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COLONIZATION OF GRAPES BERRIES AND CIDER BY POTENTIAL PRODUCERS OF PATULIN

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ABSTRACT

The aim of this study was to detect potential producers of mycotoxin patulin from grapes (berries, surface sterilized berries - endogenous mycobiota and grape juice) of Slovak origin. We analyzed 47 samples of grapes, harvested in 2011, 2012 and 2013 from various wine-growing regions. For the isolation of species we used the method of direct plating berries and surface-sterilized berries (using 1% freshly pre-pared chlorine) berries on DRBC (Dichloran Rose Bengal Chloramphenicol agar). For the determination of fungal contamination of grape juice we used plate-dilution method and DRBC and DG18 (Dichloran 18% Glycerol agar) as media. The cultivation in all modes of inoculation was carried at 25 ±1 °C, for 5 to 7 days. After incubation *Aspergillus* and *Pencillium* isolates were inoculated on the identification media. The potential producers of patulin were isolated from 23 samples berries, 19 samples of surface-sterilized berries and 6 samples of grape juice. Overall, the representatives of producers of patulin were detected in 32 (68.1%) samples (75 isolates). In this work we focused on the detection of potential producers of patulin, *Penicillium expansum* (the most important producer of patulin in fruits), *Penicillium griseofulvum* and *Aspergillus clavatus* were isolated. Chosen isolates of potential patulin producers were tested for the ability to produce relevant mycotoxins in *in vitro* conditions using thin layer chromatography method. The ability to produce patulin in *in vitro* condition was detected in 82% of isolates of *Penicillium expansum*, 65% of *Penicillium griseofuvum* and 100% of *Aspergillus clavatus*. Some isolates of *Penicillium expansum* were able to produce citrinin and roquefortine C, *Penicillium griseofulvum* cyclopiazonic acid, griseofulvin and roquefortin C, also.

Keywords: grapes; patulin; *Penicillium*; *Aspergillus*; mycotoxin

INTRODUCTION

Grapes and their derived products are important components of the human diet all over the world. The concern about mould in the vineyeard has traditionally been linked to spoilage of grapes due to the fungal growth **(Serra et al. 2006)**. Mycotoxins are secondary metabolites produced by filamentous fungi that have been detected in food commodities, including grapes and wine **(Serra et al., 2005)**. Mycotoxins are abiotic hazards produced by certain fungi that can grow on a variety of crops. Consequently, their prevalence in plant raw materials may be relatively high **(Marin et al., 2013)**. Mycotoxins are produced in plants by filamentous micro-fungi species, and naturally contaminated the food chain **(Sirot et al., 2013)**. Some mycotoxins are acutely toxic, some are chronically toxic and some are the both. Furthemore, it is possible that mixtures of mycotoxins are acting synergistically or additively, so a mycological examination of the mycobiota, to species level, is very important, as different species produce different profiles of extrolites **(Frisvad and Samson, 2002, Bürger et al., 2004)**. Patulin, a mycotoxin found in apples, grapes, oranges, pear and peaches, is a potent genotoxic compound **(Saxena et al., 2009)**. Patulin is a mycotoxin produced by several species of filamentous micro-fungi, especially within *Penicillium*, *Aspergillus* and *Byssochlamys* **(Weidenbörner, 2001, Bennett and Klich, 2003, Moake et al., 2005, Pitt and Hocking, 2009, Puel et al., 2010, Samson et al., 2010)**, from which *Penicillium expansum*, a common contaminant of damaged fruits, is the most important **(Morales et al., 2007)**. Patulin is generally very toxic for both prokaryotes and eukaryotes, but the toxicity for humans has not been conclusively demonstrated. Several countries in Europe and the USA have now set limits on the level of patulin in apple juice **(Frisvad et al., 2006, Frisvad et al., 2007b)**. Positive samples of grape juice, grape must be reported Rychlik and Schieberle; Altmayer et al.; grape wine Altmayer et al. **(Barkai-Golan, 2008)**.

The aim of our study was to detect species of genera *Aspergillus*, *Byssochlamys* and *Penicillium* from grapes of Slovak origin. The isolates of potential producers of patulin were tested for their ability to produce patulin and other mycotoxins in *in vitro* conditions.

MATERIAL AND METHODOLOGY

Samples

We analyzed 47 samples of grapes, harvested in years 2011, 2012 and 2013 from various wine-growing regions of Slovakia, from small and medium-sized vineyard. We

analyzed grape variety Alibernet (1 sample), André (2 samples), Blaufrankise (8), Cabernet Sauvignon (2), Müller Thurgau (1), Velsch Riesling (4), Grüner Veltliner (5), Pálava (1), Pinot blanc (2), Pinot gris (2), Pinot noir (2), Saint Laurent (1), Sauvignon (2), Tramin (1), Zala gyöngye (1). Samples (3 kg) were collected at the time of technological ripeness. Picked grapes were stored at 4 ±1 °C and analyzed within 24 h after harvest.

Mycological analysis

For analysis intact berries have been used. For the isolation of species we used the method of direct plating berries, surface-sterilized berries (using 1% freshly pre-pared chlorine) and non-sterilized berries on DRBC (Dichloran Rose Bengal Chloramphenicol agar). For each analysis were used 50 berries. The cultivation was carried at 25 ±1 °C, for 5 to 7 days in dark. Only undamaged berries have been used for analysis. After incubation, the colonies of *Aspergillus* and *Penicillium* were transferred onto appropriate identification media.

Dilute plate technique was used for isolation of fungi from the samples of cider according to **Samson et al. (2002)**. Sample 20 ml of cider was mixed with 180 ml of saline solution (0.85% sodium chloride) with 0.05% Tween 80 on homogenizer. Then 0.1 ml of appropriate dilutions made up to 10^{-3} was applied on DRBC and DG18 (Dichloran 18% Glycerol agar). After 5 to 7 days of incubation at 25 ±1 °C in dark, resulting colonies were transferred onto appropriate identification media.

Identification of *Aspergillus* species. Conidial suspensions were inoculated at three equidistant points both on Czapek-yeast extract agar (CYA), Czapek-yeast with 20% Sucrose (CY20S) and malt extract agar (MEA), and incubated in dark at 25 ±1 °C, 7 days. Species identification was done according to **Klich (2002), Pitt and Hocking (2009), Samson et al. (2002, 2010), Samson and Varga (2007)**.

Identification of *Penicillium* species.

Conidial suspensions were inoculated at three equidistant points on Czapek-yeast extract agar (CYA), malt extract agar (MEA) and Creatine Sucrose agar (CREA) and incubated in dark at 25 ±1 °C. Sub-cultivation on CYA at 37 ±1 °C was used as well. Species identification was done after 7 days according to **(Pitt and Hocking, 2009; Samson et al., 2002, 2010; Samson and Frisvad, 2004)**.

Obtained results were evaluated and expressed in isolation frequency (Fr) at the species level. The isolation frequency (%) is defined as the percentage of samples within which the species occurred at least once **(Gautam et al., 2009)**. These values were calculated according to **González et al. (1996)** as follows:

$$Fr\ (\%) = (ns / N) \times 100$$

where ns = number of samples with a species; N = total number of samples.

Patulin and other mycotoxins - screening by a modified agar plug method.

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

Cultivation for screening extracellular metabolites (citrinin, griseofulvin patulin) was carried out on YES (Yeast Sucrose agar) and for intracellular (cyclopiazonic acid and roquefortin C, sterigmatocystin) 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 approximately 5 x 5 mm area were cut from colonies and extracted in 1000 ml of chloroform:methanol (2:1, v/v) on vortex for 2 minutes. Then 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 toluene:ethylacetate:formic acid (5:4:1, v/v/v/). The visualisation of extrolites was carried out as follows: cyclopiazonic acid directly in daylight after spraying with the Ehrlich reagent (violet-tailed spot); patulin by spraying with 0.5% methylbenzothiazolone hydrochloride in methanol, heated at 130 °C for 8 min and then detectable as a yellow-orange spot; roquefortin C after spraying with $Ce(SO4)_2$ x 4 H2O visible as orange spot. Directly under UV light (365 nm) were visualised following mycotoxins: citrinin (yellow-green) and griseofulvin (blue spot).

RESULTS AND DISCUSSION

Mycotoxins are abiotic hazards produced by certain fungi that can grow on a variety of crops **(Marin et al., 20013)**. In the current study from 68.1% of samples were isolated potential producers of patulin. We isolated species genera *Aspergillus* (*Aspergillus clavatus*) and *Penicillium* (*Penicillium expansum* and *Penicillium griseofulvum*), as potential producers of this mycotoxin. **According Dombrink-Kurtzman and Engberg (2006)** the *Byssochamys nivea* strains produced patulin in amounts comparable to *Penicillium expansum* strains. Interest in the genus *Byssochlamys* is related to the ability of its ascospores to survive pasteurization and cause spoilage of heat-processed fruit products worldwide. Genus *Byssochlamys* was not detected in our samples. The number of isolates and isolation frequency of species recovered from the sample are listed in Table 1. According **Zouaoui et al. (2015)** patulin is a secondary metabolite, which is mainly produced by certain species of *Aspergillus* and *Penicillium* fungi.

***Penicillium* spp.**

Penicillium species are ubiquitous, opportunic saprophytes. A majority of the described species are soil fungi, and their occurrence in food is more of less accidental and rarely of consequence. However, quite numbers of species are closely associated with human food supplies. Some species are more specialised: several are destructive pathogen on fruit (e.g. *Penicillium digitatum, Penicillium expansum, Penicillium italicum*) **(Pitt and Hocking, 2009)**. *Penicillium expansum* is one of the most common fruit pathogens; it causes soft rot known as "a blue mould rot" on a variety of fruits **(Franck et al., 2005, Neri et al., 2010, Elhariry et al., 2011)**. 251 isolates from grapes berries and 180 isolates from cider (3 positive samples) have been identified as *Penicillium expansum*. **Frisvat et al. (2007b)** reported that *Penicillium expansum* is one of the most common fungal species associated with grapes. Representative isolates of *Penicillium expansum*

Table 1 Filamentous fungi – potential producers of patulin identified from grape berries and cider from 47 samples.

Species	Berries		Surface-sterilized berries		Cider	
	Number of isolates	Isolation frequency (%)	Number of isolates	Isolation frequency (%)	Number of isolates	Isolation frequency (%)
Aspergillus clavatus	23	27.7	7	10.6	25	4.3
Penicillium expansum	143	23.4	108	6.4	180	6.4
Penicillium griseofulvum	29	2.1	13	27.7	25	6.4

Table 2 Potential ability isolates to produce patulin and other mycotoxins in *in vitro* conditions, tested by TLC method.

Tested isolates	Mycotoxins				
	Patulin	**Citrinin**	**Cyclopiazonic acid**	**Roquefortine C**	**Griseofulvin**
Aspergillus clavatus	15**/15*	–	–	–	–
Penicillium expansum	37/45	22/45	–	42/45	–
Penicillium griseofulvum	11/17	–	16/17	15/17	15/17

** - number of tested isolates, * - number of isolates with ability to produce mycotoxin, TLC – thin layer chromatography

were tested for ability to produce patulin in *in vitro* condition. From 45 tested isolates 37 (82.2%) produced patulin. Postharvest diseases are the most important factors that limit commercial export of Chilean table grapes. In recent years, blue mould decay caused by *Penicillium expansum* has frequently appeared on Red Globe grapes after long period (>60 days) of cold storage, causing significant economical losses **(Franck et al., 2005)**. *Penicillium expansum* causes significant economic losses to the fruit industry and is also one of potential public health concern because it produces toxic secondary metabolites including patulin, citrinin, and chaetoglogosins **(Andersen et al., 2004)**. 48.9% of tested isolates *Penicillium expansum* produced citrinin and 93.3% roquefortine C *in vitro* conditions, as well. **Bragulat et al. (2008)** reported, that 100% isolates *Penicillium expansum*, detected from grapes, were able to produce citrinin and 60% patulin. Citrinin is a quinone methide with a powerful antibacterial effect, but toxic to humans and animals. This mycotoxin is mainly hepato-nephrotoxic **(Zaied et al., 2012)**. Roquefortine C (another mycotoxin produced by *Penicillium expansum*) is a very widespread fungal secondary metabolite. The acute toxicity of roquefortine C is not very high **(Cole and Cox, 1981)**. Furthermore, we have identified species *Penicillium griseofulvum* – other producer of patulin; 42 isolates from the berries and 25 from cider (2 positive samples). *Penicillium griseofulvum* is a very efficient producer of high levels of patulin in pure culture, and it may potentially produce patulin in cereals, pasta and similar products **(Frisvad et al., 2006, Frisvad et al., 2007b)**. 11(65%) tested isolates (Table 2) were able to produce patulin. Some isolates produced cyclopiazonic acid (94%), roquefortine C (88%) and griseofulvin (88%), also. Incidence of *Penicillium griseofulvum* on grapes also described **Serra et al. (2005)**, **Bragulat et al. (2008)** and other authors. As mentioned above, the *Penicillium griseofulvum* also produced cyclopiazonic acid. Persistently studies with this mycotoxin have shown that targets correspond to muscles, liver and spleen (Burdock and Flamm, 2000). The last detected mycotoxin was griseofulvin. Griseofulvin is active against dermatophytic fungi of different species in the genera *Microsporum*, *Trychophyton* and *Epidermophyton*. Prolonged griseofulvin treatment in experimental animals provoked biochemical changes consisting mainly of disturbances of porphyrin metabolism, variation in the microsomal cytochrome levels and formation of Mallory bodies **(De Carli and Larizza, 1988)**.

***Aspergillus* sp.**

Species of *Aspergillus* are among the most economically important fungi, on the positive side being very widely used for synthesis of chemicals, for biosynthetic transformations and enzyme production. On the negative side, they are of great importance in food spoilage and they produce important mycotoxins **(Pitt and Hocking, 2009)**. Lopez-Diaz and Flannigan reported that *Aspergillus clavatus, Aspergillus longivesica, Aspergillus giganteus* and other species are very efficient producers of patulin laboratory, but only *Aspergillus clavatus* may play role in human health **(Frisvad et al., 2007b)**. 30 isolates from grapes berries and 25 isolates from cider (2 positive samples) have been identified as *Aspergillus clavatus*. All tested isolates (Table 2) were able to produce patulin.

CONCLUSION

Potential producers of patulin were isolated from 68.1% of the analysed samples. We isolated species genera *Aspergillus* and *Penicillium*. The ability to produce patulin in *in vitro* condition was detected in 82% of isolates of *Penicillium expansum*, 65% of *Penicillium griseofuvum* and 100% of *Aspergillus clavatus*. Some isolates of *Penicillium expansum* were able to produce citrinin and roquefortine C, *Penicillium griseofulvum* cyclopiazonic acid, griseofulvin and roquefortin C, also.

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