotoxins as deoxynivalenol (DON), diacetoxyscirpenol (DAS), HT-2 (HT-2) toxin, T-2 (T-2) toxin and zearalenon (ZEA). Totally we processed 24 samples, collected from various Slovak localities in year 2012. The total and endogenous mycobiota was determined by the method of direct placing of grape berries on agar plates. Endogenous mycobiota was estimated after the superficial sterilization. The isolation frequency of the *Fusarium* genus was 83.3%, in the framework of the non-sterilized and also of sterilized berries. The average relative density was relatively low (2.2% - without sterilization, 2.3% - with sterilization). Totally we identified 11 species of the genus *Fusarium*. The most important species, on the basis of the isolation frequency and relative density, were *F. proliferatum* and *F. sporotrichioides*. Selected isolates of this two species were tested for their toxigenity, by means of thin-layer chromatography. Tests of *F. proliferatum* confirmed only sporadic production of diacetoxyscirpenol, HT-2 and T-2 toxins. Isolates of *F. sporotrichioides* have demonstrated high ability to produce diacetoxyscipenol, deoxynivalenol and T-2 toxin (100%), 73% produced HT-2 toxin and 50% synthesized zearalenon.

Keywords: *Fusarium* sp., grape berries, mycobiota, mycotoxins

INTRODUCTION

Grape is a fruit appreciated by consumers as fresh (table grapes), dried (raisins), or as processed products, such as grape juice and wine (**Magnoli *et al.*, 2003; Battilani *et al.*, 2003**). If certain physical conditions, such as moisture level, temperature and the presence of organic and inorganic substrates, are met in fungi, they can easily proliferate **(Andersen *et* Thrane, 2006)**. Contamination of grapes by different moulds occurs during preharvesting, harvesting and grape processing. The fungal growth begins in grapes if temperature and humidity are suitable. Rotting and spoilage of grape berries before harvest can be caused by a variety of fungal species (**Valero *et al.*, 2005**). Grapevine can be attacked by a number of fungi and fungus-like organisms which affect the berries and cause loss of quality and influence the taste of the wine **(König *et al.*, 2009)**. Grapes that are heavily infected with moulds alter in chemical composition and secondary metabolites such as mycotoxins (**Magnoli *et al.*, 2003; Battilani *et al.*, 2003**). Besides the pathogenic fungi causing grapevine diseases, berries are also colonized by ubiquitous epiphytic fungi which use sugar and amino acids leaking out of berries as nutrient source. Some of the fungi produce mycotoxins which are more or less human-toxic and some may release compounds which are toxic to yeasts **(König *et al.*, 2009)**.
Mycotoxins are toxic secondary metabolites produced by filamentous fungi that have been detected in food commodities, including grapes and wine (**Serra *et al.*, 2005**). Mycotoxin production can occur in the field and/or in postharvest situations. It has been found that the synthesis of mycotoxins can occur in grapes before harvest, and thus they may be present in wine (**Serra *et al.*, 2004**). Therefore, it is relevant to determine the mycoflora of grapes and the potential for mycotoxins to be present in wine.
The main objective of this work was to assess mycotoxin-producing fungi, especially from *Fusarium* genus, in grapes destined for wine production and to test the ability of selected *Fusarium* strains to produce mycotoxins as deoxynivalenol, diacetoxyscirpenol, HT-2 toxin, T-2 toxin and zearalenon.

MATERIAL AND METHODS

The study was focused on the mycological analysis of grape berries with a focus on potentially toxigenic representatives of genera *Fusarium*. For the analysis we used 24 samples collected from various Slovak localities in 2012 (Table 1). The collection of grape samples took place in the time of their technological ripeness. The grapes were picked at random by the diagonal of the land and each sample was made up of around 3 kg of grapes. Samples were collected in sterile plastic containers, stored in a cool place and transported to the mycological laboratory for analysis up to 24 hours from the collection.
The **total mycobiota** was determined by the method of direct placing of grape berries on agar plates **(Samson *et al.*, 2002b)**. Exactly 50 berries from each sample were placed on DRBC plates (agar with dichloran, rose bengal and chloramphenicol) **(Samson *et al.*, 2002a)**. Cultivation lasted from 5 to 7 days in darkness at 25 ± 1 °C.
The **endogenous mycobiota** was determined by the method of direct placing of superficially sterilized berries on agar plates **(Samson *et al.*, 2002b)**. More than 50 pieces of undamaged berries from each sample were superficially sterilized with chloramine solution, prepared from 10 ml of distilled water and 5 g of chloramine. Sterilization was carried out 2 minutes. Grains were rinsed 3 times with sterile distilled water and dried on sterile filter paper. Exactly 50 berries from each sample were placed on DRBC plates **(Samson *et al.*, 2002a)**. Cultivation lasted from 5 to 7 days in darkness at 25 ± 1 °C.
Grown micromycetes were classified into the genera and then isolated by re-inoculation on the identification nutrient media and identified through macroscopic and microscopic observation in accordance with accepted mycological keys and publications. Isolates of the genus *Fusarium* were re-inoculated on SNA – Synthetischer nährstoffarmer agar **(Nirenberg, 1976)** and PDA – potato-dextrose agar **(Samson *et al.*, 2002a)** and cultured for 7 days at room temperature and natural light. The colonies were examined according to the classification schemes proposed by **Leslie *et* Summerell (2006)**, **Samson *et al.* (2002a)**.
The obtained results were evaluated and expressed in isolation frequency (Fr) and relative density (RD) at the genus and species level. 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:

$$Fr\ (\%) = (ns / N) \times 100 \qquad RD\ (\%) = (ni / Ni) \times 100$$

where 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.

Table 1 Sampling points of mycologically analyzed Slovak grape berries and their varieties

No.	Town or village	Wine-growing rayon	Wine-growing region	Variety
1.	Svätý Martin	Senecký	Malokarpatský	André
2.	Doľany	Dolanský	Malokarpatský	Pinot noir
3.	Dolné Orešany	Orešanský	Malokarpatský	Blue Frankish
4.	Dvorníky	Hlohovecký	Malokarpatský	Sauvignon
5.	Pezinok	Pezinský	Malokarpatský	Blue Frankish
6.	Moravany nad Váhom	Vrbovský	Malokarpatský	Green Veltliner
7.	Vinica	Vinický	Stredoslovenský	Blue Frankish
8.	Šahy	Ipeľský	Stredoslovenský	Pearl of Zala
9.	Sebechleby	Hontiansky	Stredoslovenský	St. Lorenz
10.	Gajary	Záhorský	Malokarpatský	André
11.	Skalica	Skalický	Malokarpatský	Blue Frankish
12.	Zeleneč	Trnavský	Malokarpatský	Cabernet Sauvignon
13.	Nové Zámky	Palárikovský	Južnoslovenský	Green Veltliner
14.	Abrahám	Galantský	Južnoslovenský	Riesling Italico
15.	Čamovce	Fiľakovský	Stredoslovenský	Pálava
16.	Rimavská Sobota	Gemerský	Stredoslovenský	Blue Frankish
17.	Kráľ	Tornaľský	Stredoslovenský	Müller Turgau
18.	Orechová	Sobranecký	Východoslovenský	Pinot gris
19.	Vinné	Michalovský	Východoslovenský	Green Veltliner
20.	Streda nad Bodrogom	Kráľovsko-chlmecký	Východoslovenský	Traminer
21.	Hrušov	Moldavský	Východoslovenský	Alibernet
22.	Viničky	Tokaj	Tokaj	Furmint
23.	Viničky	Tokaj	Tokaj	Hárslevelű
24.	Viničky	Tokaj	Tokaj	Yellow Muskateller

For the **determination of toxigenity** we used thin-layer chromatography according to the **Samson *et al.* (2002a)**, modified by **Labuda *et* Tančinová (2006)**. A total of 25 randomly selected strains of the *F. proliferatum* and *F. sporotrichioides* (the most important species according to their occurrence) have been re-inoculated on YES (yeasts extract agar), cultured in the dark at a temperature of 25 ± 1 °C for 7-14 days and then tested for the ability to produce mycotoxins deoxynivalenol (DON), diacetoxyscirpenol (DAS), HT-2 toxin (HT-2), T-2 toxin (T-2) and zearalenon (ZEA). From the grown colonies we cut squares of the approximate size 2 x 2 cm and put them in small chunks to Eppendorf tube with 1 ml of extraction reagent chloroform : methanol, 2 : 1 (for DON, ZEA) and acetonitrile : water, 50 : 50 (for DAS, HT-2, T-2). After a 5 minute mixing was extract applied to the chromatographic plate (Alugram®SIL G, Macherey – Nagel, Germany). Subsequently, we used developing solution toluene : acetone : methanol (5 : 3 : 2). Before visualisation, chromatographic plates were processed as in Table 2. Mycotoxins have been confirmed by comparison with standards (Merck, Germany) under UV light with a wavelength of 366 nm.

Table 2 Preparation of the chromatographic plates before visualisation of the diacetoxyscirpenol (DAS), deoxynivalenol (DON), HT-2 toxin (HT-2), T-2 toxin (T-2) and zearalenon (ZEA) and the manifestations of visualization

Mycotoxin	Chromatographic plate preparation	Visualisation under UV light with a wavelength of 366 nm
DAS, DON	- application of 20% $AlCl_3$ in 60% ethanol - heating-up	- light blue fluorescent spot
HT-2, T-2	- application of 20% H_2SO_4 in water - heating-up	- green-blue fluorescent spot
ZEA	- application of 20% $AlCl_3$ in 60% ethanol - heating-up - application of 20% H_2SO_4 in water - heating-up	- yellow fluorescent spot

RESULTS AND DISCUSSION

Mycological analysis of the non-sterilized grape samples showed, that all isolates belonged to the 25 genera. The most abundant genera were *Alternaria*, *Aspergillus*, *Botrytis*, *Cladosporium*, *Epicoccum*, *Fusarium*, *Penicillium*, *Rhizopus* and *Trichoderma*. The remaining 16 genera were detected rather sporadically. The berries from the vineyards sampled were generally in good condition, without any visible signs of damage or microbiological depreciation. After sterilization of the berries, the number of isolated genera decreased to 17, but the most abundant genera remained the same.

In the next part of the study, we focused on *Fusarium*, potentially toxigenic genus. *Fusarium* (Figure 1) is a typical field fungus. The isolation frequency of this genus was 83.3%, in the framework of the non-sterilized and also of sterilized berries. **Serra *et al.* (2005)** reported, that *Fusarium* strains were primarily detected at the early maturation stages of grapes, with and without surface disinfection. Overlapping of our results with and without sterilization shows, that only 2 samples (out of 24) were free of *Fusarium* genus (samples 22. and 23.). On the other hand, the average relative density was relatively low (2.2% - without sterilization, 2.3% - with sterilization). The highest relative density was detected in the sample 19. (7.2%; without sterilization) and 4. (8.4%; after sterilization).

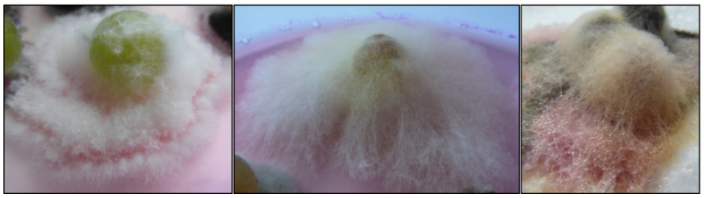

Figure 1 Grape berries of Slovak origin, colonized by *Fusarium* species (agar with dichloran, rose bengal and chloramphenicol)

After further mycological investigations, we identified totally 11 species of the genus *Fusarium*. Detected species, their isolation frequency and average relative density within the genus are presented in the Table 3. By comparison, the authors **Mikušová *et al.* (2013)** reported, that *F. subglutinans*, *F. oxysporum*, *F. proliferatum*, *F. semitectum*, *F. solani* and *F. verticillioides* were found on grape berries from Slovak vineyards with varying frequency. The most important species in our study, on the basis of the isolation frequency and relative density, were *F. proliferatum* and *F. sporotrichioides*.

F. proliferatum is particularly known for the production of fumonisins and many strains can also produce high levels of beauvericins, fusaproliferin and moniliformin. Its other known metabolites are enniatins, fusaric acid and fusarin **(Desjardins, 2006)**. In our toxicological study we analysed isolates of *F. proliferatum* for production of fumonisins, but the visualisation was unsuccessful. Tests confirmed only sporadic production of diacetoxyscirpenol, HT-2 and T-2 toxins (Table 4). **Mikušová *et al.* (2013)** reported, that *F. proliferatum*, cultured in vitro on Czapek yeast autolysate agar and yeast extract sucrose agar, produced beauvericin, in the range from 3,265 to 13,400 µg/kg, and fusaproliferin in high concentration, ranging from 49,850 to 259,500 µg/kg. They analysed also fumonisin B_1 and fumonisin B_2 and the observed levels ranged from 500 to 2,040 µg/kg. **Tamura *et al.* (2012)** mentioned, that in several samples of Japanese red wines were detected fumonisins, but they were less than limits of quantification. Another study **(Logrieco *et al.*, 2010)** also reported occurrence of fumonisins (in particular fumonisin B_2) in red wine from Italy. Authors **Wang *et al.* (2011)** have found that wine can also be contaminated

by enniatins and they confirmed presence of enniatin B in a sample of white wine.

F. sporotrichioides have been reported to produce high levels of T-2 toxin, as well as various T-2 toxin derivates and biosynthetic intermediates, such as neosolaniol and diacetoxyscirpenol **(Desjardins, 2006)**. Strains can produce also butenolid, fusarin C, moniliformin, scirpentriol, steroids, zearalenon **(Leslie *et* Summerell, 2006; Samson *et al.*, 2002a; Desjardins, 2006; Diaz, 2005)** and some isolates are able to produce HT-2 toxin, nivalenol and fusarenon X **(Pitt *et* Hocking, 1999)**. Our isolates have demonstrated high ability to produce diacetoxyscipenol, deoxynivalenol and T-2 toxin (100%), 73% produced HT-2 toxin and 50% synthesized zearalenon (Table 4).

Table 3 The isolation frequency (Fr) and the average relative density (RD; within the genus) of the *Fusarium* species isolated from non-sterilized and sterilized grape berries, originated in Slovakia, vintage 2012

Species	Non-sterilized berries		Sterilized berries	
	Fr [%]	RD [%]	Fr [%]	RD [%]
F. acuminatum	12.5	3.7	12.5	5.2
F. avenaceum	4.2	0.9	8.3	2.6
F. graminearum	12.5	5.6	12.5	9.1
F. oxysporum	12.5	7.4	4.2	1.3
F. proliferatum	66.7	50.9	37.5	20.8
F. semitectum	12.5	2.8	8.3	2.6
F. solani	8.3	5.6	4.2	2.6
F. sporotrichioides	25.0	5.6	33.3	16.9
F. subglutinans	0.0	0.0	4.2	1.3
F. tricinctum	4.2	1.9	0.0	0.0
F. verticillioides	8.3	1.9	4.2	1.3
F. sp	25.0	13.9	50.0	36.4

Table 4 The results of the testing of isolates, obtained from the grapes of Slovak origin, for the ability to produce mycotoxins diacetoxyscirpenol (DAS), deoxynivalenol (DON), HT-2 toxin (HT-2), T-2 toxin (T-2) and zearalenon (ZEA) *in vitro* by means of thin-layer chromatography

Species	Number of tested isolates / number of positive tests				
	DAS	DON	HT-2	T-2	ZEA
F. proliferatum	10 / 2	nt	10 / 1	10 /3	nt
F. sporotrichioides	8 / 8	5 / 5	15 / 11	15 / 15	4 / 2

Legend: nt – not tested

CONCLUSION

Fusarium is a genus, which is very frequent on grape berries of Slovak origin. On the other side, the relative density of the isolates is relatively low. The most frequent species are *F. proliferatum* and *F. sporotrichioides*, potential producers of many toxic metabolites. The ability of the *Fusarium* fungi to produce mycotoxins was tested to assess the potential for mycotoxin synthesis in Slovak grapes. Mainly strains of *F. sporotrichioides* showed a high potential to produce tested metabolites. *F. proliferatum* is more important because of production of fumonisins and we would like to draw the attention of their analysis.

It should be pointed out that the presence of these mycotoxins appears to be especially relevant when grapes in poor condition are used in winemaking. It is worth emphasizing that the use of good quality raw materials is essential for mycotoxin control in food products.

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REFERENCES

ANDERSEN, B., THRANE, U. 2006. Food-Borne Fungi in Fruit and Cereals and Their Production of Mycotoxins. *Advances in Food Mycology*, 571, 137-152.

BATTILANI, P., GIORNI, P., PIETRI, A. 2003. Epidemiology of toxin-producing fungi and ochratoxin A occurrence in grape. *European Journal of Plant Pathology*, 109, 715–722.

DESJARDINS, A. E. 2006. Fusarium Mycotoxins. Chemistry, Genetics, and Biology. St. Paul : The American Phytopathological Society, 260 p. ISBN 0-89054-335-6.

DIAZ, D. 2005. The mycotoxin Blue Book. University Press : Nottingham, 349 p. ISBN 1-904761-19-4.

GONZÁLES, H. H. L., PACIN, A., RESNIK, S. L., MARTINEZ, E. J. 1996. Deoxynivalenol and contaminant mycoflofa in freshly harvested Argentinean wheat in 1993. *Mycopathologia*, 135 (2), 129-134.

GUATAM, A., SHARMA, S., BHADAURIA, R. 2009. Detection of toxigenic fungi and mycotoxins in medicinally important powdered herbal drugs. *Internet Journal of Microbiology*, 7(2).

KÖNIG, H., UNDEN, G., FRÖHLICH, J. 2009. Biology of Microorganismson Grapes, in Must and in Wine. Berlin Heidelberg : Springer-Verlag, 522 p. ISBN 978-3-540-85462-3.

LABUDA, R., TANČINOVÁ, D. 2006. Fungi recovered from Slovakian poultry feed mixtures and their toxinogenity. *Annals of Agricultural and Environmental Medicine*, 13, 193-200.

LESLIE, J. F., SUMMERELL, B. A. 2006. The Fusarium Laboratory Manual. Australia : Blackwell Publishing, 388 p. ISBN 978-0-8138-1919-8.

LOGRIECO, A., FERRACANE, R., VISCONTI, A., RITIENI, A. 2010. A Natural occurrence of fumonisin B_2 in red wine from Italy. *Food Additives and Contaminants: Part A*, 27, 1136-1141.

MAGNOLI, C., VIOLANTE, M., COMBINA, M., PALACIO, G., DALCERO, A. 2003. Mycoflora and ochratoxin-producing strains of *Aspergillus* section *Nigri* in wine grapes in Argentina. *Letters in Applied Microbiology*, 37, 179–184.

MIKUŠOVÁ, P., ŠROBÁROVÁ, A., SULYOK, M., SANTINI, A. 2013. *Fusarium* fungi and associated metabolites presence on grapes from Slovakia. *Mycotoxin Research*, 29(2), 97-102.

NIRENBERG, H. I. 1976. Untersuchngen über die morphologische und biologische Differenzierung in der *Fusarium*-sektion *Liseola*. *Mitteilung aus der Biologische Bundesanstalt für Land- und Forstwirschaft*, 169, 1-117.

PITT, J. I., HOCKING, A. D. 1999. Fungi and Food Spoilage. 2. vyd. Maryland : An Aspen Publication, 593 p. ISBN 0-8342-1306-0.

SAMSON, R. A., HOEKSTRA, E. S., FRISVAD, J.C., FILTENBORG, O. 2002a. Introduction to food- and airborne fungi. Utrecht : Centraalbureau voor Schimmelcultures, 389 p. ISBN 90-70351-42-0.

SAMSON, R. A., HOEKSTRA, E. S., LUND, F., FILTENBORG, O., FRISVAD, J.C. 2002b. Method for the detection, isolation and characterisation of food-borne fungi. In Samson, R. A., Hoekstra, E. S., Frisvad, J. C. & Filtenborg, O. Introduction to food- and airborne fungi. Utrecht : Centraalbureau voor Schimmecultures, p. 283-297. ISBN 90-70351-42-0.

SERRA, R., BRAGA, A., VENÂNCIO, A. 2005. Mycotoxin-producing and other fungi isolated from grapes for wine production, with particular emphasis on ochratoxin A. *Research in Microbiology*, 156, 515–521.

SERRA, R., MENDONCA, C., ABRUNHOSA, L., PIETRI, A., VENÂNCIO, A. 2004. Determination of ochratoxin A in wine grapes: Comparison of extraction procedures and method validation. *Analytica Chimica Acta*, 513, 41–47.

TAMURA, M., TAKAHASHI, A., UYAMA, A., MOCHIZUKI, N. 2012. A Method for Multiple Mycotoxin Analysis in Wines by Solid Phase Extraction and Multifunctional Cartridge Purification, and Ultra-High-Performance Liquid Chromatography Coupled to Tandem Mass Spectrometry. *Toxins*, 4, 476-486.

VALERO, A., MARI´N, S., RAMOS, A. J., SANCHIS, V. 2005. Ochratoxin A-producing species in grapes and sun-dried grapes and their relation to ecophysiological factors. *Letters in Applied Microbiology*, 41, 196–201.

WANG, Z., LIN, X., YU, M., HUANG, Y., QIAN, X., SHEN, Y. 2011. Wine contamination by mycotoxin enniatin B from *Fusarium tricinctum* (Corda) Sacc. *Journal of Food, Agriculture and Environment*, 9,182-185.