

SURVEY OF MYCOBIOTA ON SLOVAKIAN WINE GRAPES FROM SMALL
CARPATHIANS WINE REGION

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ABSTRACT

A total of 13 samples of grapes (bunches) without apparent fungal contamination were analyzed. The samples were collected during the 2019 harvest from Vrbové village in the Small Carpathian region of Slovakia. For the isolation of fungi were used the direct plating technique on DRBC plates. The plates were incubated aerobically at 25 ± 1 °C for one week in the dark. The data obtained from the cultivation of the grape berry samples revealed a high diversity of fungal species (a total of 1044 isolates were obtained). *Alternaria* and *Rhizopus* were the main components of the wine grape mycobiota of the Vrbovský subregion at harvest time (92%, each), followed by *Cladosporium* (85%), *Penicillium* (77%), *Botrytis* and *Epicoccum* (54%, each). The most abundant genera found by descending order were *Penicillium* (25%), *Alternaria* (24%), *Cladosporium* (20%), and *Rhizopus* (12%) and only in minor percentage by *Aspergillus* (3%) among others. The main fungal species isolated from genera *Penicillium* and *Aspergillus* were *Penicillium expansum* (57% RD) and *A. section Nigri* (97% RD). Of 17 analyzed *Penicillium* strains, 65% were able to produce at least one of the six mycotoxins analyzed in *in vitro* conditions by means of thin-layer chromatography method: citrinin, griseofulvin, patulin, cyclopiazonic acid, penitrem A, and roquefortin C.

Keywords: grape; filamentous fungi; *Penicillium*; mycotoxin; Slovakian vineyard

INTRODUCTION

Viticulture is an important activity in many countries (Einloft et al., 2017). Vine growing and viticulture have a very long tradition in Slovakia and are parts of the country's cultural and historical heritage. Hundreds of years of viticulture and viniculture have created a specific type of landscape (Bezák et al., 2010), with unique cultural and aesthetic values (Salašová and Štefunová, 2009). In total there exist six viticultural regions in Slovakia with forty areas and wine-growing villages (ÚKSÚP, 2019a). Slovakia features almost 660 producers growing around 13.500 ha of vines (of a potential 15.300 ha) (ÚKSÚP, 2019b) for a production of about 300.000 hL annually, which is primarily sold within the national market.

The microflora of the grapes is highly variable, mostly due to the influence of external factors as environmental parameters, geographical location, grape cultivars, and application of phytochemicals on the vineyards (Pretorius, 2000; Pinto et al., 2014). A variety of fungal genera, mainly *Botrytis*, *Alternaria*, *Aspergillus*, *Penicillium*, and *Cladosporium*, can contribute to grape spoilage before harvest (Bellí et al., 2006; Magnoli et al., 2003; Medina et al., 2005). Filamentous fungi impact negatively in the production, sensory quality, and safety characteristics of the wine in several ways. Their

development in wine grapes brings significant yield losses for winemaking, alters the chemical composition of wine grapes, and produces secondary fungal metabolites and enzymes that together adversely affect wine flavor and color as well as yeast and lactic acid bacteria growth during vinification (Fleet, 2003). Among them, it is of great concern the presence of toxicogenic fungi in wine grapes capable of producing mycotoxins that could persist during the winemaking process up to wine, being a high risk for consumer's health (Paterson et al., 2018; Prendes et al., 2015).

The genus *Alternaria* is ubiquitously distributed and includes both saprophytic and opportunistic plant-pathogenic species, which may affect crops in the field or cause harvest and postharvest decay of plant products. Moreover, several *Alternaria* species are known to produce toxic secondary metabolites, *Alternaria* mycotoxins. The major *Alternaria* mycotoxins are the tetramic acid derivate, tenuazonic acid, and the dibenzopyrone derivatives, alternariol (AOH), and alternariol monomethyl ether (AME) (Prendes et al., 2015). Despite the toxic effects of the *Alternaria* toxins and their documented occurrence, they have not yet received the same attention as others mycotoxins and up to now, there is no regulation about them (EFSA, 2011). As an opportunistic pathogen, it has the potential to cause a grape berry rot in the field under high disease pressure

situations. Strikingly, *Alternaria* has not been extensively studied in wine grapes as a hazardous genus.

Penicillium has gained attention as grapevine pathogens. *Penicillium expansum* can cause rot in grapes, but does not usually attack grapes before harvest. Aside from losses in fruit, this species is regarded as the major producer of patulin, although this species produces many other toxic metabolites such as citrinin, roquefortine C or chaetoglobosins among others (Andersen, Smedsgaard and Frisvad, 2004).

Scientific hypothesis

Some of the fungal species occurring on grapes and grape products can produce mycotoxins, so species identification is critical to predicting the potential mycotoxin contamination of grapes and wine.

MATERIAL AND METHODOLOGY

Study area

Village Vrbové is located in the Vrbovský subregion in the Small Carpathian wine region. The Small Carpathian wine region is the most extensive of the six wine regions in Slovakia (vineyards are covering 4175 hectares) and is located in the southwestern part of Slovakia (ÚKSÚP, 2019b). Vines have been grown on the south-facing slopes of the Small Carpathian mountains in locality Záhorie for more than three thousand years. This region has a medium climate and abundant moisture.

Last year, as a whole, was extremely warm. The year 2019 had the same average annual temperature in Hurbanovo 12.42 °C as in 2018. This value is a record high for Hurbanovo since the record began. During the whole year, 2019 was only one month of the territory temperature below normal. It was May (Beránek and Faško, 2020).

Grape sampling

A total of 13 samples were taken: 3 from red varieties (Alibernet, Cabernet Sauvignon, and Blaufränkisch) and 10 from white varieties (Palava, Green Veltliner, Seteasca Regala, Chardonnay, Rheinriesling, Welschriesling, Sauvignon, Pinot Blanc, Irsai Oliver, and Müller Thurgau). The sampling was conducted at the 2019 vintage, at the end of September. Two diagonals crossing the vineyards were delimited, and five healthy and undamaged bunches from each diagonal were obtained. Each bunch was collected in a sterilized plastic bag and sent to the laboratory chilled on ice.

Mycological analysis

Fifty berries were selected randomly from each sample (totaling 650 berries) and placed in Dichloran Rose Bengal Chloramphenicol agar (DRBC) (Samson et al., 2002). Plates were incubated at 25 ± 1 °C for 7 days in darkness. Genera identification was conducted according to microscopic and macroscopic criteria using the key of Pitt and Hocking (2009). *Aspergillus* strains were isolated and cultivated on MEA (Malt extract agar) (Samson et al., 2010), CYA (Czapek yeast extract agar) (Samson et al., 2010), and CY20S (Czapek yeast extract with 20% sucrose) (Pitt and Hocking, 2009). The *Aspergillus*

colonies were identified to species level according to micro and macroscopic criteria, using the keys of Klich (2002) and Pitt and Hocking (2009). *Penicillium* strains were isolated and cultivated on MEA, CYA, Creatine-Sucrose agar (CREA) (Samson et al., 2010) and Yeast Extract agar (YES) (Samson et al., 2010). The *Penicillium* colonies were identified to species level according to Pitt and Hocking (2009) and Samson and Frisvad (2004).

Mycotoxin production

Toxinogenicity of selected isolates was screened in *in vitro* conditions by means of thin-layer chromatography (TLC) according to Samson et al. (2002), modified by Labuda and Tančinová (2006). Extracellular metabolites – citrinin, griseofulvin and patulin were carried out on YES agar and intracellular cyclopiazonic acid, penitrem A, and roquefortin C on CYA agar. At 14 days of incubation, five agar plugs (4 mm diameter) were cut from the edge of a colony (extracellular metabolites) or cut from a colony (intracellular metabolites) from each Petri plate and placed in an Eppendorf tube. The plugs were extracted in 500 µL of chloroform-methanol (2:1, v/v) (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 extract of liquid phase 30 µL along with 10 µL of standards (Sigma, Germany) was transferred to the TLC plate (Alugram® SIL G, Macherey – Nagel, Germany). The plate was put into TEF solvent (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. The identification of the metabolites was done by comparison with metabolite standards. Cyclopiazonic acid was visible directly in daylight after spraying with the Ehrlich reagent as a violet-tailed spot. Penitrem A was visible after spraying with 20% AlCl₃ in 60% ethanol and heating at 130 °C for 8 min as a dark blue spot. Roquefortin C was visible after spraying with Ce(SO₄)₂ × 4 H₂O as an orange spot. Patulin detection was achieved by spraying with 0.5% methylbenzothiazolone hydrochloride (MBTH) (Merck, Germany) in methanol and heating at 130 °C for 8 min and then detected as a yellow-orange spot under visible light. Citrinin was detected directly as an intense yellow-green streak under ultraviolet light (365 nm) as well as griseofulvin, which was visible as a blue spot.

Statistical analysis

The obtained results were evaluated and expressed according to relative density (RD) and isolation frequency (Fr). The relative density (%) is defined as the percentage of isolates of the species or genus, occurring in the analyzed sample (Guatam, Sharma and Bhadauria, 2009). These values were calculated according to González et al. (1999) as follows:

$$RD (\%) = (ni/Ni) \times 100$$

where ni – number of isolates of a species or genus; Ni – total number of isolated fungi.

The isolation frequency (%) is defined as the percentage of samples within which the species or genus occurred at

least once. These values were calculated according to **González et al. (1999)** as follows:

Fr (%) = (ns/N) x 100; where ns – number of samples with a species or genus; N – total number of samples.

RESULTS AND DISCUSSION

Fifteen fungal genera were identified from the grape samples: *Alternaria*, *Aspergillus*, *Aureobasidium*, *Botrytis*, *Cladosporium*, *Epicoccum*, *Fusarium*, *Mucor*, *Penicillium*, *Phoma*, *Rhizopus*, *Syncephalastrum*, *Trichoderma*, *Trichothecium*, and *Ulocladium*. About 2 % of the isolated fungi did not produce conidiophores or conidia on the tested conditions and were nominated as non-sporulated fungi *Mycelia sterilia*. The number of isolates within the several genera found on grapes from different varieties are shown in Table 1. The highest number of isolates (from 101 to 199) with 7, 8, or 9 genera were isolated from varieties Müller Thurgau (13), Irsai Oliver (12), Blaufränkisch (3), Palava (4), and Alibernet (1). The lower number of isolates (26, 28, respectively) were isolated from the white variety Rheinriesling (8) with the number of genera 6 and Pinot Blanc (11) with the number of genera 4. It is interesting to note the absence of isolates belonging to microscopic filamentous fungi in one sample Green Veltliner (5). This wine grape was colonized only by yeasts. All samples (except sample 5) were colonized by genera *Alternaria* and *Rhizopus*. Genus *Alternaria* was dominated in samples Palava (4), Cabernet Sauvignon (2) and Welschriesling (9), genus *Rhizopus* in sample Alibernet (1), genus *Penicillium* in samples Müller Thurgau (13), Irsai Oliver (12), Blaufränkisch (3) and Welschriesling (9) and genus *Cladosporium* in samples Irsai Oliver (12) and Sauvignon (10).

Thirty of the 32 *Aspergillus* isolates were identified as *A. section Nigri* and 1 isolate as *A. ochraceus*. Sixteen black aspergilli were isolated from the Blaufränkisch variety (3), 6 from Cabernet Sauvignon (2), 5 from Irsai Oliver (12), 2 from Palava (4), and 1 from Chardonnay (7). Among section *Nigri*, *A. carbonarius* is considered the predominant species responsible for the occurrence of OTA in wine grapes and derivatives (**Ponsone et al., 2010; Visconti et al., 2008**). A low occurrence of this fungus was previously reported in Argentina (**Chiotta et al., 2009; Ponsone et al., 2010**), Brazilia (**Einloft et al., 2017**) and Lebanon (**El Houry et al., 2006**), and the absence of this fungus was observed in cold regions, like Germany, North Hungary, Czech Republic and Portugal (**Abrunhosa et al., 2001; Ostrý et al., 2007; Varga et al., 2005**).

Three species of *Penicillium* were isolated from grapes. Species *Penicillium expansum* were dominated from the Müller Thurgau variety (13), Blaufränkisch (3), Seteasca Regala (6), Rheinriesling (8), and Palava (4). *Penicillium expansum* has a high incidence in certain wine regions such as bordering regions of North Portugal and Galiza (Spain) (**Serra et al., 2006**). The incidence of *P. expansum* in some wine regions is high, but the attack of this fungus to vineyards, is rare, being *B. cinerea* the most common disease. **Morales et al. (2013)** observed that, *in vitro*, the

presence of *P. expansum* spores enhanced *B. cinerea* growth, while the latter avoided patulin accumulation.

The data in Table 2 obtained from the cultivation of the berry samples revealed a high diversity of fungal species (a total of 1044 isolates were obtained). *Alternaria* and *Rhizopus* were the most frequently occurring genera (92%, each), followed by *Cladosporium* (85%), *Penicillium* (77%), *Botrytis*, and *Epicoccum* (54%, each). *Penicillium* spp. was predominant in terms of relative abundance (25%), followed by *Alternaria* (24%), *Cladosporium* (20%), *Rhizopus* (12%), and *Botrytis* (6%). Besides, a minor portion (<5%) of *Aspergillus* and other genera was found.

Alternaria genus was the main component of the wine grape mycobiota of the Vrbovský subregion (Small Carpathian wine-growing region) at harvest time, which is in agreement with previous studies carried out in several winemaking regions worldwide, e.g. from Uruguay (**Garmendia and Vero, 2016**), Argentina (**Magnoli et al., 2003; Prendes et al., 2015**), Spain (**Medina et al., 2005**), Slovakia (**Felšöciová et al., 2015c; Felšöciová, Mašková and Kačániová, 2018; Felšöciová and Kačániová, 2019a; Felšöciová and Kačániová, 2019b**).

It was followed by *Penicillium*, which recorded a frequency of 77% and a high relative density of 25%. From the previous study by **Felšöciová and Kačániová (2019a)**, *Penicillium* contributed a small proportion (21% Fr, <1% RD) from mycobiota associated with grapevine in Vrbové. The *Botrytis* genus, which is regarded as the main spoilage cause in wine grapes, was isolated in this study, but the absence of this genus has already been reported by **Magnoli et al. (2003)** in Argentina, and **Medina et al. (2005)** in Spain. Grey mold *Botrytis cinerea* is responsible for severe economic loss. Musts obtained from botrytized grapes are more liable to oxidation because of the polyphenol oxidizing activity of *B. cinerea* laccase and are not suitable for wine production (**Morales et al., 2013**).

Aspergillus was one the less common genera (46% Fr, 3 % of all fungi). These results differ from those obtained by other authors, who reported a much higher frequency from this genus, ranging from 70% to 95% (**El Houry et al., 2008; Magnoli et al., 2003; Medina et al., 2005**).

Data in Table 3 show that, 32 *Aspergillus* species were identified from grape samples. The section *Nigri* was predominant within the *Aspergillus* genus, representing 94% of species isolated from this genus with 38% frequency. Certainly, the *Aspergillus* species are present worldwide, in all the grape products and under all environmental conditions (**Somma, Perrone and Logrieco, 2012**). From the 12 vineyards in the Small Carpathian area (14 samples), 79% of the samples were colonized by the genus *Aspergillus* (**Felšöciová et al., 2015c**). During the 3 years survey (2011, 2012, and 2013), 37 isolates belonging to 7 *Aspergillus* species (*A. clavatus*, *A. flavus*, *A. section Nigri*, *A. ostianus*, *A. parasiticus*, *A. versicolor* and *A. westerdijkiae*) were isolated. The main occurring *Aspergillus* species of the samples were *A. section Nigri* (64%), as in our research. On the other hand, the most species were not been isolated from any of the samples analyzed in the present study.

Table 1 Fungi identified in Slovak wine grapes from exogenous mycobiota in 2019 by direct plating method.

Fungal taxa	Grape varieties												
	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.
<i>Alternaria</i>	17	28	36	77	-	13	10	7	25	7	2	4	26
<i>Aspergillus</i>	-	7	16	2	-	-	1	1	-	-	-	5	-
<i>A. ochraceus</i>	-	1	-	-	-	-	-	-	-	-	-	-	-
<i>A. section Nigri</i>	-	6	16	2	-	-	1	-	-	-	-	5	-
<i>A. sp.</i>	-	-	-	-	-	-	-	1	-	-	-	-	-
<i>Aureobasidium</i>	-	-	-	-	-	-	-	-	-	-	-	-	1
<i>Botrytis</i>	17	8	-	-	-	-	10	-	-	7	6	4	12
<i>Cladosporium</i>	14	7	14	2	-	5	5	-	6	22	9	67	55
<i>Epicoccum</i>	5	3	1	-	-	3	-	-	-	1	-	6	5
<i>Fusarium</i>	2	-	2	12	-	-	-	-	-	1	-	-	-
<i>Mucor</i>	-	4	-	3	-	1	4	2	-	-	-	3	-
<i>Penicillium</i>	19	5	48	5	-	12	-	10	23	1	-	51	90
<i>P. crustosum</i>	-	-	-	-	-	-	-	-	-	-	-	-	1
<i>P. expansum</i>	-	-	41	5	-	12	-	10	6	-	-	15	61
<i>P. griseofulvum</i>	-	-	-	-	-	-	-	-	-	-	-	10	-
<i>P. sp.</i>	19	5	7	-	-	-	-	-	17	1	-	26	28
<i>Phoma</i>	1	-	-	-	-	-	1	-	1	-	-	-	-
<i>Rhizopus</i>	26	23	6	6	-	6	2	1	15	3	9	18	8
<i>Syncephalastrum</i>	-	-	-	-	-	-	-	-	-	1	-	-	-
<i>Trichoderma</i>	-	-	1	-	-	2	5	-	-	-	-	1	2
<i>Trichothecium</i>	-	-	-	-	-	-	-	1	-	-	-	-	-
<i>Ulocladium</i>	-	-	-	2	-	1	-	-	-	-	-	-	-
<i>Mycelia sterilia</i>	-	-	-	-	-	9	4	4	2	2	2	2	-
Total isolates	101	85	124	109	-	52	42	26	72	45	28	161	199

Note: 1. Alibernet, 2. Cabernet Sauvignon, 3. Blaufränkisch, 4. Palava, 5. Green Veltliner, 6. Seteasca Regala, 7. Chardonnay, 8. Rheinriesling, 9. Welschriesling, 10. Sauvignon, 11. Pinot Blanc, 12. Irsai Oliver, 13. Müller Thurgau.

Table 2 The occurrence, isolation frequency and relative density of filamentous microscopic fungi in surface mycobiota of grapes (n = 13) harvested in Small Carpathian region.

Fungal taxa	No.	Fr (%)	RD (%)
<i>Alternaria</i>	252	92	24
<i>Aspergillus</i>	32	46	3
<i>Aureobasidium</i>	1	8	<1
<i>Botrytis</i>	64	54	6
<i>Cladosporium</i>	206	85	20
<i>Epicoccum</i>	24	54	2
<i>Fusarium</i>	17	31	2
<i>Mucor</i>	17	46	2
<i>Penicillium</i>	264	77	25
<i>Phoma</i>	3	23	<1
<i>Rhizopus</i>	123	92	12
<i>Syncephalastrum</i>	1	8	<1
<i>Trichoderma</i>	11	38	1
<i>Trichothecium</i>	1	8	<1
<i>Ulocladium</i>	3	15	<1
<i>Mycelia sterilia</i>	25	54	2
Total isolates	1044		

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

Table 3 The occurrence, isolation frequency and relative density of *Aspergillus* species in surface mycobiota of grapes (n = 13) harvested in Small Carpathian region.

<i>Aspergillus</i> species	No.	Fr (%)	RD (%)
<i>A. ochraceus</i>	1	8	3
<i>A. section Nigri</i>	30	38	94
<i>A. sp.</i>	1	8	3
Total isolates	32		

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

Table 4 The occurrence, isolation frequency and relative density of *Penicillium* species in surface mycobiota of grapes (n = 13) harvested in Small Carpathian region.

<i>Penicillium</i> species	No.	Fr (%)	RD (%)
<i>P. crustosum</i>	1	8	<1
<i>P. expansum</i>	150	54	57
<i>P. griseofulvum</i>	10	8	4
<i>P. sp.</i>	103	54	39
Total isolates	264		

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

Table 5 Toxinogenicity of selected *Penicillium* strains, isolated from exogenous mycobiota of wine grapes.

Species	C	G	P	CA	PA	RC
<i>P. crustosum</i>					1/1	0*/1**
<i>P. expansum</i>	13/14		11/14			9/14
<i>P. griseofulvum</i>		2/2	2/2	1/2		1/2

Note: * – number of isolates with ability to produce mycotoxin, ** - number of tested isolates, C – citrinin, G – griseofulvin, P – patulin, CA – cyclopiazonic acid, PA – penitrem A, RC – roquefortin C.

Data in Table 4 show 3 different *Penicillium* species from the 264 fungal strains. *Penicillium expansum* was predominant within the *Penicillium* species, representing 57% of the isolates and 54% frequency, which agrees with previous publications by Felšöciová and Kačániová (2019a), Felšöciová and Kačániová (2019b). The predominant species of *Penicillium* isolated from grapes differs between vineyards. For example, *Penicillium expansum* is the species most frequently isolated in South Slovak wine region (28% RD) (Felšöciová et al., 2017), *P. aurantiogriseum* in East Slovak wine region (34% RD) (Felšöciová et al., 2015a), *P. chrysogenum* in Small Carpathian wine region (64% RD) (Felšöciová et al., 2015c), in Nitra wine region (28% RD) (Felšöciová et al., 2013), in Central Slovak wine region (53% RD) (Felšöciová et al., 2014) and Tokaj (39% RD) (Felšöciová et al., 2015b). *Penicillium expansum* was found frequently in botrytized grapes (Morales-Valle et al., 2011). This species was the second frequent (after *P. chrysogenum*) in Tokaj (33% RD).

The toxicogenic profile of the 17 *Penicillium* isolates representing *P. crustosum*, *P. expansum* and *P. griseofulvum* from the Slovak grapes is shown in Table 5.

The 65% of the 17 analyzed *Penicillium* strains were able to produce at least one of the six mycotoxins tested (citrinin, griseofulvin, patulin, cyclopiazonic acid, penitrem A, and roquefortin C). Citrinin was the toxin produced by the majority of the strains *P. expansum* (93%). It was followed by patulin produced by 79% of the strains *P. expansum*, and roquefortin C produced by 64% of the strains. *Penicillium crustosum* produced only penitrem A, did not produce roquefortin C. Two strains of *Penicillium griseofulvum* produced griseofulvin and patulin, the production of cyclopiazonic acid and roquefortin C was confirmed by one isolate.

Almost 100% of *Penicillium expansum* strains are patulin producers (Andersen, Smedsgaard and Frisvad, 2004; Morales et al., 2008), which does not fully correspond to our results. *Penicillium expansum* is commonly associated with apple rot, production of geosmin – a well-known compound with a strong earthy smell, and patulin contamination in apple derivatives (Morales-Valle et al., 2011). However, patulin has been reported in grapes (Moake, Padilla-Zakour and Worobo, 2005), processed grape juice (Scott, Fuleki and Harwig, 1977), and fermenting wine (Majerus, Hain and Kölb, 2008; Bragulat, Abarca and Cabañes, 2008), although the occurrence in wine is low because it is well-known to be degraded partially by the fermentation process (Moss and Long, 2002). Patulin mainly induces gastrointestinal disorders including ulceration, distension, and bleeding. The compound provokes congestion and oedema of pulmonary, hepatic, and gastrointestinal blood vessels and tissues. Subcutaneous injection of patulin produced local sarcomas in rats and is classified in group 3 as not classifiable as to its carcinogenicity to human by IARC (Varga et al., 2015).

CONCLUSION

Our results indicate a high diversity of fungal species with a high incidence of *Alternaria* genus. Out of 17 potentially toxigenic *Penicillium* strains isolated from exogenous mycobiota, namely *P. crustosum*, *P. expansum* and *P. griseofulvum*, 65% produced at least one mycotoxin by thin-layer chromatography method. The occurrence of the potentially toxigenic fungus *Aspergillus* was overall very low what indicates the high quality of the wine grapes produced in Slovakia.

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