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MICROSCOPIC FUNGI ISOLATED FROM DIFFERENT SLOVAK GRAPE VARIETIES

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ABSTRACT

The aim of this study was to isolate and identify microscopic fungi in different grape samples. We collected 13 grapes varienties samples (9 white and 4 red) from local Slovak winemakers in the end of the September 2017. Used 13 grape samples in this study: Alibernet, Irsai Oliver, Dornfelder, Blue Frankish, Feteasca regala, Green Veltliner, Pálava, Müller Thurgau, Rhinriesling, Cabernet Savignon, Pinot Blanc, Savignon Blanc and Welschriesling. Microscopic fungi in grape samples were detected on Malt extract agar by spread plate method. The number of microscopic fungi ranged from 2.85 log cfu.g^{-1} in Cabernet Savignon to 4.83 log cfu.g^{-1} in Feteasca regala. A total of 627 isolates of microscopic fungi were obtained in this study. The most abundant fungi belonged to genera *Alternaria* and *Penicillium* (100% frequency). The high frequency was also detected for *Aspergillus* (76.92%) and *Cladosporium* (76.92%) but with lesser relative density. *Alternaria* sp., *Aspergillus niger*, *Aspergillus* sp., *Botrytis cinerea*, *Cladosporium* sp., *Penicillium expansum*, *Phoma* sp., *Rhizopus* sp. and *Trichoderma* sp. species were isolated from grape berries.

Keywords: grape; microscopic fungi; *Alternaria; Penicillium*; the Lesser Carpathian region

INTRODUCTION

Grape berries are colonized by a complex microbial ecosystem, which consists of epiphytic microorganisms represented by bacteria, yeast, and filamentous fungi **(Barata et al., 2012)**. This microflora plays a major role in crop health and in winemaking process, affecting the wine quality, as reported by **Barbe et al. (2001), Nisiotou et al. (2011)** and **Verginer et al. (2010)**. The filamentous fungi and yeast of grapes has been intensevily studied due to the impact on wine quality **(Pretorius, 2000)**. Research has also covered the pathogenic fungi affecting grapes, including *Erysiphe necator* (the causal agent of grapevine powdery mildew), *Botrytis cinerea* (gray rot) and *Plamospara viticola* (downy mildew). However, saprophytic molds, like *Aspergillus* spp., *Cladosporium* spp., and *Penicillium* spp. are also responsible for grape rots. They also were involved in food poisoning by their mycotoxin production **(Martin set al., 2014)**.

During the harvest period grapes were affected by insects, yeasts, or bacteria. The most damaging form of attack is linked to gray mold, *Botrytis cinerea*, eventually associated with various fungi and typical for temperate climate **(Ribereau-Gayon et al., 1998)**.

A variety of grapevines are grown in specific wine producing regions, which results in high variability of physical and chemical characteristics of grapes, and the wines produced thereof **(Abe et al., 2007)**. The grapes may be susceptible to infection by filamentous fungi from the initial stages of maturation **(Bau et al., 2005)**. The filamentous fungi are able to produce an enzyme complex responsible for the degradation of specific substrates, production of secondary metabolites and volatile compounds **(Medina et al., 2015)**. Grapes contamination by fungi may promote the productions of mycotoxins, which may develop at pre-harvest or during the harvesting leading to vinification **(Freire et al., 2017)**.

Identification of filamentous fungi of post-harvest fruits and their storage environments, lifestyle and pathogenicity are essential to develop strategies to prevent and control thier distribution **(Narayanasam, 2006)**. This information is important to understand the fungal contamination of withered grapes. Previous investigations indicated that the fruit-drying rooms showed the diversity of several fungal groups. Notably that intraspecific variability in the ability to infect grapes under different withering conditions was found **(Lorenzini et al., 2015; Lorenzini and Zapparoli, 2014a, 2014b, 2015)**.

The aim of our study was to identify the filamentous microscopic fungi on the grape berries.

Scientific hypothesis

The scientic hypothesis of this study was that the grapes were colonized with different microscopic fungi species, which could be identified with MALDI-TOF method.

MATERIAL AND METHODOLOGY

Grape samples collection

Threeteen grape samples from 2017 year were used in this experiment. Ripe grape bunches were collected into sterile polyethylene bags and transported to laboratory for the next microbiological analysis. The grape samples were collected from the Lesser Carpathian wine region (n = 13). We investigated grape samples of the following varieties: Alibernet, Irsai Oliver, Dornfelder, Blue Frankish, Fateasca reagla, Green Veltliner, Pálava, Mūller Thurgau, Rheinriesling, Cabernet Savignon, Pinot Blanc, Savignon Blanc and Welschriesling. Eache grape was one sample.

Characterisation of „Lesser Carpathian" wine region

The Lesser Carpathian wine region is located in the southwestern part of Slovakia. Vines are grown for more than three thousand years on southern, southwestern and south-eastern slopes and plains of the Lesser Carpathians and in locality Záhorie. Geological substrate is predominantly formed by detrial cones of Lesser Carpathian rivers, soils are silty sands, medium skeletal, in the peripheral parts eventually drifting sands. Vineyards are covering 5 588 hectares within 132 specified regions. Wine from the Lesser Carpathian wine region is a product obtained exclusively by a total or partial fermentation of grapes or grape must, which originates in this region. Grapes are rich in high sugar content, wines are full bodied, with intensive taste and pleasant level of acidity, suitable for longer cellar maturation. Lesser Carpathian wine region has continental climate. The total volume of rainfall is 650 mm distributed fairly evenly throughout the year. Altitude of vineyards in the area is from 100 to 250 metres above sea level. Average air temperature from May to September ranges from 13 °C to 20 °C and is 17.5 °C in growing season. Average annual sunshine duration is 2100 hours, the sum of active air temperature during vegetation is at least 3000 °C. This area is characterized by at least 15 °C temperature difference between day and night during the vegetation, with skeletal base allowing the grapes to produce white varieties of higher acidity, while malolactic fermentation is eliminated to occur when the grapes are still on plants.

The vineyards are mainly trained on medium or high techniques of vine training system. There is up to 10 000 vines/hectare density of vineyard. Number of vine buds shall not exceed 80 000 per one hectare of vineyard for production of wine, quality wine, sparkling wine of wine region, grower's sparkling wine or liqueur wine. Maximum of 65 000 vine buds for the production of quality wines with attribute. Treatment of the white grapes varieties during processing is very fast and tactful, white wines are typically with higher acidity, wine is extracted with optimum ratio of sugars and acids. Production of white wines in the Lesser Carpathian wine region is done in reductive way, without or with only minimal access of air. Temperature is controlled during fermentation and it does not exceed 15 °C. Controlled fermentation involves possible use of indigenous or commercial preparations of isolated strains of yeast *Saccharomyces cerevisiae*. Sulfur dioxide is used as a chemical preservative.

Microbiological analyses of grape berries samples

Five gram of berries from each grape variety were diluted in 45 ml sterile physiological saline (0.85%) and stirred on a horizontal shaker for 30 minutes. The suspension was used for preparation of dilutions of 10^{-2} and 10^{-3} and 0.1 ml of each dilution (10^{-2}, 10^{-3}) was plated onto Malt extract agar (base, Oxoid, UK supplemented with bromocresol green (0.020 g/l), Centralchem®, Slovakia). Microscopic fungi were cultivated at 25 °C for five days in aerobic conditions and identified to species level according to the manuals of **Samson et al. (2002a)**, **Samson and Frisvad (2004)**, **Pitt** and **Hocking (2009)**.

The obtained results were evaluated and expressed according to isolation frequency (Fr) and relative density (RD). 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. (1999)** as follows:

Fr (%) = (ns / N) x 100;

RD (%) = (ni / Ni) x 100

Where: ns – number of samples within a species or genus; N – total number of samples; ni – number of isolates of species or genus; Ni – total number of isolated fungi.

RESULTS AND DISCUSSION

Filamentous fungi are the main pathogens of post-harvest fruits and can cause heavy economic losses. The type of fruit, maturity stage, pre-harvest and storage conditions are known to affect the fungal contamination and growth of saprophytic microorganisms (**Narayanasam, 2006**).

Numbers of microscopic fungi in grape beries varietes isolated are shown in Table 1. The number of microscopic fungi ranged from 2.85 log cfu.g^{-1} in Cabernet Savignon to 4.83 log cfu.g^{-1} Feteasca regala.

A total of 627 isolates of microscopic fungi were obtained in this study. The most abundant moulds belonged to genera *Alternaria* and *Penicillium* and their frequency comprised 100%. The higher frequency was also detected for *Aspergillus* (76.92%) and *Cladosporium* (76.92%) but with lesser relative density. Table 2 shows the fungal isolates from grape berries.

Felsöciová et al. (2017) isolated a total of 1377 cultures of microscopic fungi and the most abundant moulds were *Alternaria*, *Cladosporium* and *Penicillium*. The frequency found was similar to those in our study (100%). The higher frequency was detected for *Fusarium* (100%), *Epicoccum*, *Rhizopus* (87.5%), *Botrytis*, *Aspergillus* (75%) and *Mucor* (62.5%) but with lesser relative density. Authors found different genera of fungi with higher frequency in comparison with our study.

The *Aspergillus*, *Botrytis* and *Penicillium* strains were identified on species level and the isolation rate for *Aspergillus* was 76.92% but the relative densiy was low (20.42%, Table 2). Figure 1 shows the isolated microscopic filamentous fungi species.

Table 1 Number of microscopic filamentous fungi in grape varieties (log cfu.g^{-1}).

Grape variety	average	SD %
Alibernet	4.70	0.12
Blue Frankish	4.69	0.17
Cabernet Savignon	2.85	0.10
Dornfelder	4.15	0.03
Feteasca regala	4.83	0.07
Green Veltliner	4.63	0.02
Irsai Oliver	4.61	0.10
Mūller Thurgau	4.28	0.21
Pálava	4.09	0.02
Pinot Blanc	4.29	0.04
Savignon Blanc	4.32	0.07
Rheinriesling	4.41	0.05
Welschriesling	4.31	0.04

Table 2 Fungi identified in Slovak grape beries.

Fungal taxa	No.	Fr	RD
Alternaria	253	100.00	40.35
Aspergillus	128	76.92	20.42
Botrytis	142	53.85	22.65
Cladosporium	22	76.92	3.51
Penicillium	35	100.00	5.58
Phoma	12	15.38	1.91
Rhizopus	15	38.46	2.39
Trichoderma	20	15.38	3.19
Total isolates	627		

Note: No. – number of isolates, Fr – isolation frequency, RD – relative density.

B. cinerea, the fungus responsible for gray mold, was the most recurrent species isolated every year on grapes containing geosmin from all samples sites. This fungus is responsible for gray mold on many fruits, and notably grapes **(La Guerche et al., 2005)**. Several species of *Penicillium* were found in association with *B. cinerea: P. expansum, P. thomii, P. purpurogenum, P. glabrum, P. brevicompactum* and *P. carneum*. This fungal genus has already been described on grapes **(Abrunhosa et al. 2001)** and is responsible for blue mold.

In our study, 8 genera and 9 species of microscopic filamentous fungi were found. The grape rotting and spoilage can be caused by a variety of fungal species, including *Penicillium, Aspergillus, Alternaria, Cladosporium* and *Rhizopus*. *Aspergillus* and *Alternaria*, followed by *Penicillium*, were the most frequently reported genera on grapes. The genus *Penicillium* was more frequently found in temperate and cold climates typical for northern Europe, whereas *Aspergillus* was more frequently associated with warmer and wetter regions **(Serra et al., 2006)**.

Mikušová et al. (2010) identified the fungi in grapes of three out of six the most important Slovakia wine making areas – Small Carpathian, Nitrian and South Slovakian in harvest year the 2008. *Cladosporium*, *Epicoccum*, *Rhizopus*, *Ulocladium*, *Trichoderma* and *Trichothecium* were identified in range of 1 – 4%. The genera *Aspergillu*s (11.4%), *Fusarium* (11.4%), *Penicillium* (29.7%) and *Alternaria alternata* (14.8%) were considered to be predominant among the toxigenic fungi. These genera were the most frequently distributed also in our study, but the incidence was significantly higher and ranged from 75% to 100%. The results of **Mikušová et al. (2010)** showed that the relative density was lower and did not exceeded 2%, while this reached the limit of 34.3% in our study.

There were comparatively few species of *Penicillium* and *Aspergillus* identified in our research compared to those recorded previously during the vineyard sampling **(Rousseaux et al., 2014; Sage et al., 2004; Serra et al., 2005)**. The lower species diversity could be an outcome of several factors, such as high osmosis, low temperature and reduced water activity during withering. This could favour the selectivity of certain species and the prevalence of *Aspergillus* species in section *Nigri* and *Penicillium* in raisins and sun-dried grapes in relation to their growth response to water activity and temperature has been documented **(Romero et al., 2007; Valero et al., 2005)**.

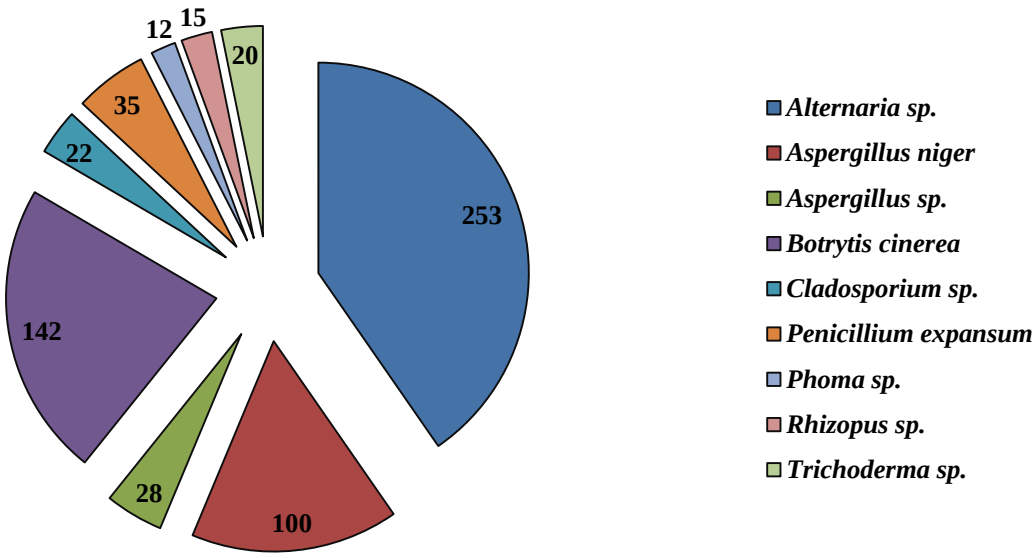

Figure 1 Number of isolated species of microscopic filamentous fungi.

Filamentous fungi were the main pathogens of withered grapes destined for passito wine production.**(Lorenzini et al., 2016)**.

The high prevalence of fungi from genera *Penicillium*, *Alternaria*, *Aspergillus* and *Botrytis* on withered grapes is the result of their generally high incidence on grapes in vineyards **(Rousseaux et al., 2014)**. The occurrence of common saprophytic fungi and their capacity to colonize berries change the fruit-drying room environment as compared with the field conditions, influencing the withering process **(Mencarelli and Tonutti, 2013)**.

Beside the most common necrotrophic-saprophytic species of *Penicillium*, *Aspergillus*, *Alternaria* and *Botrytis* species responsible for fruit rot, other identified saprobic species, e.g. *Trichoderma atroviride*, *Sarocladium terricola*, *Arthrinium arundinis* and *Diaporthe eres*, generally were not associated with post-harvest fruit diseases **(Lorenzini et al., 2016)**.

CONCLUSION

Alternaria and *Penicillium* were the most frequent genera of filamentous fungi found in the Lesser Carpathian wine region. The high frequency (100%) of those species may be attributed to the specific climatic conditions in particular wine-making region and the association with grapes on wineyards.

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