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Original Article

Berries contamination by microfungi in Slovakia vineyard regions: Impact of climate conditions on microfungi biodiversity

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ABSTRACT

Background: Weather conditions can selectively promote the growth of particular fungal species, which cause rotting and spoilage of grape berries before harvest. The presence of pathogenic fungi can lead to the development of opportunist microfungi that can produce mycotoxins and cause grapes and wine contamination.
Objective: The variation of climatic conditions allowed to design a pilot study and address relevant questions for risk assessment of climate related mycotoxins production in grapes and in wine.
Methods: Microfungi contaminating berries during the vegetative period of year 2008 and 2009 in vineyards regions of Slovakia at the early veraison and at the ripening in harvest time have been identified.
Results: Spoilage fungi were more abundant in veraison for both years in all the studied geographical regions, with an average temperature of 20 °C, humidity between 60 and 80%, and precipitation in the range 6–5 mm. Much more strains of toxigenic fungi were found during veraison and ripening in the case of a year temperature variation in the range 17–22.5 °C, humidity values ranging from 58 to 80% and precipitations in the range 0.7–8.4 mm.
Conclusions: The results suggest that there is an effect of the climatic conditions on microfungi biodiversity.

Contaminación de las bayas por hongos en los viñedos de Eslovaquia: influencia de las condiciones climáticas en la biodiversidad de los hongos

Palabras clave:
Viñedos
Uva
Eslovaquia
Hongos toxigénicos
Hongos no toxigénicos
Fusariotoxinas
Ocratoxina

RESUMEN

Fundamento: Las condiciones climáticas pueden favorecer selectivamente el crecimiento de determinadas especies de hongos que causan la podredumbre y el deterioro de las bayas antes de la vendimia. La presencia de hongos patógenos puede dar lugar al crecimiento secundario de hongos oportunistas que pueden producir micotoxinas y causar contaminación tanto de la uva como del vino.
Objetivo: La variación de las condiciones climáticas nos permitió el diseño de un estudio piloto, así como abordar las cuestiones pertinentes para la evaluación de los riesgos de producción de micotoxinas relacionadas con el clima en la uva y en el vino.
Métodos: Durante el período vegetativo del año 2008 y 2009, en las regiones de viñedos de Eslovaquia, se han identificado hongos contaminantes de bayas al inicio del envero y durante la maduración y la vendimia.
Resultados: En todas las regiones geográficas estudiadas, los hongos toxigénicos fueron más abundantes en el período de envero de ambos años, con una temperatura media de 20° C, una humedad del 60-80%, y una precipitación de 5-6 mm. Durante el período de envero y de maduración, se detectó un mayor número de cepas de hongos productores de toxina en el caso de una variación anual de la temperatura de 17 a 22,5 °C, de la humedad del 58 al 80% y de la precipitación de 0,7 a 8,4 mm.
Conclusiones: Los resultados del presente estudio sugieren que las condiciones climáticas producen efectos sobre la biodiversidad de los hongos.

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Slovakia is the northern boundary of the grape-growing areas for wine production in Europe, and its geographical location presents advantages as well as some disadvantages, since the northern areas of vine ripe are different from the southern areas of Europe. In Spain and Italy for example, that are among the main wine producers countries in Europe, complaints are raised because wines often do not reach a complex aromatic structure. This could be attributed to the relatively high temperature and weather condition contributing to the fast grapes maturation and to the berries aroma release. Climatic conditions in Slovakia are relatively moderate during the critical stages of maturation and harvesting of the grapes, as it can be common in the Middle Europe areas.

Weather conditions may be different from one season to another, selectively promoting the growth of particular moulds.[17] Cooler temperatures and high relative humidity and/or moisture are suitable conditions for the development of fungal species, which cause rotting and spoilage of grape berries before harvest. This situation represents a serious phytopathological problem, especially for the operators in the wine.[25]

The presence of pathogenic fungi can lead to the development of opportunist microfungi like *Aspergillus* and other moulds that can produce mycotoxins and cause grapes and, consequently, wine contamination.[24] Grapes that are heavily infected with fungi alter their chemical composition and their enzymes content, adversely affecting wine flavour and colour and causing the growth of yeasts during alcoholic fermentation.[9] The potential mycotoxigenic fungi grown on/in grapes or grape musts are used for grape juices or wine production and the related exposure risks should also be evaluated.[3] After the first detection of mycotoxins in wine[29] several studies, particularly in Europe,[10] were conducted. The climatic conditions are considered a critical factor for the mycotoxins onset, and a clear relationship between geographical location and toxins accumulation in red wines has been demonstrated in Germany,[16] Italy,[18] and Greece.[26] Several factors, e.g. agronomic and genetic, can influence the qualitative and quantitative variability of the mycotoxins present in the grapes and can contaminate not only the wine, but also other grape based foodstuffs, e.g. raisins.[12]

In this work, for the first time, the native microfungi in Slovak vineyards have been identified. Two vegetative seasons have been evaluated, namely during the year 2008 and 2009. The variation of climatic conditions allowed to design a pilot study to address relevant questions for risk assessment of climate related mycotoxins production in grapes and consequently in wine.

Material and methods

Study area

Three winemaking regions ca. 69 km^2 (out of a total 89 km^2) were chosen for this study based on their climatic differences and national economical importance, in particular: Little Carpathian: Modra, Šenkvice, Pezinok (HMD-Slovenský Grob); Nitria: Malanta, Hrnčiarovce, Nitra (HMD in Nitra-Janíkovce); South Slovakian region: Radošina, Galanta, Kralova (HMD Žiharec). A total of 9 localities and 18 vineyard areas were investigated, in each year, during the 2008 and 2009 seasons. Meteorological data were considered and, in addition, average values for each season were also determined. Meteorological data for the two weeks before taking samples at the stage of veraison and ripening have been also considered, considering that these information could have been relevant for the inoculums to be firmly attached to the grapes.

Ecophysiological factors, identified as important during the growing season, have been considered during two steps of berries growth in a two year-period of time. The source of meteorological data has been the Hydro Meteorological Data (HMD) for each locality of the three regions where grape samples were collected, namely Little Carpathian, Nitria, and South Slovakian. Average meteorological seasonal data were considered, and also values of meteorological data in the two weeks before samples collection (veraison and ripening) were taken into account.

Sample collection

The grape berries used for this work have been sampled according to methods suggested for other grapes and wine producing countries.[4,5] Grapes were collected twice during the year: early veraison in July, and ripening before harvest in September for the years 2008 and 2009. After each sampling and in each vineyard, samples were taken to the laboratory in sealed paper bags, transported in cooled boxes, and analysed within 24 h from the collection. Each sample from different locations represents one occurrence of fungi.

Mycological analysis of the grapes

From each bunch, 10 berries were randomly selected, cut in half and aseptically plated on Czapek Dox Agar medium, CDA, (Biomark Laboratories, Pune, India). For each sampling period, a total of 50 berries were collected per vineyard and, as reference, a mixed berries sample was pressed against a plate of the same medium, in order to compare the fungal species isolated in the two sample collection periods. The plates were incubated for 7 days at 25 °C.[19] From the second day of incubation, the plates were monitored with a stereo microscope for the presence of toxigenic moulds and other species. When the moulds were present in a grape sample, they were re-isolated on CDA and cultivated for seven days for *Aspergillum* and *Penicillium*, and from 10 to 14 days at a temperature of 25(±2) °C for the other fungi species.

Staining and microscopic identification of fungi

For a first microscope observation, cotton blue with lactophenol was used. Fungi have been identified according to the spore, measure of the spore and shape and colour of the culture. In doubtful cases, the form of spore was used as a discriminating criterium. The identification of the fungi has been made according to suggested procedures and methods.[15,20,21,27]

Preservation of the isolated strains

All the fungi and representative strains of the moulds found were preserved as a suspension of spores in a 10% glycerol solution and maintained at a constant temperature of −8 °C. Fifteen strains were stored in the collection of agro-food important toxigenic fungi at the Institute of Sciences of Food Production (ISPA), of the National Research Council (CNR) in Bari, Italy. This Institute is part of the World Federation for Culture Collection.

Results and discussion

Occurrence of spoilage fungi

Comparing average meteorological data between single regions during the year 2008, the temperature fluctuated from 19 to 21.5 °C in all regions as shown in Fig. 1. Small Carpathian region and South Slovakia region had a similar humidity value (ranging between 60 and 71%), while the Nitrian region had humidity values from 58 to 66%. Concerning precipitations data, a descending order 0.7, 3, and 6 mm was observed for South Slovakia, Small Carpathian

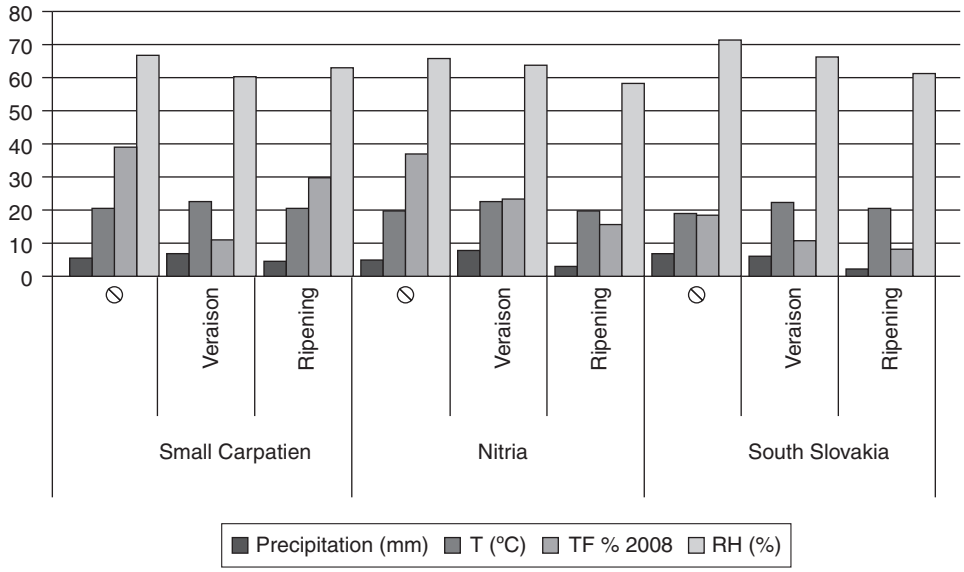

Fig. 1. Weather parameters, year 2008: precipitation (mm), temperature (*T*, °C), humidity (*H*, %), and number of toxigenic fungi (TF). Average data, taken during the vegetative season, and two weeks before sampling, are reported. Data collected by Hydro Meteorological Station (HMD) from Small Carpatien; HMD from Slovenský Grob, Nitria; HMD from Nitra-Velké Janíkovice, South Slovakia; HMD from Žiharec.

and Nitrian regions, respectively. In the year 2009, as shown in Fig. 2, the South Slovakia region was the warmest one with an average temperature of 22.5 °C when comparing with the Carpathian region, where a temperature of 17 °C was measured. Accordingly, relative humidity and precipitation were in descending order from Small Carpathian region, South Slovakia, and Nitrian region, with values of 81, 68 and 67%, respectively.

Non-toxigenic fungi that cause the disease particularly at the stage of veraison, may affect wine the most. The development of grape fungal diseases that can cause rots such as *Plasmopara*, *Uncinula*, *Botrytis*, *Coniella*, *Guinardia*, *Elsinoë* and *Saccharomycetes* sp., as shown in Table 1, were frequent in veraison, and occurred in both years with an about 75% percentage. *Botrytis*, *Coniella*, *Rosellinia* and *Sacharomycetes* sp., occurred during the harvest time in the year 2009 for the 25%, as shown in Table 1. These infections are well recognised and they cause sporadic damage in seasons when conditions like cooler temperature and more humidity in July, and before harvesting stage in September, can favour the disease. In this case, fungicides should be used. However, changes in berries due to fungal infection may, in turn, alter the properties of the wine,[8] while fungicides may change the aroma of the wine.[8]

Toxigenic microfungi occurrence

The number of toxigenic microorganisms of grapes is important to determine since it is considered a relevant indicator for the presence of mycotoxins on the grapes. Our study of microfungi revealed that toxigenic fungi occur in all vineyards of the 69 km^2 of land out of the total 89 km^2 intensively cultivated with grapes in Slovakia. Comparing sampling made during the year 2008, when 93 strains of toxigenic fungi *Aspergillus*, *Penicillium*, *Alternaria*, and *Fusarium* were identified, and year 2009, when 57 strains were observed, it can be observed that much more strains were found in Small Carpathian region during harvesting, and only in 2009. On the contrary strains were observed during the early veraison in the other regions and year.

In particular, as shown in Table 2, the *Aspergillus* section *Nigri*, "black aspergillosis",[5] was isolated with a relatively high frequency. Ochratoxin A producers[4] were isolated in 24.74% of the cases during the 2008, and only one species in the year 2009, accounting for the 7.01%. The higher presence of *Aspergillus carbonarius* (9.68%) was observed only in year 2008.

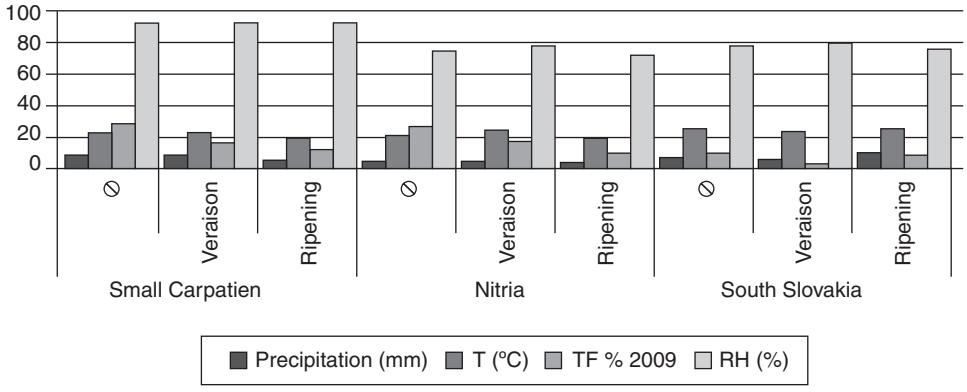

Fig. 2. Weather parameters, year 2009: precipitation (mm), temperature (*T*, °C), humidity (*H*, %), and number of toxigenic fungi (TF). Average data, taken during the vegetative season, and two weeks before sampling, are reported. Data collected by Hydro Meteorological Station (HMD) from Small Carpatien; HMD from Slovenský Grob, Nitria; HMD from Nitra-Velké Janíkovice, South Slovakia; HMD from Žiharec.

Table 1
Spoilage occurrence percentage and potentially non-toxigenic fungi sampled twice during the year 2008 and 2009.

Potential non-toxigenic species	% veraison		% harvest		% total	
	2008	2009	2008	2009	2008	2009
Botrytis cinerea Pers.	9.52	3.33	14.29	0	23.8	3.33
Coniella diplodiella (Speg.) Petr. & Syd.	11.11	16.67	4.76	0	15.87	16.67
Elsinoë ampelina Shear	3.17	0	0	0	3.17	0
Guignardia bidwelli (Ellis) Viala & Ravaz	12.07	6.67	0	0	12.07	6.67
Plasmopara viticola (Berk. & M.A. Curtis) Berl. & De Toni	19.04	20	0	0	19.04	20
Saccharomycetes sp.	1.59	8.33	6.35	16.67	7.94	25
Rosellinia necatrix Berl. ex Prill.	0	15	0	8.33	0	23.33
Uncinula necator (Schwein.) Burrill	17.46	5	0	0	17.46	5
Total	74.6	75	25.4	25	100%	100%

A novel species present in five isolates was identified in the 2008 season as *Aspergillus uvarum* sp. nov., described within *Aspergillus* section *Nigri*. This species present in the 5.37% of the samples collected during the year 2008 can be distinguished from other black aspergilli, based on internal transcribed spacers (ITS).[17] *A. uvarum* sp. nov. produces secalonic acid, geodin, erdin, and dihydrogeodin, that are not produced by any other *black Aspergillus* producer.

The isolates of Aspergillus flavus produce *in vitro* small amounts of aflatoxin.[7] The largest source of wine grapes contamination is the raw material, so its health conditions are a very important factor. In fact, the ochratoxin-producing fungi can easily colonize the damaged grapes before harvest and easily at the end of the vegetative session and then produce and accumulate ochratoxin A.[1] The concentration of ochratoxin A in wine, in the 2005 harvest, were well below the proposed European limit of 2 mg L^{-1}.[2] According to these authors, little changes in the concentration of ochratoxin A were found and recorded during the 2006 winemaking season in Slovakia. This was observed in very rainy years, nevertheless there is a need to monitor the rain levels every year, since they may be very different from one year to another. In our study, for both years, in veraison and in harvest time, the same rain amount was observed. During harvest time, in year 2009, only one *Aspergillus niger* was identified, probably because of high temperature and high humidity measured in South Slovak region. *Penicillium* sp. represents a high percentage in microfungi, 29.5% in the year 2008 and 28.05% in 2009. In the 2008 season, small differences between the Nitrian and Little Carpathien regions were observed, and, as expected, differences have been observed for regions with warmer weather conditions like South Slovakia at the time of harvesting. Data represented in Fig. 1 and reported in Table 2 confirm these observations. For example, *Penicillium expansum*, a well described citrinin and patulin producer,[13,23,28] is a common fungus in all samples before the harvest. *Penicillium glabrum*, *Penicillium citrinum* and *Penicillium verrucosum*, ochratoxin producers, are present in samples collected in rainy days during early veraison in July for both years.

In the year 2009, *Penicillium* spp., as shown in Fig. 2 and Table 2, were present only in two samples, and significant changes in the number of species could be observed. In the veraison time, only two species were present, namely *Penicillium crustosum* and *Penicillium*

Table 2
Spoilage occurrence percentage and potentially toxigenic fungi sampled twice during the year 2008 and 2009.

Potentially toxigenic species	% veraison		% harvest		% total	
	2008	2009	2008	2009	2008	2009
Aspergillus carbonarius Bainier	6.45	0	3.23	0	9.68	0
Aspergillus niger van Tieghem	5.38	3.5	3.23	3.5	8.6	7.01
Aspergillus uvarum Perrone. Varga et Kozakiewicz sp. nov.	4.3	0	1.08	0	5.37	0
Aspergillus flavus Link	0	0	1.08	0	1.08	0
Alternaria alternata (Fr.) Keissl.	6.45	8.77	7.53	5.26	13.98	14.04
Alternaria tenuissima (Kunze) Wiltshire	1.08	5.26	1.08	0	2.15	5.26
Cladosporium cladosporioides (Fresen) G.A. de Vries	0	7.01	2.15	3.5	2.15	10.53
Epicoccum nigrum Link	0	0	1.08	0	1.08	0
Fusarium oxysporum Schlecht	0	5.26	1.08	1.75	1.08	7.01
Fusarium proliferatum Matsushima	0	1.75	1.08	3.5	1.08	5.26
Fusarium semitectum Wollenweber	2.15	0	1.08	0	3.23	0
Fusarium solani (Mart.) Sacc.	0	0	1.08	0	1.08	0
Fusarium subglutinans Wolenweber & Reinking	2.15	0	0	0	2.15	0
Fusarium verticillioides Sacc.	1.08	7.01	1.08	0	2.15	7.01
Penicillium brevicompactum Dierckx	0	0	0	1.75	0	1.75
Penicillium chrysogenum Thom	0	0	0	1.75	0	1.75
Penicillium citrinum Thom	2.16	0	2.15	0	4.31	0
Penicillium crustosum Thom	0	1.75	1.08	3.5	1.08	5.26
Penicillium expansum Link	0	0	21.5	14.04	21.5	14.04
Penicillium glabrum (Wehmer) Westling	1.08	0	0	0	1.08	0
Penicillium palitans Westling	0	0	0	1.75	0	1.75
Penicillium polonicum K. Zaleski	0	1.75	0	1.75	0	3.5
Penicillium verrucosum Dierckx	0	0	1.08	0	1.08	0
Rhizopus stolonifer (Ehrenb.Fr.) Lindner	3.23	1.75	0	1.75	3.23	3.5
Ulocladium chartarum (Preuss) E.G. Simmons	4.3	7.01	3.23	0	7.53	7.01
Trichoderma atroviride P. Karst	4.3	3.5	0	0	4.3	3.5
Trichothecium rosem (Pers.) Link	0	0	1.08	1.75	1.08	1.75
Total	44	54.5	56	45.5	100%	100%

polonicum, with a relatively low frequency (2.16%). During the harvest time, a number of *Penicillium* strains belonging to species with ability to produce mycotoxins (*P. crustosum*, *P. expansum*, *Penicillium brevicompactum*, *Penicillium chrysogenum*, *Penicillium palitans*, *P. polonicum*) were present with a high frequency (24.5%). The most abundant *Penicillium* species, producer of citrinin and ochratoxins, were observed in regions with higher precipitations, and with high humidity, compared to Small Carpathien, where low precipitation occurred, as can be seen in Figs. 1 and 2.

Fusarium fungi were more frequently identified. Data relative to the identification of this species are found anywhere in the Mediterranean areas: the fusariotoxin fumonisin B2 was present in black *Aspergillus* and its natural occurrence has been observed in must from grape berries.[11] In recent years, a widespread occurrence of the mycotoxin fumonisin B_2 in wine has been reported.[14] *Fusarium* fungi in a quantity from 11% up to 19% were present at both sampling times. More fungi were present during the ripening time, in year 2009, when higher humidity values were observed.

The occurrence of strains of *Alternaria* isolates, present in a range from 16 to 19%, which are capable of producing a range of secondary metabolites harmful for plants, animals and humans[6] is also remarkable. The strains were represented by species *Alternaria alternata* and *Alternaria tenuissima*, in both stadium and years of sampling. Species *Trichothecium roseum* present only from 1.08 to 1.75% of the cases at the harvest time, is another mould that causes bitter rot on apples, while on grapes, it appears as a secondary infection, after *Botrytis cinerea*. It is able to produce mycotoxins, trichothecenes, trichotecolon, and rosenon. Even if present in a quantity of only 2.1 mg L^{-1}, trichothecenes can inhibit alcohol fermentation remaining unchanged.[22]

Table 2 shows also that the potential toxigenic genera *Trichoderma* is represented only by one species, *Trichoderma atroviride*, and only in the veraison time. Other toxigenic species, e.g. *Cladosporium cladosporioides*, *Epicoccum nigrum*, *Rhizopus stolonifer*, *Ulocladium chartarum*, *Trichothecium roseum*, were present, but with a low incidence; only *C. cladosporioides* was present in a higher amount (10.50%) and only in the year 2009.

The suitability of berries sampling stadium

Comparing average meteorological data in the two weeks before the berries veraison stadium, it can be observed that the temperature was approximately the same for the two years 2008 and 2009. Although precipitation was higher in 2008, relative humidity had a higher value in year 2009. Comparing the meteorological data during ripening in harvest time, the temperature was different in each region in an average range of $\geq 5\,^{\circ}C$. During two weeks at the time of ripening, the average higher humidity percentage and more precipitation have been measured in the South Slovakia region, with differences being ≥ 1 and up to 8 mm. Considering the occurrence of both type of fungi, spoilage fungi were much more severe in veraison time and it is possible to correlate the number of toxigenic fungi to the average meteorological data of the season in each region and to the time of ripening or to the time of veraison. They occurred more frequently in one region during harvesting time and, in other regions, in the veraison but always where there was an average higher temperature and a higher humidity during the two weeks before the sampling.

Conclusions

The occurrence of spoilage fungi was common in all regions where sampling has been made, and high in the stadium of early veraison for the berries. Representation of potentially toxigenic species was high and higher in first year of study, 2008, while minor in the year 2009, when climatic data changed during berries harvesting stadium. Toxigenic species represent a small amount in regions like South Slovakia where, on the average, higher temperature values have been observed. With respect to the occurrence of toxigenic fungi, no dependency in each stage of sampling from the climatic conditions has been found, but the average values of all three parameters, temperature, humidity, and precipitations in the course of the season are important. Much more strains of toxigenic fungi were observed in the veraison and ripening, when during the year temperature fluctuated from 17 to 22.5 °C, humidity from 58 to 80%, and precipitations from 0.7 to 8.4 mm. The most important mycotoxin-producing fungi were *Aspergillus* (*A. carbonarius*, *A. niger*, *A. uvarum*, *Aspergillus citrinum*, and *A. flavus)* contributing for about 33%, while *Fusarium* fungi (*Fusarium semitectum*, *Fusarium subglutinans*, *Fusarium verticillioides*, *Fusarium oxysporum*, *Fusarium proliferatum*, *Fusarium solani*) constituted up to the 19% of all the toxigenic fungi. The genera *Penicillium* (present in the range 27–54%) was predominant in harvest time, and it was represented by *P. brevicompactum*, *P. chrysogenum*, *P. crustosum*, *P. expansum*, P. palitans, P. polonicum, P. verrucosum, P. citrinum, and P. glabrum.

Alternaria accounted by itself for the 21%. Other potential toxigenic species, e.g. *Trichoderma*, *Cladosporium*, *Epicoccum*, *Rhizopus*, *Ulocladium*, *Trichothecium* were present, but with a minor contribution.

This could indicate that the danger connected to the occurrence of toxins is common, and that the microfungi presence at the stage of ripening always needs to be evaluated. Moreover, it should be stressed that a very high percentage of *Fusarium* fungi has been identified, but the presence of fusariotoxins has been observed only sporadically. These observations could be helpful in evaluating the danger and the risk related to grape contamination and risk assessment also for other countries that have climate conditions similar to Slovakia.

Conflict of interest

Authors have no conflict of interest.

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