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ENDOGENAL COLONIZATION OF GRAPES BERRIES

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ABSTRACT

The aim of study was to detect the microscopic filamentous fungi from wine surface of sterilized grapes berries of Slovak origin. We analyzed 21 samples of grapes, harvested in the year 2012 of various wine-growing regions. For the isolation of species we used the method of direct plating surface-sterilized berries (using 0.4% freshly pre-pared chlorine) on DRBC (Dichloran Rose Bengal Chloramphenicol agar). The cultivation was carried at 25±1°C, for 5 to 7 days. A total number of 2541 fungal isolates pertaining to 18 genera including *Mycelia sterilia* were recovered. Isolates of genus *Alternaria* were found in all of tested samples with the highest relative density 56.4%. The second highest isolation frequency we detected for genus *Fusarium* (90.48% positive samples), but with low relative density (31 isolates and 2.99% RD). Another genera with higher isolation frequency were *Cladosporium* (Fr 85.71%, RD 14.6%), *Mycelia sterilia* (Fr 85.71%, RD 4.25%), *Penicillium* (Fr 80.95%, RD 13.42%), *Botrytis* (Fr 71.43%, RD 2.95%) *Rhizopus* (Fr 66.66%, RD 1.34%), *Aspergillus* (Fr 57.14%, RD 0.87%), *Epicoccum* (Fr 47.62%, RD 1.22%), *Trichoderma* (Fr 42.86%, RD 1.26%). Isolation frequency of another eight genera (*Arthrinium, Dichotomophtora, Geotrichum, Harzia, Chaetomium, Mucor, Nigrospora* and *Phoma*) was less than 10% and relative density less than 0.5%. Chosen isolates of potential producers of mycotoxin (species of *Alternaria, Aspergillus, Fusarium* and *Penicillium*) were tested for the ability to produce relevant mycotoxins in *in vitro* conditions using TLC method. None isolate of *Aspergillus niger* aggregate (13 tested) did not produce ochratoxin A – mycotoxin monitored in wine and another products from grapes berries. Isolates of potentially toxigenic species recovered from the samples were found to produce another mycotoxins: aflatoxin B_1, altenuene, alternariol, alternariol monomethylether, citrinin, diacetoxyscirpenol, deoxynivalenol, HT-2 patulin, penitrem A and T-2 toxin in *in vitro* conditions. In conclusion, another research should be performed to detect the occurrence of these mycotoxins in grapes, must, wine and another products from grape.

Keywords: Grapes, endogenal colonization, ochratoxin A, mycotoxins

INTRODUCTION

The grape microbiota is complex and includes filamentous fungi, yeasts and bacteria with different physiological characteristics and effects on wine production **(Rousseaux *et al.*, 2014; Barata *et al.*, 2012)**. 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 such as *Alternaria* spp., *Aspergillus* spp., *Botrytis cinerea*, *Cladosporium* spp., *Eurotium* spp., *Penicillium* spp. and *Rhizopus* spp. **(Magnoli *et al.*, 2003; Rousseaux *et al.*, 2014).** The concern about filamentous fungi in the vineyard has been traditionally linked to spoilage of grapes due to fungal growth. However, the discussion in the European Union concerning the establishment of a maximum limit for the presence of the mycotoxin ochratoxin A (OTA) in wines has increased concern about mycotoxin production. Mycotoxins are secondary metabolites produced by filamentous fungi that have been detected in food commodities, including grapes and wine **(Serra *et al.*, 2005).** Ochratoxin A is a secondary metabolite produced by filamentous fungi of the genera *Aspergillus* and *Penicillium* present in a wide variety of foodstuffs **(Amézqueta *et al.*, 2012).** It has been classified as a possible human carcinogen (group 2B) by the International Agency of Research of Cancer **(IARC, 1993)**. Black aspergilli were the dominant genus among the filamentous fungi isolates from grapes and were the only potential OTA-producing fungi found **(Lasram *et al.*, 2007)**.

The aim of this study was to investigate endogenous microscopic filamentous fungal colonization of grapes grown in small and medium-sized vineyards in Slovakia in year 2012 with the focus on genera *Aspergillus, Alternaria, Fusarium* and *Penicillium*. The ability of isolates of potentially toxigenic species to produce the most important mycotoxins was determined by the means of thin layer chromatography.

MATERIAL AND METHODS

Samples

We analyzed 21 samples of grapes, harvested in year 2012 from various wine-growing regions of Slovakia, from small and medium-sized vineyard. We analyzed grape variety Alibernet (1 sample), André (2 samples), Blaufrankise (5), Cabernet Sauvignon (1), Müller Thurgau (1), Velsch Riesling (1), Grüner Veltliner (3), , Pálava (1), Pinot gris (1), Pinot noir (1), Saint Laurent (1), Sauvignon (1), Tramin (1), Zala gyöngye (1). Samples (3 kg) were collected at the time of technological ripeness.

Mycological analysis

For the isolation of species we used the method of direct plating berries, surface-sterilized berries (using 0.4% freshly pre-pared chlorine) on DRBC (Dichloran Rose Bengal Chloramphenicol agar). The cultivation was carried at 25±1°C, for 5 to 7 days in dark. After incubation colonies of *Aspergillus, Alternaria, Fusarium* and *Penicillium* were transferred onto appropriate identification media.

Identification of Alternaria species

Isolates were re-inoculated on PCA - potato-carrot agar **(Samson *et al.*, 2002)** and cultured for 7 days at room temperature and natural light. Main used identification keys were **Andersen *et al.* (2001)**, **Andersen *et al.* (2002)**, **Simmons (1994),** and **Simmons (2007)**.

Identification of Aspergillus species

Conidial suspensions were inoculated at three equidistant points both on Czapek-yeast extract agar (CYA) **(Samson *et al*., 2002)**, Czapek-yeast with 20% Sucrose (CY20S) **(Samson *et al*., 2002)** and malt extract agar (MEA) **(Samson *et al*., 2002)**, and incubated in dark at 25 °C, 7 days. Species identification was done according to **Klich (2002), Pitt *et* Hocking (2009), Samson *et al.* (2002, 2010), Samson *et* Varga (2007)**.

Identification of Penicillium species

The penicillia belonging to Aspergilloides, Furcatum and Biverticillium subgenera were inoculated at three equidistant points both on Czapek-yeast extract agar (CYA), malt extract agar (MEA) and Creatine Sucrose agar (CREA) **(Samson *et al*., 2002)** and incubated in dark at 25 °C. Sub-cultivation on CYA at 37 °C was used as well. Species identification was done after 7 days according to **Pitt *et* Hocking (2009), Samson et al. (2002, 2010) and Frisvad *et* Samson (2004)**.

Identification of Fusarium species

Potato Dextrose agar (PDA) **(Samson *et al*., 2002)** was used for observation of colony characteristics. "Synthetischer nährstoffarmer agar" (SNA) **(Samson *et al*., 2002)** was used for micromorphological features. Cultures were incubated at 25 °C in dark (PDA) and UV-light 365 nm (SNA). Species identification was done after 7 days according to **Leslie *et* Summerell (2006), Nelson *et al*. (1983), Pitt *et* Hocking (2009) and Samson *et al*. (2002, 2010).**

The obtained results were evaluated and expressed in isolation frequency (Fr) and relative density (RD) at the genus or 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 **(Gautam *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.

Mycotoxins screening by a modified agar plug method

The abilities of selected isolates of potentially toxigenic species to produce relevant mycotoxins in *in vitro* conditions were screened by the means of thin layer chromatography (TLC) according to **Samson *et al*. (2002)** modified by **Labuda *et* Tančinová (2006)**.

Cultivation for screening of extracellular metabolites (aflatoxin B_1, aflatoxin G_1, altenuene, alternariol, alternariol monomethylether, citrinin, diacetoxyscirpenol, deoxynivalenol, HT-2, patulin, T-2 toxin ochratoxin A) were carried out on YES (Yeast Sucrose agar) (**Samson *et al*., 2002)** and for intracellular (cyclopiazonic acid and penitrem A) on CYA (Czapek-yeast extract agar); conditions of cultivation in dark at 25 °C, 14 days. In each tested isolate, 3 pieces of mycelium together with cultivation medium of area of approximately 5 x 5 mm were cut from colonies and extracted in 1000 ml of extraction agents (as referred to Table 1) on vortex for 2 minutes. 20 µl of liquid phase of extracts along with standards (Sigma, Germany) were applied on TLC plate (Marchey-Nagel, Germany) and consequently developed in solvent system (see Table 1). Visualisation of extrolites was carried out referred on Table 1 and compared with standards.

Table 1 Extraction agent, solvents and visualisation of mycotoxins as determined by the agar plug method

Mycotoxin	Extraction agent	Solvents	Treatments of visualisation
Aflatoxin B1	ch:m	TEF	UV light (365 nm) (blue spot)
Aflatoxin G1	ch:m	TEF	UV light (365 nm) (green spot)
cyclopiazonic acid	ch:m	TEF	directly in daylight after spraying with the Ehrlich reagent (violet -tailed spot)
Patulin	ch:m	TEF	by spraying with 0.5 % methylbenzothiazolone hydrochloride in methanol, heated at 130 °C for 8 min and then detectable as a yellow-orange spot on daylight
Penitrem A	ch:m	TEF	after spraying with 20 % $AlCl_3$ in 60 % ethanol, heated at 130 °C for 8 min and then detectable as a dark green to black spot on daylight
Citrin	ch:m	TEF	UV light (365 nm) (yellow-green tailed spot)
Altenuene	ch:m	TEF	UV light(365 nm) (blue spot)
Alternariol	ch:m	TEF	UV light (365 nm) (blue spot)
Alternariol monomethylether	ch:m	TEF	UV light (365 nm) (blue spot)
Deoxynivalenol	ch:m	TAM	after spraying with 20 % $AlCl_3$ in 60 % ethanol, heated at 130 °C for 8 min and then detectable as a blue spot under UV light (365 nm)
Diacetoxyscripenol	ch:m / a:w	TAM	after spraying with 20 % $AlCl_3$ in 60 % ethanol, heated at 130 °C for 8 min, then spraying with 20 % H_2SO_4 in water heated at 130 °C for 8 min, and then detectable as a blue green spot under UV light (365 nm)
HT-2 toxin	a:w	TAM	spraying with 20 % H_2SO_4 in water heated at 130 °C for 8 min, and then detectable as a blue green spot under UV light (365 nm)
T-2 toxin	a:w	TAM	spraying with 20 % H_2SO_4 in water heated at 130 °C for 8 min, and then detectable as a blue green spot under UV light(365 nm)

ch:m - chloroform-methanol (2:1, v/v) **(Samson *et al*., 2002)**, a:w – acetonitril : water (50:50) (**Mubatanhema *et al*., 1999**), TEF – toluene/ethyl acetate/formic acid (90 %) 5:4:1 **(Samson *et al*., 2002)**, TAM – toluene/acetone/methanol 5:3:2 **(Samson *et al*., 2002)**

RESULTS AND DISCUSSION

Sound grapes are an essential prerequisite for the production of high-quality wines. However, pricing of grapes is so far mainly based on the must weight of grape deliveries, although e.g. highly botrytised grapes become raisined bringing about higher contents of soluble solids than sound ones. Besides the desired "noble rot", in particular infection of unripe fruits by grape rot decreases the perceptual quality by destroying fruit flavours typical of the grape variety, furthermore leading to off-flavours, off-odour, bitterness and colour-loss. Moreover, the formation of mycotoxins, particular ochratoxin A, associated with fungal infestation highly affects food safety of the products **(Porep *et al*., 2014).** The filamentous fungi identified from surface disinfection grapes berries are indicated in Table 2 and relative density of isolated genera in Figure 1. Altogether 2541 isolates were recovered and assigned to 18 fungal genera, including *Mycelia sterilia* (isolates without sporulation).

Table 2 Filamentous fungi identified from surface disinfected grape berries

Genera / species	Number of isolates	Isolation frequency (%)	Genera / species	Number of isolates	Isolation frequency (%)
Alternaria	**1433**	**100**	***Geotrichum***	**4**	**9.52**
Al. alternate	196	80.95	***Harzia***	**3**	**9.52**
Al. arborescens	168	47.62	***Chaetomium***	**1**	**4.76**
Al. infectoria	51	23.81	***Mucor***	**3**	**9.52**
Al. tenuissima	944	100	***Mycelia sterilia***	**108**	**85.71**

Al. sp.	74	80.95	***Nigrospora***	**1**	**4.76**
Arthrinium	**2**	**9.52**	***Penicillium***	**341**	**80.95**
Aspergillus	**22**	**57.14**	*P. aurantiogriseum*	31	4.76
A. clavatus	2	4.76	*P. brevicompactum*	12	4.76
A. flavus	6	19.05	*P. canescens*	1	4.76
A. niger aggregate	13	47.62	*P. citrinum*	2	9.52
A. sp.	1	4.76	*P. corylophilum*	16	4.76
Botrytis	**75**	**71.43**	*P. crustosum*	5	14.29
Cladosporium	**371**	**85.71**	*P. expansum*	95	38.1
Dichotomophtora	**1**	**4.76**	*P. funiculosum*	2	9.52
Epicoccum	**31**	**47.62**	*P. glabrum*	1	4.76
Fusarium	**76**	**90.48**	*P. griseofulvum*	1	4.76
F. acuminatum	4	14.29	*P. chrysogenum*	128	33.3
F. avenaceum	2	9.52	*P. oxalicum*	5	4.76
F. graminearum	7	14.29	*P. polonicum*	1	4.76
F. oxysporum	1	4.76	*P. solitum*	2	4.76
F. proliferatum	15	38.1	*P. variabile*	1	4.76
F. semitectum	2	9.52	*P.* sp.	38	33.3
F. solani	2	4.76	***Phoma***	**3**	**9.52**
F. sporotrichioides	13	38.1	***Rhizopus***	**34**	**66.66**
F. subglutinans	1	4.76	***Trichoderma***	**32**	**42.86**
F. verticillioides	1	4.76	**Total identified isolates**		**2541**
F. sp.	28	57.14	**Total berries analysed**		**1050**

Legend: *Al. – Alternaria, A. Aspergillus, F. – Fusarium, P. – Penicillium*, sp. – species

Isolates of the genera *Aspergillus*, *Alternaria*, *Fusarium* and *Penicillium* - significant producers of mycotoxins have been identified to the species level. Isolates of genus *Alternaria* were found in all of tested samples with the highest relative density 56.4%. The second highest isolation frequency we detected for genus *Fusarium* (90.48% positive samples), but with low relative density (31 isolates and 2.99% RD). Another genera with higher isolation frequency were *Cladosporium* (Fr 85.71%, RD 14.6%), *Mycelia sterilia* (Fr 85.71%, RD 4.25%), *Penicillium* (Fr 80.95%, RD 13.42%), *Botrytis* (Fr 71.43%, RD 2.95%) *Rhizopus* (Fr 66.66%, RD 1.34%), *Aspergillus* (Fr 57.14%, RD 0.87%), *Epicoccum* (Fr 47.62%, RD 1.22%), *Trichoderma* (Fr 42.86%, RD 1.26%). Isolation frequency of another eight genera (*Arthrinium, Dichotomophtora, Geotrichum, Harzia, Chaetomium, Mucor, Nigrospora* and *Phoma*) was less than 10% and relative density less than 0.5%. According to the results **Serra *et al.* (2005)** the most frequent genera isolated from grapes for wine production were *Cladosporium* (25% RD), *Alternaria* (24%), *Botrytis* (15%), *Penicillium* (9%) and *Aspergillus* (8%). **Rousseaux *et al*. (2014)** report the occurrence of 70 genera of filamentous fungi in different countries. For example the region of origin markedly influenced the spoilage fungal population to which berries are exposed **(Serra *et al.*, 2006).** Predominant mycobiota in our study belong to genus *Alternaria*. Similarly, **Bau *et al.* (2005)** classify this genus among the dominant grapes, isolated from 75.6% of plated berries. **Magnoli *et al.* (2003)** determined this genus from 80% analysed samples of wine grape varieties from Mendoza, Argentina. We identified four group of *Alternaria*: *Al. alternata*, *Al. arborescens*, *Al. infectoria* and *Al. tenuissima*. The selected isolates were tested for ability to produce mycotoxins in *in vitro* conditions by using TLC method (Table 4). The ability to produce altenuene, alternariol and alternariol monomethylether was found. **Ostrý *et al.* (2007)** tested presence of some *Alternaria* mycotoxins in fresh grape juice, must and wine (Czech origin). Occurrence of *Alternaria* mycotoxins was not proved.

Two species (*A. clavatus, A. flavus*), and *Aspergillus niger* aggregate were identified (1 isolate was not determinate to species level). Black aspergilli (mainly *A. niger* aggregate and *Aspergillus carbonarius*) are important producers of OTA in grapes **(Lasram *et al.*, 2007, Chulze *et al.*, 2006, EL Khoury *et al.*, 2008**, **Amézqueta *et al.*, 2012).** The ecological parameters of black aspergilla are not completely known, but some results are available and this knowledge is critical in the development and prediction of the risk models of contamination of grapes and interacting environmental parameters **(Battilani *et al.*, 2006).** Presence of *A. niger* aggregate was detected in 47.62% of samples, but none of them does not produce OTA in *in vitro* conditions (Table 3). **Serra *et al.* (2005)** from the *Aspergillus* strains identified, the most frequent were from section *Nigri* (84%), namely bisseriate species *A. carbonarius* and *A. niger* aggregate. Producing of aflatoxin B_1 (*A. flavus*) and patulin (*A. clavatus*) by tested isolates in *in vitro* conditions was found. **EL Khoury *et al.* (2008)** reported ability of 43.4% of tested isolates of *A. flavus* (isolated from wine-grapes or musts) to produce aflatoxin B_1. Three *Aspergillus* spp. (*A. flavus* and *A. parasiticus*) isolates from grapes had evidence of aflatoxin B_1 production **(Chunmei *et al.*, 2013).**

Fifteen species of penicilia were identified (Table 2). *Penicillium* is described as being frequent in soils and temperate regions **(Serra *et al.*, 2006)**. Blue mould, caused by *P. expansum*, is one of the most economically damaging postharvest diseases of pome fruits, although it may affect a wider host range, including grapes **(Sanzani *et al.*, 2013)**. Isolates of this species were detected from 38.1% our samples. Species of genus *Penciillium* (including *P. expansum*) are important producers of mycotoxins. The ability to produce mycotoxins (Table 3) in *in vitro* conditions was detected follows: *P. citrinum* – citrin, *P. crustosum* - penitrem A and *P. expansum* - patulin and citrinin. *P. verrucosum* potential producer of OTA was not detected in sample. Potential producers (*Aspergillus* and *Penicillium* species) of patulin from grapes detected **Serra *et al.* (2005)**, too.

Ten species of fusaria were identified (Table 2). **Serra *et al.* (2005)** shown that *Fusarium* strains were primarily detected at the early maturation stages of grapes, with and without surface disinfection. Some of the tested isolates (Table 4) have been able to produce selected trichothecenes (diacetoxyscirpenol, deoxynivalenol, HT-2 and T-2 toxin) in *in vitro* conditions.

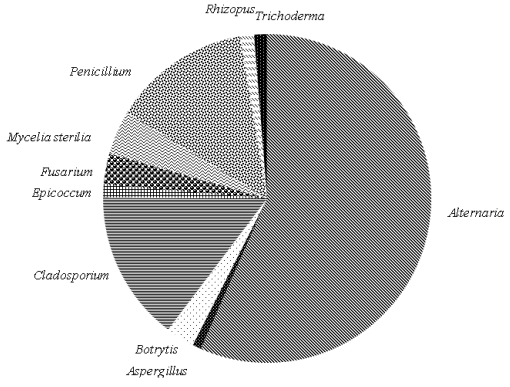

Figure 1 Relative density (RD) (%) isolated genera from surface-sterilized grapes berries (relative densities genera *Arthrinium, Geotrichum, Harzia, Chaetomium, Mucor, Nigrospora* and *Phoma* were less 0.5%)

Table 3 Potential ability isolates of species of genera *Aspergillus* and *Penicillium* to produce relevant mycotoxins in *in vitro* conditions, tested by TLC method

Tested isolates	AFB_1	AFG_1	CPA	C	OTA	PAT	PA
Aspergillus clavatus							
Aspergillus flavus	1*/2**	0/2	0/2				
Aspergillus niger aggregate					0/13		
Penicillium citrinum				1/1			
Penicillium crustosum							2/2
Penicillium expansum				3/12		10/12	

Legend: ** – number of tested isolates, * – number of isolates with ability to produce mycotoxin, OTA – ochratoxin A, AFB1 – aflatoxin B_1, AFG1 – aflatoxin G_1, CPA – cyclopiazonic acid, C – citrinin, PA – penitrem A, PAT – patulin, TLC – thin layer chromatography

Table 4 Potential ability isolates of species of genera *Alternaria* and *Fusarium* to produce relevant mycotoxins in *in vitro* conditions, tested by TLC method

Tested isolates	ALT	AME	AOH	DAS	DON	T-2	HT-2
Alternaria alternata	4*/8**	7/8	7/8				
Alternaria arborescens	6/7	7/7	7/7				
Alternaria infectoria	0/1	0/1	0/1				
Alternaria tenuissima	8/18	17/18	17/18				
Fusarium oxysporum						1/1	1/1
Fusarium proliferatum				0/1		0/1	0/1
Fusarium sporotrichioides				4/4	2/2	4/4	2/4

Legend: ** – number of tested isolates, * – number of isolates with ability to produce mycotoxin, ALT – altenuene, AOH – alternariol, AME – alternariol monomethylether, DAS – Diacetoxyscripenol, DON – deoxynivalenol , T-2 – T-2 toxin, HT-2 – HT-2 toxin, TLC – thin layer chromatography

CONCLUSION

From the 1050 surface-sterilized (21 samples) grape berries have been isolated 2541 strains of microscopic filamentous fungi. The highest relative density and isolation frequency was determined for species genus *Alternaria*. In wine the most important mycotoxin is the ochratoxin A which is not appreciably degraded during wine making, fermentation process and storage. This toxin is the only one mycotoxin monitored under EU law. *Aspergillus niger* aggregate isolates did not produce OTA in *in vitro* conditions. There were found out the ability to produce following mycotoxis: aflatoxin B_1, altenuene, alternariol, alternariol monomethylether, citrinin, diacetoxyscirpenol, deoxynivalenol, HT-2 patulin, penitrem A and T-2 toxin in *in vitro* conditions by TLC method of chosen strains of genera *Alternaria, Aspergillus*, *Fusarium* and *Penicillium*. In another research would be advisable to follow occurrence of these mycotoxins in grapes, must, wine and another products from grape.

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