JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

MICROFUNGI AND MYCOTOXINS OF GRAPES FROM TOKAJ WINE REGION

Soňa Felšöciová, Ľubomír Rybárik, Dana Tančinová, Zuzana Mašková, Miroslava Kačániová*

Address(es): Ing. Soňa Felšöciová, PhD.,
Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Microbiology, Tr. A. Hlinku 2, 949 76 Nitra, Slovak Republic; + 421 37 641 5813.

*Corresponding author: sona.felsociova@uniag.sk

doi: 10.15414/jmbfs.2015.4.special1.16-18

ARTICLE INFO

Received 12. 11. 2014
Revised 25. 12. 2014
Accepted 29. 12. 2014
Published 2. 2. 2015

Regular article

OPEN ACCESS

ABSTRACT

The aim of this study was to investigate a surface mycobiota of grapes and detection a potentially pathogenic isolates to produce selected mycotoxins. Three samples of wine grapes Furmint, Lipovina and Yellow muscat were collected in the Tokaj wine region in Viničky during the November harvest in 2012. Seven/eight berries from each sample were placed on a Dichloran Rose Bengal Chloramphenicol agar in a total of 50 and incubated at 25 °C for 5 - 7 days. After incubation from the samples were isolated following filamentous fungi: *Alternaria*, *Aspergillus*, *Botrytis*, *Cladosporium*, *Mucor*, *Penicillium*, *Phoma*, *Rhizopus*, *Trichoderma* and *Mycelium sterillium*. The most frequent were genera *Aspergillus*, *Botrytis* and *Penicillium* with 100 % frequention. The largest number of isolates belonged to *Penicillium* (368 isolates) and *Botrytis* (334 isolates). For that reason the relative density of both genera were the highest 47 % and 43 %, respectively. The selected isolates - *Aspergillus* section *Nigri*, *Penicillium crustosum*, *Penicillium expansum* and *Penicillium chrysogenum* were tested for patulin, citrinin, penitrem A, roquefortin C and ochratoxin A producing ability. A higher percentage of *Penicillium crustosum* isolates (79 %) were positive for penitrem A and all were positive for roquefortin C. A higher percentage of *Penicillium expansum* (83 %) were positive for patulin, 72 % for citrinin and they were all positive for roquefortin C. All isolates of *Penicillium chrysogenum* produced roquefortin C. As would be expected, ochratoxin A was not detected from *Aspergillus* section *Nigri*.

Keywords: Mycobiota, *Botrytis cinerea*, *Penicillium* sp., wine grapes, mycotoxins

INTRODUCTION

Tokaj wine region lies in the Lower Zemplín in the northeastern corner of Slovakia. It follows a wine region, most of which are located in Hungary. This area is also the smallest but most attractive wine-growing region of Slovakia. It consists of the southern slopes of the Zemplín hills planted with vines after two millennia. Tokaj area is one of the few areas in the world where the grown grapes for the production of natural sweet wines. On the specific and unique taste of Tokaj wine has its share of both heavy sunlight, but also soil of volcanic origin. Very important is role of weather, long sunny autumn, which will enable aged berries attacked noble *Botrytis cinerea* mould, and dried berries that remain cibeba - raisins, without which the Tokaj wine quality can not be manufactured (**Farkaš, 1983**). Under these specific environmental conditions, pathogenesis is severely constrained, resulting in partial fruit dehydration and chemical transformation. These noble-rotted grapes produce some of the most sought-after, delicious, and expensive dessert wines (**Jackson, 2014**). Contamination by different moulds occurs during preharvesting, harvesting and grape processing. During these periods, temperature and humidity are important factors in mycelium growth and conidia germination **(Lozada, 1995)**. The mycotoxins of greatest significance in grapes and wine grapes are produced by *Aspergillus* and *Penicillium*, include aflatoxins, citrinin and ochratoxin A (**Walker, 2002**). Basis for the production of Tokaj wines are by centuries tried grape varieties - Furmint, Lipovina and Muscat yellow, which must be used in a defined ratio. Other specificities include the original technological process of production and maturation of wines (**Farkaš, 1983**).
The objective of this study was to determine the composition of mould biota present on the surface of grape berries collected from the Tokaj wine region and to analyse recognized mycotoxin-producing fungi for ability to produce some selected mycotoxins.

MATERIAL AND METHODS

Mycological analysis of grapes

Three wine grapes varieties Furmint, Lipovina and Muskat yellow were harvested in village Viničky in Tokaj wine region. The samples were collected the first days of November 2012 and on the other day they were analysed. A total of 50 intact berries (7 – 8 healthy berries per bunch) from each samples were plated on Dichloran Rose Bengal Chloramphenicol agar medium (DRBC, MERCK, Germany) and incubated at 25 °C in the dark for one week. Subsequently, moulds isolates were cultured by individual genera on: MEA (Malt extract agar, **Pitt and Hocking, 1997**), CYA (Czapek yeast agar, **Samson *et al.*, 2002a**), CY20S (Czapek yeast extract with 20 % sucrose agar, **Pitt and Hocking, 1997**), YES (Yeast Extract agar, **Samson *et al.*, 2010**), CREA (Creatine-Sucrose agar, **Samson *et al.*, 2010**) and identified to species level according to the manuals of **Klich (2002), Samson *et al.* (2002a), Pitt and Hocking (1997).**

Results evaluation

Occurrence of micromycetes was expressed according to isolation frequency (Fr) and relative density (RD), which are calculated as followed (**González *et al.*, 1999**):
Fr (%) = (ns/N) x 100; RD (%) = (ni/Ni) x 100
Legend: ns – number of samples in which the genus or species is detected; N – total number of samples; ni – number of isolates of a species or genus, Ni – total number of isolated fungi.

Toxinogenity analysis

For the determination of toxinogenity was used thin layer chromatography (TLC) according to **Samson *et al.* (2002b)**, modified by **Labuda and Tančinová (2006).** Extracellular metabolites – ochratoxin A, citrinin and patulin were carried out on YES agar and intracellural penitrem A and roquefortin C 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, Inc. – Carlsbad, CA, USA). The volume 30 µL of liquid phase of extracts along with 10 µL standards (Sigma, Germany) was applied on chromatographic plate (Alugram ® SIL G, Macherey – Nagel, Germany). The plate was put into developing solution (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. Identification of the metabolites was done by comparison with standards (Merck, Germany). Roquefortin C was visible after spraying with $Ce(SO_4)_2$ x 4 H_2O as an orange spot. Penitrem A after spraying with 20 % $AlCl_3$ in 60 % ethanol and heating at 130 °C for 8 min as a dark blue 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 was visualized citrinin as a yellow-green-tailed spot and ochratoxin A as a blue-green spot.

RESULTS AND DISCUSSION

Surface fungal contamination

Moulds that contaminate grapes include species of *Botrytis, Penicillium, Aspergillus, Mucor, Rhizopus, Cladosporium, Uncinula* and *Plasmopara* (**Doneche, 1993**). In our research the mycological survey of the 3 samples of wine grapes indicated the presence of nine genera of filamentous fungi, most of them which were mentioned above (Tab. 1). Among them *Aspergillus* spp., *Botrytis cinerea* and *Penicillium* spp. were the most frequent mould of the microfungi that occurred in 100 % of samples. In relation to the isolated genera, the results obtained in this study showed small differences with those obtained from previous work with sterilized grapes from Tokaj wine region. There were isolated 12 genera of filamentous fungi. The most frequent mould were *Alternaria, Aspergillus, Botrytis, Penicillium* and *Trichoderma* that occured in all 3 grape varieties Furmint, Lipovina and Muskat yellow (**Felšöciová *et al.*, 2013**). *Aspergillus* species can infect grapes (**Snowdon, 1990**), on the other hand *Penicillium* species apparently do not attack grapes before harvest (**Pitt and Hocking, 1997**) but are prevalent in stored grapes (**Snowdon, 1990**). *Botrytis cinerea* is regarded as the most serious cause of spoilage in grapes (**Doneche, 1993**). One intriguing phenomenon associated with the infection of grapevine by *Botrytis cinerea* is the ability of this fungus to live both pathogenically and saprophytically on the attacked host. It can cause the crop-destroying grey rot but it can also have desired effects when resulting is noble rot in grapes. Grey rot (or grey mould) causes heavy losses of yield in wine grapes and is one of the most serious threats for vine growers. Noble rot is a highly specific process resulting in "botrytised" grapes that produce the greatest white wines (**Sipiczki *et al.*, 2010**). The genera isolated in smaller frequency were *Alternaria* spp., *Cladosporium* spp. and *Rhizopus* spp. in 67 %, among others. *Aspergillus* section *Nigri* was the only group indicated from *Aspergillus* genus. *Aspergillus niger* is by far the most common *Aspergillus* species responsible for postharvest decay of fresh fruit including grapes (**Snowdon, 1990**). Six species of *Penicillium* were identified. The predominant were *Penicillium expansum* and *Penicillium chrysogenum* (100 %), followed by *Penicillium crustosum* and *Penicillium glabrum* (67 %). *Penicillium corylophilum* and *Penicillium polonicum* were in smaller frequency, isolated in 33 % of the samples. According to **Snowdon (1990)** *Penicillium expansum* is the most common contaminant species in stored grapes (**Snowdon, 1990**). From the endogenous microfungi were identified 8 species of *Penicillium.* The most frequent species (100 %) was also in this case *Penicillium expansum* (**Felšöciová *et al.*, 2013**). The relative density is defined as the percentage of isolates of the species or genus occurring in the analyzed sample (**Guatam *et al.*, 2009**). The most presented genera of all tested isolates (780) were *Penicillium* spp. (47 %) *and Botrytis cinerea* (43 %). The most presented species was *P. chrysogenum* (18 %) and *P. expansum* (15 %). Another isolates were present only under 5 %. *Botrytis cinerea* was also the most prevalent species from endogenous contamination (40.5 %), followed by *Penicillium* spp. (40 %) and *Alternaria* spp. (11 %). From the *Penicillium* species it was *Penicillium expansum* (24 %) and *Penicillium chrysogenum* (8 %) (**Felšöciová *et al.*, 2013**).

Table 1 The isolation frequency and the relative density of the microfungi isolated from grape berries from Tokaj wine region

Microfungi	Furmint	Lipovina	Muskat yellow	Total	Frequency (%)	Density (%)
Alternaria		8	19	27	67	3.5
Aspergillus	1	1	7	9	100	1.1
A. section *Nigri*	1	1	3	5	100	0.6
Aspergillus sp.			4	4	33	0.5
Botrytis cinerea	115	111	108	334	100	43
Cladosporium		8	8	16	67	2
Mucor			9	9	33	1.1
Penicillium	110	154	104	368	100	47
P. corylophilum			2	2	33	0.3
P. crustosum	77		17	94	67	12
P. expansum	30	43	47	120	100	15
P. glabrum		3	3	6	67	0.8
P. chrysogenum	2	106	35	143	100	18
P. polonicum		2		2	33	0.3
Penicillium sp.	5			5	33	0.6
Phoma			1	1	33	0.1
Rhizopus		1	9	10	67	1.3
Trichoderma			1	1	33	0.1
Mycelium sterillium	5			5	33	0.6

In total 52 exogenous strains representing 4 potentially toxigenic species were tested for their toxigenic ability, namely *Aspergillus* section *Nigri*, *Penicillium crustosum*, *Penicillium expansum* and *Penicillium chrysogenum* (Tab. 2).

Ochratoxin A production was tested in 5 strains belonging to section *Nigri*. Ochratoxin A is a mycotoxin produced by *Aspergillus* and *Penicillium* species. In the Mediterranean region of Europe, the black *Aspergillus* species are the sources of ochratoxin contamination of grape products (**Varga *et al.*, 2004**). It is produced by *Penicillium verrucosum* in cereal grains in cold climates but not from wine grape samples, by *Aspergillus carbonarius* in grapes, wines and vine fruits, and by *Aspergillus ochraceus* sometimes in coffee beans (**Pitt, 2000**). This mycotoxin is a common contaminant of various foods including cereal products, spices, dried vine fruits, coffee, cocoa, beer and wine. Wine is a widely consumed product by adult individuals in both developed and developing countries and, due to its high frequency of contamination with OTA, it may represent, after cereals, a major source of daily OTA intake for this population. Ochratoxin A is a kidney toxin and probable carcinogen. Potential ochratoxin producing fungi were identified in grapes. Among the 5 black *Aspergillus* strains isolated, the production of ochratoxin A was not confirmed.

From fourteen *Penicillium crustosum* tested isolates eleven produced penitrem A. The production of roquefortin C was confirmed in all tested isolates.

Penicillium expansum produced patulin (15 out of 18 strains screened), citrinin (13 out of 18 strains screened) and all of them produced roquefortin C. *Penicillium expansum* is believed to be the major fungal species contributing to patulin in apple, apple products or in another fruit. Surveys of the presence of patulin in many mouldy fruits including cherries, strawberries, raspberries, mulberries, peaches, grapes, pears, mangoes, plums, apricots and other fruits indicated low incidence and levels of the toxin (**Drusch and Ragab, 2003, Piemontese *et al.*, 2005).** Its production in grapes has been associated with mouldy berries (**Abrunhosa *et al.*, 2001**) and in ciders is degraded by the fermentation process. Patulin causes gastrointestinal problems, skin rashes, and is known to be mutagenic (**Scott *et al.*, 1977; Moss, 1998**).

All fifteen *Penicillium chrysogenum* tested isolates produced roquefortin C. 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.*, 2000**). Potentially toxigenic fungi including *Aspergillus* section *Nigri, Penicillium crustosum, Penicillium expansum* and *Penicillium chrysogenum* were also isolated in previous work (**Felšöciová *et al.*, 2013**). The results obtained in this study showed no differences with those obtained from previous work with sterilized grapes from Tokaj wine region. Out of 8 *Aspergillus* section *Nigri* species did not produced ochratoxin A. The production of patulin, citrinin, penitrem A and roquefortin C was confirmed.

Table 2 Toxinogenity of selected strains, isolated from exogenous mycobiota of wine grapes

Species	Patulin	Citrinin	Penitrem A	Roquefortin C	Ochratoxin A
Aspergillus section *Nigri*					0*/5**
Penicillium crustosum			11/14	14/14	
Penicillium expansum	15/18	13/18		18/18	
Penicillium chrysogenum				15/15	

Legend: * positive isolates; ** number of tested isolates

CONCLUSION

Three grape samples were collected from Furmint, Lipovina and Musacat yellow grape varieties in the Tokaj wine growing region. Mycological survey indicated the presence of nine genera: *Alternaria, Aspergillus, Botrytis, Cladosporium, Mucor, Penicillium, Phoma, Rhizopus, Trichoderma* and one unidentified genus *Mycelium sterillium* without creation fruiting bodies. *Aspergillus, Botrytis* and *Penicillium* were the most frequent isolated in 100 % of the samples, followed by *Alternaria, Cladosporium* and *Rhizopus* in 67 % frequency. The species isolated in higher frequency were *Aspergillus* section *Nigri, Penicillium expansum, Penicillium chrysogenum* (100 %). The largest number of isolates belongs to *Penicillium* (368 isolates) and *Botrytis* (334 isolates). From this point of view, the relative density of both genera was the highest 47 % and 43 %, respectively. Four potentially toxigenic species isolated from exogenous mycobiota were tested for their toxigenic ability by thin layer chromatography, namely *Aspergillus* section *Nigri*, *Penicillium crustosum, Penicillium expansum* and *Penicillium chrysogenum*. Out of 52 strains 90 % produced at least one mycotoxin. In our research ochratoxigenic microfungi (some species of *Aspergillus* section *Nigri*) were found in grape samples but without production of OTA.

Acknowledgments: This work was co-funded by VEGA 1/0611/14 and European Community under project no 26220220180: Building Research Centre Agrobiotech.

REFERENCES

ABRUNHOSA, L., PATERSON, R.R.M., KOZAKIEWICZ, Z., LIMA, N., VENÂNCIO, A. 2001. Mycotoxin production from fungi isolated from grapes. *Letters in Applied Microbiology*, 32(4), 240-242. http://dx.doi.org/10.1046/j.1472-765x.2001.00897.x

DONÈCHE, B. 1993. Botrytized wines. In FLEET, G.H. (ed). Wine Microbiology and Biotechnology. Chur, Swizerland : Harwood Acadedemic Publishers, 327-351.

DRUSCH, S., RAGAB, W. 2003. Mycotoxins in fruits, fruit juices, and dried fruits. *Journal of Food Protection*, 66(8), 1514-27.

FARKAŠ, J. 1983. Biotechnológia vína. Bratislava : Alfa, 984 p.

FELŠÖCIOVÁ, S., RYBÁRIK, Ľ., TANČINOVÁ, D., MAŠKOVÁ, Z., KAČÁNIOVÁ, M. 2013. Mykocenóza hrozna z tokajskej vinohradníckej oblasti. Vinohradníctvo, vinárstvo a agroturistika (Zborník vedeckých, odborných a diskusných príspevkov z 1. ročníka konferencie „Tokaj 2013“) Trebišov : Regionálna rozvojová agentúra pre rozvoj Dolného Zemplína, 32-36. ISBN 978-80-971514-9-2

GONZÁLEZ, H.H.L., MARTÍNEZ, E.J., PACIN, A., RESNIK, S.L. 1998. Relationship between *Fusarium graminearum* and *Alternaria alternata* contamination and deoxinivalenol occurrence on Argentinian durum wheat. *Mycopathologia*, 144(2), 97-102. http://dx.doi.org/10.1023/a:1007020822134

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). http://dx.doi.org/10.5580/104b

JACKSON, R.S. 2014. Botrytis. In BATT, C.A., TORTORELLO, M.L. Encyclopedia of food microbiology. Academic Press, 2nd ed., 288-296. ISBN: 978-0-12-384733-1. http://dx.doi.org/10.1016/b978-0-12-384730-0.00042-2

KLICH, M.A. 2002. Identification of common *Aspergillus* species. Utrecht : Centraalbureau voor Schimmelcultures, 116 p. ISBN 90-70351-46-3. http://dx.doi.org/10.1017/s0269915x03243123

LABUDA, R., TANČINOVÁ, D. 2006. Fungi recovered from Slovakian poultry feed mixtures and their toxinogenity. *Annals of Agricultural and Environmental Medicine*, 13 (2), 193-200.

LOZADA, A.F. 1995. Isolation and identification of mycotoxigenic fungi in selected foods and feeds. *Food Additives and Contaminants*, 12 (3), 509-514. http://dx.doi.org/10.1080/02652039509374337

MOSS, M.O. 1998. Recent studies of mycotoxins. *Journal of Applied Microbiology Symposium Supplement*, 84(1), 62-76. http://dx.doi.org/10.1046/j.1365-2672.1998.0840s162s.x

PIEMONTESE, L., SOLFRIZZO, M., VISCONTI, A. 2005. Occurrence of patulin in conventional and organic fruit products in Italy and subsequent exposure assessment. *Food Additives Contaminants*, 22(5), 437-442. http://dx.doi.org/10.1080/02652030500073550

PITT, J. I., HOCKING, A. D. 1997. Fungi and food spoilage. 2nd ed. London : Blackie Academic & Professional, 593 p. ISBN 0-8342-1306-0. http://dx.doi.org/10.1007/978-1-4615-6391-4

PITT, J.I. 2000. Toxigenic fungi: which are important? *Medical Mycology*, 38(1), 17-22. http://dx.doi.org/10.1080/mmy.38.1.17.22

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. http://dx.doi.org/10.1007/s11046-005-4201-1

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, 283-297, ISBN 90-70351-42-0. http://dx.doi.org/10.1007/s11046-005-4201-1

SAMSON, R. A., HOUBRAKEN, J., THRANE, U., FRISVAD, J. C., ANDERSEN, B. 2010. Food and Indoor Fungi. Utrecht : CBS – KNAW Fungal Biodiversity Centre, 390 p. ISBN 978-90-70351-82-3.

SCOTT, P.M. 2001. Analysis of agricultural commodities and food for *Alternaria* mycotoxins. *Journal of AOAC International*, 84(6), 1809-1817.

SCOTT, P.M., FULEKI, T., HARWIG, J. 1977. Patulin content of juice and wine produced from moldy grapes. *Journal of Agricultural and Food Chemistry*, 25(2), 434-437. http://dx.doi.org/10.1021/jf60210a024

SIPICZKI, M., CSOMA, H., ANTUNOVICS, Z., PFLIEGLER, W. 2010. Biodiversity in yeast populations associated with botrytised wine making. *Mitteilungen Klosterneuburg*, 60, 387-394.

SNOWDON, A.L. 1990. A Color Atlas of Post-Harvest Diseases and Disorders of Fruits and Vegetables. Vol. 1. General introduction and Fruits. London : Wolfe Scientific, 302 p. http://dx.doi.org/10.1017/s0014479700018573

VARGA, J., KONCZ, Z., KOCSUBÉ, S., MÁTRAI, T., TÉREN, J., OSTRÝ, V., ŠKÁRKOVÁ, J., RUPRICH, J., KUBÁTOVÁ, A., KOZAKIEWICZ, Z. 2007. Mycobiota of grapes collected in Hungarian and Czech vineyards in 2004. *Acta Alimentaria*, 36 (3), 329-341. http://dx.doi.org/10.1556/aalim.2007.0016

WALKER, R. 2002. Risk assessment of ochratoxin: current views of the European Scientific Committee on Food, the JECFA and the Codex Committee on Food Additives and Contaminants. *Advances in Experimental Medicine and Biology*, 504, 249-255. http://dx.doi.org/10.1007/978-1-4615-0629-4_26