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SPECIES OF GENUS *ASPERGILLUS* ON GRAPE SLOVAK ORIGIN

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ABSTRACT

The aim of this study was to detect species of genus *Aspergillus* from wine grapes (berries, surface sterilized berries - endogenous mycobiota, from damaged berries and grape juice) of Slovak origin. We analyzed 20 samples of grapes, harvested in 2011 from various wine-growing regions. For the isolation of species we used the method of direct plating berries, surface-sterilized berries (using 0.4% freshly pre-pared chlorine), and damaged berries on DRBC (Dichloran Rose Bengal Chloramphenicol agar). For the determination of fungal contamination of grape juice, we used plate-dilution method and DRBC as medium. The cultivation in all modes of inoculation was carried at 25±1°C, for 5 to 7 days. After incubation *Aspergillus* isolates were inoculated on the identification media. Representatives of the genus *Aspergillus* were isolated from 13 samples berries, 7 samples of surface-sterilized berries, 4 samples of damaged berries and 9 samples of grape juice. Overall, representatives of aspergilli were detected in 90% of samples (75 isolates). In this work we focused on the detection of potential producers of ochratoxin A belonging to the genus *Aspergillus*. Isolates, potential producers of ochratoxin A (*Aspergillus niger* aggregate and *Aspergillus westerdijkiae*), were after their identification inoculated on YES medium (Yeast Extract Sucrose Agar) and after 14 days of incubation at 25±1°C, in the dark, we tested them for their ability to produce ochratoxin A using thin layer chromatography. Out of the 16 isolates from isolated potential producers of ochratoxin A none of the isolates of *Aspergillus niger* aggregate (13 tested) produced ochratoxin A. The isolate of *Aspergillus westerdijkiae* (1), isolated from the surface-sterilized berries, produced ochratoxin A.

Keywords: *Aspergillus*, ochratoxin A, grape

INTRODUCTION

The fungal genus *Aspergillus* was established in 1729, and includes species that are adapted to a wide range of environmental conditions. Many aspergilla produce mycotoxins in food that may be toxic, mutagenic or carcinogenic in animals **(Wilson *et al.*, 2002).**
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**). 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. **(Valero *et al.*, 2005; Magnoli *et al.*, 2003).** Grapes that are heavily infected with moulds alter in chemical composition and secondary metabolities such as mycotoxins. These mycotoxin of great significance in grapes and grape products produced by *Aspergillus* and *Penicillium* spp., include ochratoxin A, aflatoxins, patulin and citrinin **(Magnoli *et al.*, 2003; Battilani *et al.*, 2003).** The mycotoxin production is characteristic for the species and therefore by identifying the species one can predict potential mycotoxin hazards **(Serra *et al.*, 2006)**. Mould growth in wine is strongly inhibited by ethanol and anaerobic conditions, during the fermentation process **(Otteneder *et* Majerus, 2000)**, but possible occurrence of mycotoxins in final products from grapes is high. The occurrence of mycotoxin in wine and fruit juices is a result of poor agricultural practices **(Otteneder *et* Majerus, 2000; Zimmerli *et* Dick, 1996)**. In wine the most important mycotoxin is the ochratoxin A (OTA) which is not appreciably degraded during wine making, fermentation process, and storage **(Delage *et al.*, 2003**). OTA was first detected in wines by **Zimmerli and Dick (1995, 1996)**. Since then, the presence of OTA in imported and locally produced wines has been reported from a number of European and other countries **(Varga *et* Kozakiewicz, 2006)**. According to studies, the source of OTA in this products are OTA-producing strains from the group *Aspergillus* section *Nigri* (*A. carbonarius* and *A. niger* aggregate) **(Valero *et al.*, 2005; Hocking *et al.*, 2007**). OTA is produced primarily when *A. carbonarius* infects berries before harvest. The relatively few toxigenic strains of the relates species, *A. niger*, may also contribute to OTA contamination, as *A. niger* is by far the most common species of *Aspergillus* present on grapes **(Chulze *et al.*, 2006).**
The aim of our study was to detect species of genus *Aspergillus* from wine grapes of Slovak origin. The isolates of potential producers of ochratoxin A were tested for their ability to produce this mycotoxin *in vitro*.

MATERIAL AND METHODS

Samples

We analyzed 20 samples of grapes, harvested in year 2011 from various wine-growing regions of Slovakia. We analyzed grape variety Chardonnay (number of samples 1, 3, 5), mix (2), Velsch Riesling (4, 7, 11, 12), Riesling (6 and 13), Grűner Veltliner (8), Pinot blanc (9 and 17), Konkordia (10), Pinot gris (14), Pinot noir (15), Sauvignon (16), Cabernet Sauvignon (18), Tramin (19) and Limberger (20).

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), and damaged berries on DRBC (Dichloran Rose Bengal Chloramphenicol agar). For the determination of fungal contamination of grape juice, we used plate-dilution method and DRBC as medium. The cultivation in all modes of inoculation was carried at 25±1°C, for 5 to 7 days. After incubation *Aspergillus* isolates were inoculated on the identification media. We use CYA (Czapek Yeast Extract agar), MEA (Malt Extract agar), CY20S (Czapek Yeast Extract agar with 20 % Sucrose) as the identification media. In all cases, cultivation proceeded for 7 days in the dark at 25 ± 1°C. To determine particular species, diagnostic literature was used as follows: **Klich (2002), Samson *et al.* (2002, 2010), Samson *et* Varga (2007).**
The results were expressed according to isolation frequency (Fr):
Fr(%) = (ns/N) x 100

Ns – number of samples with a species or genus, N – total number of samples.

Production of ochratoxin A

The ability of selected isolates of potentially toxigenic species to produce ochratoxin A 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)**. The cultivation for screening of ochratoxin A was carried out on YES (Yeast Extract agar). Isolates were cultivated for 14 days in the dark at 25 ± 1°C. In each tested isolate, 3 pieces of mycelium together with the cultivation medium on area of approximately 5 x 5 mm were cut from colonies and extracted in 1000 ml of chloroform-methanol (2:1, v/v) on vortex for 2 minutes. 20 µl of liquid phase from extracts along with standard ochratoxin A (Sigma, Germany) were applied on TLC plate (Marchey-Nagel, Germany) and consequently developed in solvent system toluene:ethylacetate:formic acid (5:4:1, v/v/v/). Ochratoxin A was visualized directly under UV light (365 nm) as a bluish-green spot.

RESULTS AND DISCUSSION

Table 1 shows the results from investigation of the colonization of grapes and grape juice by species of genus *Aspergillus*. We isolated 74 isolates of genus *Aspergillus* – *A. clavatus, A. fumigatus, A. flavus, A.* niger aggregate, *A. versicolor* and *A. vesterdijkiae*. In Table 2 is shown mycological colonization and isolation frequency of analyzed samples. We detected species of genus *Aspergillus* from 85 % samples. Thirty-six species of *Aspergillus* have been isolated from grapes in vineyards around the world (**Rousseaux *et al.*, 2014**). The most significant potential mycotoxin producers occurring in wine - ochratoxin A were detected representatives of *Aspergillus niger* aggregate (12 isolates), which were detected in all methods of isolation and *A. westerdijkiae* (1 isolate) from the surface sterilized berries. *A. carbonarius,* most important producer of ochratoxin A in wine, has not been identified. **Romero *et al.* (2005)** identified Aspergillus as a predominat genus from berries. *Aspergillus niger* was the most common species but only 3 of 293 isolates screened were ochratoxin A producers. *Aspergillus carbonarius* was less common but 96 % of 48 strains screened were ochratoxigenic **(Romero *et al.*, 2005).** The highest number of isolates, also the highest isolation frequency was observed in *A. clavatus*. Isolation from grape berries from Portugal described **Serra *et al.* (2005). Tančinová *et* Labuda (2009)** in all tested isolates indicate ability to produce mycotoxin patulin**.** *A. flavus* (1 isolat) is potential producer of aflatoxins. Ability of isolates from wine grapes identified **Chunmei *et al.* (20013).** *A. fumigatus* (5 isolates) was isolated from 5 samples. According Doaré-Lebrun (source: **Rousseaux *et al.*, 2014)** this species is able to produce off-flavors – geosmin ane earthy odor on grapes. Species *A. versicolor* (4 isolates) was presented in 3 samples. Occurrence of this species in the berries is reported by Serra et al. (2005, 2006). *A. westerdijiae* was detected only in one sample (1 isolate). **Diaz *et al.* (2009)** reported the presence of this species on berries, also. Species was separated from species *A. ochraceus* in 2004 and it is an important producer of ochratoxin A **(Frisvad *et al.*, 2004).**

Table 1 Species of genus *Aspergillus* isolated from grape and grapes juice

Species	Berries	Surface sterilized berries	Grape juice	Damages berries	Total
	Number of isolates				
A. clavatus	10	5	3	1	19
A. flavus	1	-	-	-	1
A. fumigatus	3	1	1	-	5
A. niger aggregate	8	-	1	3	12
Aspergillus sp.	8	3	18	3	32
A. versicolor	3	-	1	-	4
A. vesterdijkiae	-	1	-	-	1
Total	33	10	24	7	74

Legend: *A. – Aspergillus*, sp. - species

Table 2 Species of genus *Aspergillus* isolated from analysed samples

	Samples																				Fr
Species	**1.**	**2.**	**3.**	**4.**	**5.**	**6.**	**7.**	**8.**	**9.**	**10.**	**11.**	**12.**	**13**	**14**	**15.**	**16.**	**17.**	**18.**	**19**	**20.**	**(%)**
	Number of isolates																				
A. clavatus	2		1		1			3	2	2						1		2	2	3	50
A. flavus										1											5
A. fumigatus										1		1		1	1				1		25
A. niger aggregate										1				2	4	2		3			25
A. sp.	1		1	1			11	3	6	2	1			1		1	3			1	60
A. versicolor						1									2			1			15
A. westerdijkiae																		1			5
Total	3		2	1	1	1	11	6	8	7	1	1		4	7	4	3	7	3	3	85

Legend: *A. – Aspergillus*, Fr – isolation frequency, sp. - species

Production of ochratoxin A

Ochratoxin A was first detected as a wine contaminant in 1996 and the role of *Aspergillus* section *Nigri* and *A. carbonarius* in ochratoxin A production discovered in Europe in 1999 **(Battilani *et al.*, 2006).** We isolated *A. niger* aggregate (12 isolates) and *A. westerdijkiae* (1 isolates) as a potential producers of ochratoxin A. Isolat *A. westerdijkiae* was detected as real producer of ochratoxin A detected by TLC method in *in vitro* conditions. **Labuda *et* Tančinová (2006), Dovičičová *et al.* (2009)**, **Tančinová *et al.* (2012)** similarly referred to the inability of isolates obtained from samples of Slovak origin to produce ochratoxin A. *A. westerdijkiae* was separated from *A. ochraceus* **(Frisvad *et al.*, 2004),** and neither of them is referred to as producer of ochratoxin A in grapes and in wine.

CONCLUSION

Representatives of the genus *Aspergillus* were isolated from 85% of the analysed samples. We isolated by species *A. clavatus, A. fumigatus, A. flavus, A. niger* aggregate, *A. versicolor* and *A.vesterdijkiae*. Representatives of *A. niger* aggregate did not produce the most significant mycotoxin studied in vine - ochratoxin A. This mycotoxin was produced only by isolates of *A. westerdijkiae*. The occurrence of potential producers of mycotoxins as well as their mycotoxins in grapes and the vine should be paid more attention to.

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