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Survey of viable airborne fungi in wine cellars of Tokaj, Hungary

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Abstract The composition of fungal biota and air quality of five traditional subterranean wine cellars and one store building of a modern wine production facility were examined in the Tokaj wine region (northeastern Hungary). Air samples were collected with SAS IAQ sampler onto PDA, MEA and RBA. Strains representing morphotypes were isolated from colonies formed on agar plates from either air or surface samples. The internal transcribed spacer (ITS) region of the rRNA gene cluster was amplified with primers ITS1 and ITS4. Altogether 90 morphotypes were isolated, 48 and 12 strains (43 species) from the air and surfaces, respectively. The number of spore-forming species generated high diversity of indoor fungi and differences between the cellars' fungal compositions; however, their dominant species were proved to be the same. Among the isolated strains *Penicillium* spp. were the most frequent. The walls of cellars were covered by colonies of *Zasmidium* (*Cladosporium*) *cellare* often referred to as a noble mold. Even so, this mold has been found only at a small concentration in the air samples (10–30 CFU/m^3). The walls of the modern store were free of molds. Diversity of fungi of the examined wine cellars was influenced by environmental conditions to a certain degree, such as elevation (height above sea level), age, reconstruction time of cellars, indoor ethanol concentration and the number of chimneys. The location of cellars poorly influenced the concentration of fungi of the air inside cellars, contrary to outdoors where the air

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of the municipal area contained more CFUs than that of rural spaces.

Keywords Air sampling · Molds · Traditional wine production

1 Introduction

Microfungi often colonize subterranean spaces like wine cellars. Their spores are dominant components of the indoor air. Surface and air samplings were carried out at different wine-producing areas in Austria (Clemenz et al. 2008; Haas et al. 2010), France (Simeray et al. 2001), Italy and Switzerland (Picco and Rodolfi 2004), Japan (Goto et al. 1989), to determine fungal composition in wine cellars. In these studies, several fungal species were detected, including pathogens.

Some of them may occasionally have an influence on the health of workers by inducing the development of respiratory diseases (allergy, asthma), non-specific respiratory symptoms and irritation (Douwes et al. 2003; Fung and Hughson 2003; Flannigan et al. 2011; Mendell et al. 2011); furthermore, their presence is proposed as a cause of biodeterioration. Microfungi habiting in wine cellars excrete volatile organic compounds that may also negatively affect the quality of wine (Moularat et al. 2011). Tokaj is one of the world's great historical wine-producing regions, located in northeastern Hungary with strong viticulture traditions which have survived for over 1000 years (Nyizsalovszki and Fórián 2007; Boatto et al. 2011; UNESCO 2015). The vegetation period is determined by the sunny, south-facing slopes and the proximity of the Tisza and Bodrog rivers. The wine quality depends largely on the specific climate of foothills being conducive to the proliferation of *Botrytis* (noble rot) colonizing and subsequently desiccating the grapes that promote the production of 'aszú' (botrytized) wine. This process depends largely on meteorological conditions during September (Makra et al. 2009). The terroir consists of clay or loess soil on volcanic subsoil (rhyolite) suitable for digging out the cellars without support system. The vast system of cellars with long and narrow corridors has been carved out of solid rock between 1400 and 1600 AD. The cellars have long-term constant temperatures of 10–12 °C (except for the differences in summer and winter temperatures of ca. 1.5 °C) as well as air humidity of 85–95% (Gašinec et al. 2012). In these traditional wine cellars, all available surfaces—walls, barrels, bottles, etc.—are covered with a characteristic blackish-gray layer of *Zasmidium* (*Cladosporium*) *cellare* (noble mold, Fig. 1). This fungus has no moldy odor and allegedly contributes to the creation of extraordinary climatic environment for faster wine aging and its bouquet. Local winemakers suppose that the fungus filters out unwanted yeasts and bacteria and consumes airborne alcohol, aldehydes and other organic substances evaporated during the aging of Tokaj wine (Tóth 2006; Gašinec et al. 2012). The positive effects of *Z. cellare* have not been proven by scientific methods, and some superstitions aroused related to its harmful effects on workers. Consequently, *Z. cellare* has been removed recently in some cellars due to these hygiene considerations. Studies are therefore required to evaluate whether there are any positive or negative effects of fungal colonization and traditional versus new wine aging techniques on air quality in wine cellars. To our knowledge, the fungi of wine cellars in Hungary have never been studied before, although fungi derived from grapes (e.g., Magyar and Bene 2006; Sipiczki 2016) and fermentation (e.g., Miklós et al. 1994; Sipiczki et al. 2001; Naumov et al. 2002; Magyar and Tóth 2011) are widely studied.

The aim of this study was to collect data on the composition of the fungal biota of wine cellars in Tokaj as well as their effect on air quality.

2 Materials and methods

The Tokaj wine region in Hungary consists of 27 villages and 11,149 h of classified vineyards, of which an estimated 5500 h are currently planted. It lies in the close proximity of the northernmost climatic limit of wine production. The microclimate is cold and temperate (the average temperature is 9.9 °C). There is significant rainfall throughout the year (averages 611 mm). Samples were collected in four communities in the Southern part of the Tokaj wine district with five traditional (subterranean) wine cellars (Tolcsva, Szegi, Tarcal 1–3) and one building with modern wine production technology (Erdőbénye) in November 2012 (Table 1). Both

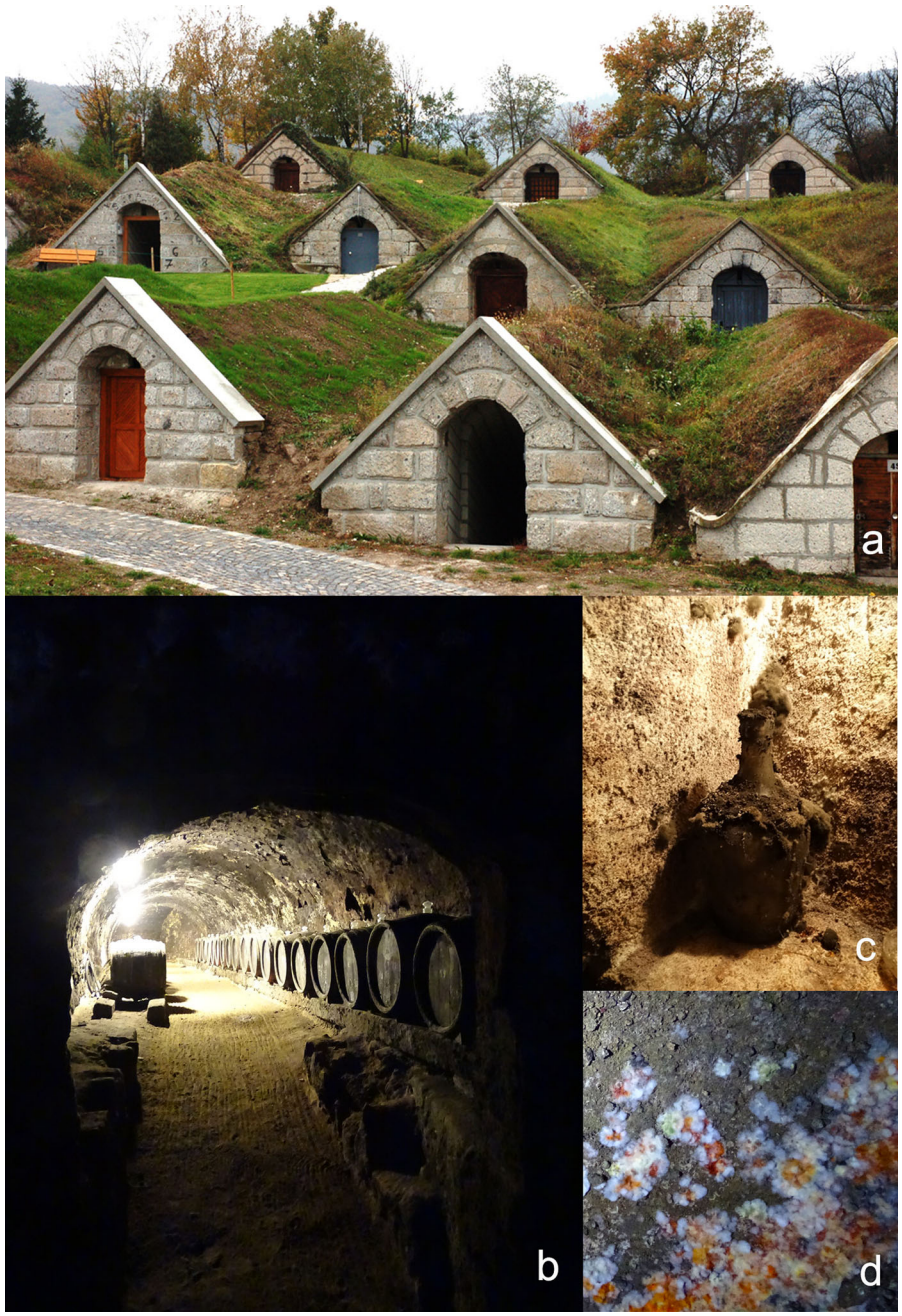


Fig. 1 **a** Landscape of Tokaj with the entrances of cellars, **b** Traditional wine cellar, **c** Colony of *Zasmidium cellare* on a wine bottle, **d** Colonies of *Aspergillus spelunceus* on the floor of a cellar. Photographs: **a** Bihari Z., **b–d** Magyar D

outdoor and ambient conditions in each winery area were measured parallel with biological samplings (Tables 2 and 3, respectively).

2.1 Examination of air quality

Air samples were collected in November 2012 at a height of 1.2 m above ground level with a radial diffusive sampler (Radiello® BTEX/VOC's cartridge CS2 solvent desorption), activated

Table 1 Characteristics of sampling locations

Parameters		Wine cellars					
		A	B	C	D	E	F
Location		Tolcsva	Erdőbénye	Szegi	Tarcal	Tarcal	Tarcal
Coordinates	North	48°16′20″	48°15′59″	48°12′24″	48°7′42″	48°7′42″	48°7′44″
	East	21°22′29″	21°21′42″	21°23′19″	21°20′47″	21°20′54″	21°20′54″
Elevation (m)		135	299	146	104	181	110
Surroundings[a]		W	W + F	W + F	M	M	M
Age (years)		800	10	416	316	566	216
Reconstructed (years)		56	0	76	116	29	36
Depth (m)		15	0	30	13	5	11
Ventilation[b]		1	2	1	1	3	1
Number of chimneys		20	0	10	6	0	40
Tunnels length (m)		700	0	5000	200	50	700
Tunnels width (m)		3	0	4	3	6	3
Mold removal (years)		55	1	46	10	6	200
Wine capacity (hl)		1600	800	20,000	200	140	60
Current wine load (%)		80	95	40	80	30	3

[a]The surrounding locations W, F and M mean vineyard, forest and municipal areas, respectively. [b]1: ventilation passively through chimneys, 2: HVAC system, 3: no ventilation

Table 2 Environmental parameters outdoors of the wine cellars sampled

Parameters	Wine cellars locations						C.V.%
	A	B	C	D	E	F	
Air pressure (hPa)	992.3	986.5	990.0	994.5	994.5	994.5	0.3
Temperature (°C)	15.0	15.5	12.4	13.1	13.1	13.1	9.1
Relative humidity (%)	59.3	58.1	67	62.7	62.7	62.7	5.0
Dew point (°C)	7.1	6.0	6.3	6.0	6.0	6.0	7.1
Evaporation temperature (°C)	10.7	9.9	9.1	9.3	9.3	9.3	6.3
Moisture content (g/kg)	6.4	6.0	6.0	5.9	5.9	5.9	6.0
Absolute humidity (g/m^3)	7.8	7.3	7.4	7.5	7.5	7.5	7.5

The capital letters A–F mark wine cellars, see Table 1

charcoal (30–50 mesh, Fondazione Salvatore Maugeri, Tradate, Italia) to detect airborne ethanol and methanol. Exposition time of tubes was 7 days. The samples were analyzed according to standards (MSZ EN 1076/1999, NIOSH 2000:1998, OSHA 100:1993, NIOSH 1405:200). Concentration levels were expressed in μg/m^3.

2.2 Examination of mycota

Two sampling techniques were used for the examination of fungal communities residing in wineries. Reference air samples were collected outdoors, at the entrance of wine cellars.

2.2.1 Surfaces

Surfaces were examined visually, and samples were collected from mold-covered walls and other objects with sterile cotton swabs and spread subsequently on MEA plates. The plates were incubated at 25 °C.

Table 3 Indoor conditions in wine cellars sampled

Parameters	Wine cellars						C.V.%
	A	B	C	D	E	F	
Air pressure (hPa)	992.9	987.3	990.3	995.1	994.8	993.1	0.3
Temperature (°C)	17.7	16.5	14.8	14.6	13.8	13.7	10.5
Relative humidity (%)	74.1	52.4	72.9	85.4	89.5	84.8	17.7
dew point (°C)	13.1	6.5	9.9	12.3	12.7	11.2	22.5
evaporation temperature (°C)	14.8	10.9	12	13.3	13.5	12.3	10.6
moisture content (g/kg)	9.6	6.1	7.8	9.2	9.4	8.4	15.7
absolute humidity (g/m^3)	11.4	7.6	9.5	11.1	11.3	10.2	14.4
Surface temperature (°C) min	12.3	18.3	10.8	14.1	13.6	11.6	19.9
Surface temperature (°C) max	14.3	14.3	11.3	17.0	14.1	12.4	14.0
Ethanol (μg/m^3)[a]	n.d.	136.8	206.6	16.8	105.2	1.6	91.4
Methanol (μg/m^3)[b]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	–
Average RBA (CFU/m^3)[c]	2145	75	8190	2535	1825	1955	99.7
I/O ratio RBA[d]	0.65	0.05	9.05	1.40	1.01	1.08	153.4

The capital letters A–F mark wine cellars, see Table 1

[a,b] detection limits, a: 0.21 μg/m^3, b: 0.17 μg/m^3

[c] average number of total colony-forming units (CFU) per m^3 ($F_{\text{cellar}} = 11.28$, $p = 0.042$), samples collected onto Rose Bengal agar (RBA) ($F_{\text{replication}} = 0.48$, $\text{LSD}_{0.05} = 350$, $p > 0.1$)

[d] indoor versus outdoor ratio of average number of total CFU per m^3 ($F_{\text{I.O}} = 6.69$, $p < 0.05$)

n.d.: not detected

2.2.2 *Air samples*

Air samples were taken 1.2 m above ground level, using a one-stage volumetric sieve sampler (SAS IAQ, International PBI S.p.A., Milan, Italy) at a flow of 100 l/min. Three types of culture media [Potato Dextrose Agar (PDA), malt extract agar (MEA, Samson 2010) and Askew agar with Rose Bengal (RBA, Askew and Laing 1993)] supplemented with chloramphenicol have been applied for sampling. The plates were incubated at 25 °C. Qualitative and quantitative evaluation of CFUs (colony-forming units) were carried out following 3–8 days of culturing; 15 days for slow-growing species. CFUs were divided into different 'morphotypes' based on similarity of cultural characteristics, e.g., colony color, texture, and growth rates. Sporulating morphotypes were identified at the genus level by their microscopic and macroscopic morphology using standard mycological literature to validate the molecular identification (Raper and Fennell 1977; Von Arx 1981; Singh 1991; Klich 2002; Samson 2010). The airborne fungal concentration was calculated using the correction factors according to Macher (1989) and expressed in CFU/m^3 air.

2.3 Isolation and maintenance of fungal strains

Strains representing m orphotypes were isolated from colonies formed on agar plates from either air or surface samples and maintained on PDA slants at ambient temperature until their identification.

2.3.1 *Molecular identification*

For the molecular identification of the isolates, mycelia were grown in 250-ml flasks in 50 ml potato dextrose broth (PDB, Scharlau Microbiology, 4 gL^{-1} potato peptone, 20 gL^{-1} glucose in distilled water) by shaking at 120 rpm at 20 °C for 10 days. The mycelial mat was ground to fine powder under liquid nitrogen with a pestle in a mortar. Genomic DNA was isolated with the GenElute^{TM} Plant Genomic DNA Miniprep Kit (Sigma-Aldrich Ltd.) according to the manufacturer's protocol. Then the internal transcribed spacer (ITS) region of the rRNA gene cluster was amplified

with primers ITS1 and ITS4 (White et al. 1990) and sequenced. A Bioer Life ECO Thermal cycler was used for PCR amplification with the following conditions: 2-min initial denaturation at 94 °C, followed by 35 cycles of 1-min denaturation at 95 °C, 1-min primer annealing at 60 °C, 2-min elongation at 72 °C and a final extension at 72 °C for 15 min. The amplified DNA products were purified using Gel/PCR DNA Fragment Extraction Kit (Geneaid) according to the manufacturer's protocol. Sequencing was carried out by Microsynth Austria GmbH. With the sequences obtained, we carried out similarity searches using the BLAST network service of the NCBI database (http://www.ncbi.nlm.nih.gov/blast.cgi). After the database search, pairwise BLAST comparisons were performed with each sequence and the sequences of the type strains of the species which gave the most similar matches. When the ITS sequence analysis gave ambiguous results, we also amplified and sequenced the D1/D2 domains of the large-subunit 28S rRNA gene with the primers NL-1 and NL-4 (O'Donnell 1993) under the following conditions: 2-min initial denaturation at 94 °C, followed by 35 cycles of 1-min denaturation at 94 °C, 1-min primer annealing at 51 °C, 2.5-min elongation at 72 °C and a final extension at 72 °C for 15 min.

2.4 Data analysis

Fisher's test was applied to evaluate significance of differences between variants (sampling, sampling points, wine cellars and composition of fungal consortia) at $p = 0.05$ level, and the interpretation was made according to Sváb (1979). The coefficient of variation [$(100 \times LSD_{0.05})$/Average)] was used for the characterization of individual parameters of ambient and outdoor environmental conditions. The data of observations were transformed into numerical values (1 and 0, presence or absence, respectively), and the resulted data matrix (in our case 18 sampling points and 43 fungal strains as varieties and observations, respectively) was analyzed by multivariate techniques to disclose patterns of distribution of species and similarities in diversity of fungal consortia in cellars. The dummy variables were used to avoid heterogeneous errors of variation originated of alterations in sampling methods. Relationships between varieties were analyzed with nonlinear mapping (sampling points) and cluster analysis (fungal species) applying appropriate modules of Statistica 5 program (StatSoft 5.0., Tusla, USA). Graphic plotting and regression analysis were used to disclose influence of environmental factors (Tables 1, 2, 3); the acceptance level was set to 95% significance level. Details of schemes followed were delineated previously (Magyar et al. 2011; Magyar and Oros 2012). The graphical presentation of the result of data analysis was edited uniformly in MS Office PowerPoint 2003.

3 Results and discussion

No extreme weather events happened in the season of sampling. The state of vegetation corresponded to usual conditions of the season. Surroundings of the cellars were well kept and typical for the area. Insides outlooked and smelled as usual for the well-managed wine cellars. Dark, spongious colonies of *Zasmidium* (*Cladosporium*) *cellare* covered the walls of all subterranean cellars; however, it was not found in the modern building in Erdőbénye. The basidiomycetous fungus *Bjerkandera adusta* (Polyporales) colonized frequently some surfaces in subterranean cellars. Also, red colonies of *Aspergillus spelunceus* covered large areas on the floor of subterranean corridors of the cellar *E*. Visually distinguishable spots on surfaces were randomly found and sampled as well. Presence of 43 fungal species could be detected at the sampling sites (Table 4); however, none of them was represented in all samples. Moreover, some species occurred only in single samples. The difference between subterranean cellars and the modern building is due to the diverse indoor conditions, especially ventilation and surfaces types. Ventilation of the modern building is mechanical, therefore indoor air quality of its open space could be controlled more effectively than that of the passively ventilated, irregularly carved tunnels of subterranean cellars having numerous stagnant airspaces and dead ends. Surface types are also different: rough, porous and often earthy and leaking (wet) walls and wood offer better conditions for fungal growth in the subterranean cellars than concrete walls and containers from steel of the modern building.

Table 4 Fungal taxa isolated in wine cellars and surrounding outdoor air

No.	Species	Cellars									
		A		B		C		D	E	F	D–F
		In	Out	In	Out	In	Out	In	In	In	Out
1	*Absidia psychrophilia*	s	–	–	–	–	–	–	–	–	–
2	*Acremonium brachypenium*	–	–	–	–	–	–	s	–	–	–
3	*Alternaria alternata*	–	–	–	–	–	–	–	a	–	–
4	*Aphanocladium album*	a	–	–	–	–	–	–	–	–	–
5	*Aspergillus niger*	–	–	–	–	–	a	a	–	–	–
6	*Aspergillus spelunceus*	–	–	–	–	–	–	–	as	–	–
7	*Aspergillus varians*	–	–	–	–	as	–	–	–	–	–
8	*Aspergillus versicolor*	–	–	–	–	–	–	a	–	–	–
9	*Aspergillus* spp.	–	–	–	–	a	–	a	–	–	a
10	*Bjerkandera adusta*	–	–	–	–	–	–	–	–	s	–
11	*Botrytis cinerea*	–	a	–	–	–	–	a	as	a	–
12	*Candida membranifaciens*	s	–	–	–	as	–	–	–	–	–
13	*Cladosporium ossifragi*	–	–	a	–	–	–	–	–	–	–
14	*Cladosporium* spp.	a	a	a	a	a	a	as	a	a	a
15	*Debaryomyces subglobosus*	a	–	–	–	–	–	–	–	–	–
16	*Emericella nidulans*	a	–	–	–	–	–	–	–	a	–
17	*Epicoccum nigrum*	a	a	–	a	–	a	a	–	a	a
18	*Mortierella alpina*	s	–	–	–	–	–	–	s	–	–
19	*Mucor* sp.	–	–	–	a	a	–	–	–	a	–
20	*Penicillium adametzioides*	–	–	–	–	–	–	–	a	–	–
21	*Penicillium brevicompactum*	–	–	–	–	–	–	a	a	–	–
22	*Penicillium chrysogenum*	a	–	–	–	–	–	–	–	–	a
23	*Penicillium citreonigrum*	–	–	–	–	as	–	a	–	–	–
24	*Penicillium citrinum*	–	–	–	–	s	–	–	–	–	–
25	*Penicillium commune*	–	–	–	–	a	–	–	–	–	–
26	*Penicillium echinulatum*	–	–	–	–	–	–	–	–	as	–
27	*Penicillium expansum*	–	–	a	–	–	–	as	a	a	–
28	*Penicillium glandicola*	–	–	–	–	–	–	–	–	s	–
29	*Penicillium oxalicum*	–	–	–	–	–	a	–	–	–	a
30	*Penicillium roqueforti*	–	–	–	–	–	–	s	–	–	–
31	*Penicillium solitum*	–	–	–	–	–	–	–	–	a	–
32	*Penicillium spinulosum*	as	–	a	–	as	–	as	as	as	–
33	*Penicillium thomii*	as	–	–	–	–	–	–	a	–	–
34	*Penicillium* spp.	a	–	a	–	a	a	a	a	a	a
35	*Phanerochaete chrysosporium*	–	–	–	–	–	–	s	–	–	–
36	*Rasamsonia brevistipitata*	–	–	–	–	–	–	–	–	a	–
37	*Scopulariopsis* sp.	a	–	–	–	–	–	–	–	–	–
38	*Talaromyces diversus*	–	–	–	–	–	–	–	s	–	–
39	*Talaromyces rugulosus*	–	–	–	–	–	–	s	s	–	–
40	*Umbelopsis isabellina*	s	–	–	–	–	–	–	–	–	–
41	*Zasmidium cellare*	s	–	–	–	s	–	as	as	s	–
42	Yeasts	a	–	–	a	a	–	as	–	–	–
43	Non-sporulating strains	as	a	a	a	s	a	as	a	a	a

The capital letters A–F mark wine cellars, see Table 1

a air sample, *s* surface sample, *In* indoor, *Out* outdoor sampling

3.1 Environmental conditions

The outdoor conditions varied to a certain degree (Table 2), however, the coefficient of variation (*c.v.*) did not surpassed 10%, indicating that there were no great differences in this respect. However, the indoor parameters varied at a higher degree (Table 3). Except from air pressure, the *c.v.* surpassed 10% having maximum in the case of dew point. Correlations between outdoor and indoor parameters revealed dependence between several environmental factors. The high correlation in air pressure ($r_{i,o} = 0.97$, $p < 0.001$) demonstrates the rapid equilibration that signalizes the perpetuous airflow between outer and inner airspaces. The absolute humidity also related positively but to a lesser extent ($r_{i,o} = 0.67$, $p < 0.1$), most probably due to the buffering capacity of insides. The latter can explain the absence of correlation in temperature parameters. A study performed in traditional wine cellars in Spain showed that interior air temperature is fundamentally conditioned by the temperature of the ground and the outdoor air (Mazarrón and Cañas 2009). Ground temperature has a major influence. In the autumn and winter, the influence of ground temperature decreases while that of the outside air increases, because the higher ventilation reduces the stability of the wine cellar. In the case of other parameters, the relationship also turned to be strictly linear ($r_{i,o} > 0.84$, $p < 0.05$) when either cellar *A* or *F* outlying of regression was omitted from the calculations. These two cellars differed from the others with high number of chimneys that underlines the dominant role of ventilation in the determination of conditions in cellars. A Spanish study performed in the main wine-producing regions of the world showed that underground cellars have advantages for the aging and conservation of wine in all regions studied, because of the thermal inertia of the ground and its ventilation behavior (Mazarrón et al., 2012).

3.2 Analytical determinations

3.2.1 Airborne alcohol content

In all six wine cellars studied, airborne methanol was below the detection limits (Table 3). The aerial ethanol content of cellars varied between 1.6 and 206.8 μg/m^3 (Table 3). Evaporation from the barrels (old wooden barrels let more gaseous ethanol to leak than modern metal containers) and from the surfaces (due to the tradition of pouring wine onto the walls in Tokaj cellars) are the main sources of gaseous ethanol, while minor amounts could be emitted by some fungal species, e.g., *Penicillium expansum* (as MVOC, see Fiedler et al. 2001). The ethanol consumption of fungi is also well known; *Zasmidium cellare* (Goodwin et al. 2016) is seemingly the main utilizer in the sampled cellars. Development of young colonies of *Z. cellare* was often observed near ethanol sources, e.g., on corks of wine bottles and near frequently used wine thieves. The effect of ethanol vapor on the growth of *Baudoinia compniacensis*, a fungus colonizing building walls near sources of ethanol emissions (e.g., cognac aging warehouses, commercial bakeries and printing industry), was studied in detail (Scott et al. 2007; Ewaze et al. 2008). Amendment of growth medium with 5 ppm ethanol greatly increased the success of isolation of this fungus. Optimal germination of dormant mycelia was seen at a low level of ethanol vapor (10 ppm). It was concluded that ethanol may play a stimulatory role in the growth of colonies. *Zasmidium cellare* and other frequent taxa may also prefer high ethanol concentrations; therefore, further studies are needed to test whether the isolated fungi have a more intensive growth on media supplemented with ethanol.

3.2.2 Density of fungal populations

The variation in CFU numbers on parallel plates in samplers varied to a low extent ($F_{\text{repl.}} = 0.41$, $p > 0.1$); furthermore, the position of sampler in various locations caused seemingly negligible effect on collection ($F_{\text{exp}} = 1.64$, $p > 0.1$), supporting the reliability of measurements. However, the number of countable colonies grown up on various selective media placed in the sampler altered significantly ($F = 6.69$, $p < 0.05$), which can be explained with differences in growth intensity of filamentous fungi on various media. Certain species (e.g., *Alternaria*, *Botrytis*, *Epicoccum*) rapidly overgrew the plate, suppressing the formation of visible colonies of other species. For this reason, the CFUs counted on RBA (Table 3) were taken as a measure of concentration of fungi in the air of the cellars as the fungal colonies developing on this medium showed moderate growth rate (no overgrowing colonies). It has to be mentioned

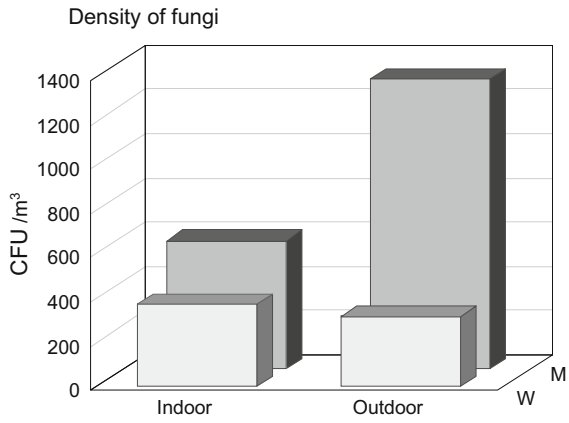


Fig. 2 Influence of the environment around cellars on the concentration of airborne fungi. M and W = municipal = downtown, wild = rural area

that Rose Bengal, a member of the anthracene dye family, exhibiting noticeable antimicrobial activities (Oros et al. 2003), can efficiently suppress both conidium germination and hyphal growth of fungal species from the orders Sphaeriales and Helotiales (Oros and Cserháti 2009); consequently, the densities of *Cladosporium* and *Botrytis* in the sampled area might be underestimated. Furthermore, spreading the swab samples directly on MEA plates may underestimate fungal diversity, as the dominant species may suppress slow-growing taxa. The sampling method allowed an insight into the fungal composition of the cellars, but several taxa remained undiscovered by the culturing method, as it is suggested by direct microscopical observations of the surfaces (i.e., tape lift samples or plating serial dilutions onto different culture media including media supplemented with ethanol). Therefore, future studies should be focused on the development of a more efficient technique based on monospore DNA analysis.

Significant differences were disclosed in CFU numbers in the air of six cellars ($F_{\text{cellars}} = 11.28$; $p < 0.01$), and to a lesser degree between CFU numbers of the samples ($F_{\text{outdoors}} = 11.28$; $p < 0.01$) collected in their outdoors. Significant differences were found also between the concentrations of airborne propagules measured outdoors and inside cellars ($F_{\text{o.i}} = 5.86$; $p < 0.05$). The concentration of airborne propagules measured either outside or inside the cellars was significantly higher in municipal areas than in rural ones (Fig. 2). One can assume that the traffic and the diverse flora of parks and gardens in municipal areas are responsible for these differences.

The outdoor samples were characterized by the presence of *Cladosporium* and *Epicoccum* spp., which were found in the air of the cellars as well. However, the frequency of propagules of fungal species in various sampling points varied substantially ($F = 24.43$, $p < 0.001$). For example, *Botrytis cinerea* had high outdoor concentration in Tolcsva only (140 CFU/m^3 on RBA, but more common on MEA and PDA: 640 and 870 CFU/m^3, respectively), while in Tarcal this fungus was not found in the samples collected outdoors.

Indoor samples were dominated by penicillia: a total of 15 species (including those referred to genera *Rasamsonia* and *Talaromyces*) were present. The most frequent species was *P. spinulosum* detected in five cellars; its highest concentration was above 13,000 CFU/m^3 in cellar *C*, while 47% of the penicillia were found in one cellar only; among them *P. adametzioides*, *P. solitum* and *P. thomii* had high levels (710, 2120 and 760 CFU/m^3 in cellars *A*, *E* and *F*, respectively). *Penicillium expansum*, being absent in outdoor samples collected in Tarcal, was common in the indoor samples collected in the cellars of this village and had also low level in cellar C. The genus *Penicillium* was richest in species in Tokaj, but also in certain wineries in Austria (Haas et al. 2010), France (Simeray et al. 2001), Italy and Switzerland (Picco and Rodolfi 2004) and Japan (Goto et al. 1989). *Penicillium expansum, Botrytis cinerea* and some other fungal species could produce geosmin (*trans*-1,10-dimethyl-*trans*-9-decalol, La Guerche et al. 2005), while some *Penicillium* (and *Trichoderma*) species may contribute to the formation of 2,4,6-trichloroanisole (TCA, Prak et al. 2007). These fungal metabolites could cause bad flavor of wines. Geosmin is an earthy-musty compound. TCA contamination is recognized by cork taint, the most frequent wine fault ('wet dog or wet cardboard smell'), which makes wine mostly unpalatable (Tanner and Zanier 1981). TCA was detected by Haas and coworkers (2010) in the air of Austrian wine cellars with visible moldy patches. Further studies are therefore needed to see whether these metabolites are present in Tokaj wine cellars.

Two members of the genus *Aspergillus* were also common inside the Tokaj cellars (*A. nidulans* in cellar *A*: 110 and *F*: 90 CFU/m^3; *A. spelunceus* in cellar *E*: 490 CFU/m^3). Moreover, *Aphanocladium album* was

an abundant fungus but only in cellar *A* (980 CFU/m^3). Among yeasts, *Candida membranifaciens* (in cellar *C*: > 13,000 CFU/m^3) and *Debaryomyces hansenii* (in cellar *A*: 760 CFU/m^3) were detected in high numbers.

The samplings revealed high diversity of fungi attached to the surfaces in traditional cellars. Most of the isolated strains have been identified at the species level (Table 4). Besides the aforementioned *Z. cellare*, *A. spelunceus* and *B. adjusta*, 18 other species occurred in surfaces, among them 11 were found in air samples as well. *Penicillium spinulosum* was present in all traditional cellars, although its inconspicuous colonies could not be perceived with naked eyes before the evaluation of the samples. Although *Z. cellare* was present in all subterranean cellars, it was detected in the air of only two of them (in cellar *D* and *E*: 30 and 10 CFU/m^3, respectively). Simeray et al. (2001) had similar results in French cellars, where *Zasmidium* mycelia commonly cover the walls but the fungus is rarely isolated from the air samples, possibly due to poor sporulation.

As demonstrated in Fig. 3, the outside environment did not significantly influence the number of species habiting on surfaces of cellars located either in rural or municipal areas; the ratio of frequent and sporadic species was similar in both locations with higher number of the latter group. Contrarily, great differences manifested in the composition of airborne species in cellars: the consortia indoors were more diverse than outdoors, and the highest number of species was

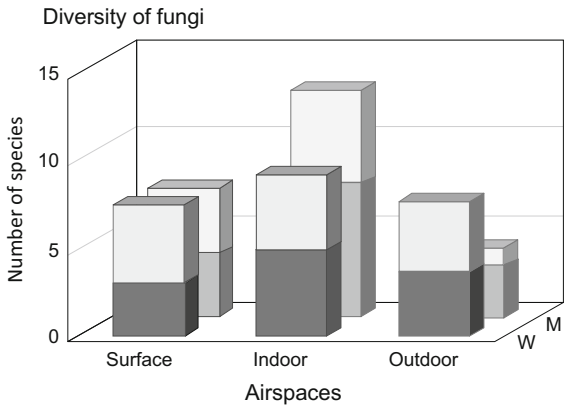


Fig. 3 Influence of the environment around cellars on the diversity of airborne fungi. M and W = municipal = downtown, wild = rural area. The lower (dark gray) and upper (light gray) parts of the columns are proportional to the number of frequent and rare species trapped

Fig. 4 Cluster-diagram of the interrelationships among fungal species presented in Tokaj wine cellars. Grouping of species based on their presence (+) or absence (−) in sampling points (Table 4). Unweighted pair group average method based on Pearson's correlation coefficients was applied to construct the clusterogram. Codes of cellars (A-F) are the same as in Table 1. A and B: superclusters; a1-3, b1-6: subclusters. The species marked with symbol filled circle produce hydrophilic spores. The black, gray and white prisms on the histogram refer to the number of species present in surface, indoor and outdoor air samples, respectively

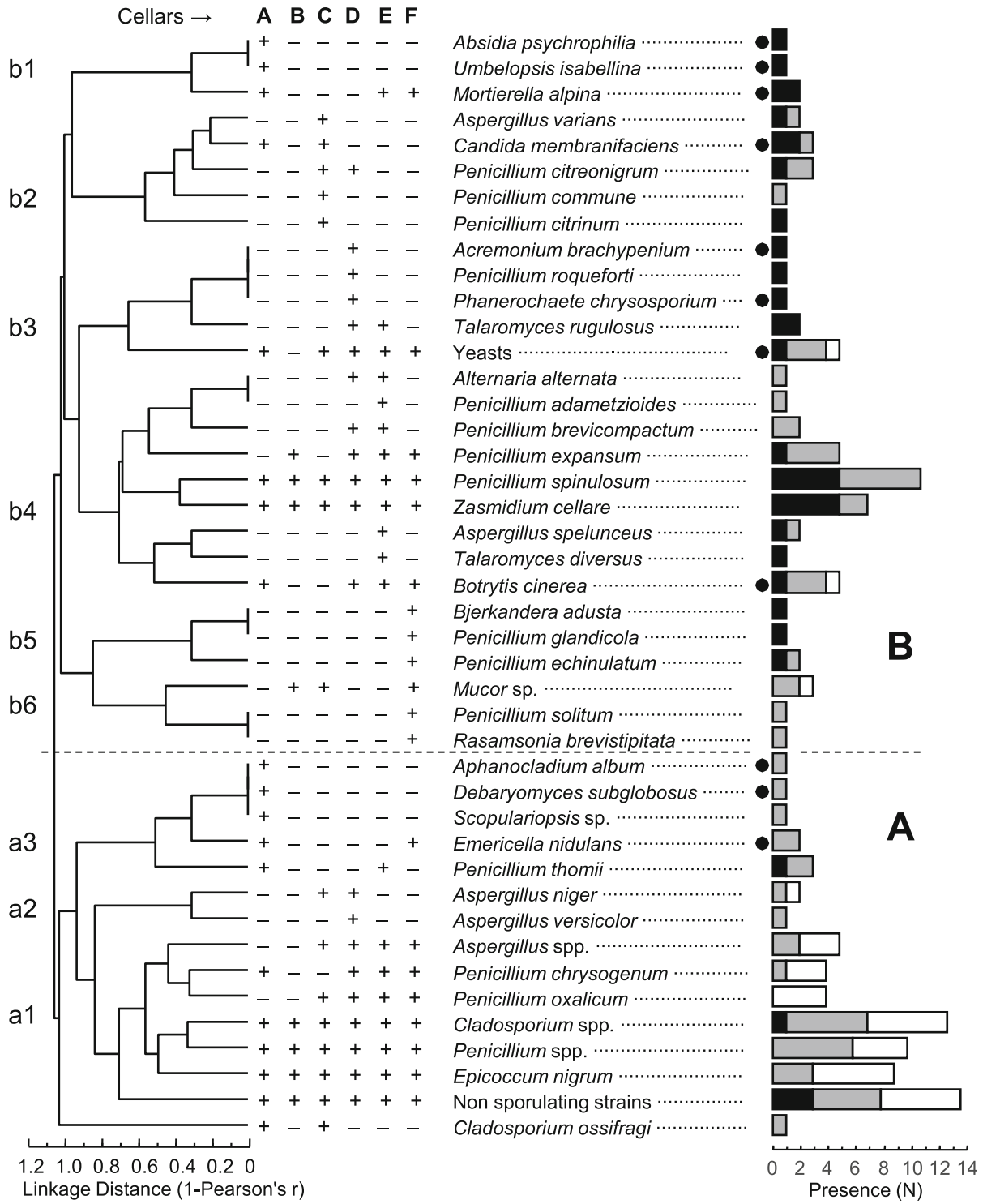
identified in airspaces of cellars located in municipal areas, where common species dominated over rare ones. Although the number of propagules was the highest in outdoor air of municipal areas (Fig. 2), the species diversity was below the one detected at other sampling points; moreover, the number of sporadic species was also the lowest in this case (Fig. 3).

3.3 Diversity of fungal consortia

The MANOVA of population density data revealed significant differences ($p < 0.01$) between either outdoor or indoor sampling points of various cellars (Table 3). To disclose patterns of species distribution and similarities in the diversity of fungal consortia in cellars, the data of Table 4 were transformed into numeric values on the basis of presence/absence of species to correct for heterogeneous variances originating from differences in samplings. The resulting data matrix was subjected to nonlinear mapping (Sammon 1969) and cluster analysis (unweighted pair group average method based on Pearson's correlation coefficients).

The species formed two large clusters (Fig. 4), both of them enclosing several subclusters ($p = 0.01 - 0.05$). The first supercluster (A), dominated by airborne species, comprises mainly fungi producing dry (hydrophobic) spores, and only one of the three subclusters included some species having readily wettable (hydrophilic) spores. Spores grouped according to their wettability (see Mason 1937; Magyar et al. 2016).

Most species isolated from surfaces belonged to the other supercluster (B), where three out of six subclusters included only species producing hydrophobic spores. The majority of the hydrophilic-spored species agglomerated into other three subclusters was isolated



from surface samples, and most of them were found only in one cellar. Hydrophilic species have hydrophylic spores and these spores could hardly be aerosolized (they liberate if the surface dries out).

Consequently, their airborne dispersal could be limited in the moist environment of subterranean cellars.

Fungal diversity in various sampled locations, i.e., on surfaces and in either indoor or outdoor air was significantly different ($F = 12.19$, $p < 0.001$). Assuming that propagules collected demonstrate the diversity of resident fungal consortia in situ, the position of sampling points plotted as variables of nonlinear map (Fig. 5) relates to the similarity of fungal populations in the proper locations, i.e., distances between points are proportional to their similarity. The sampling locations formed well-separated clusters, among them the outdoor air samples being the most compact one. The group of indoor airspaces formed a less compact cluster with strictly separated cellar *C*, which is the most spacious among the cellars examined. The surface samples of various cellars showed low similarity to the respective airspaces.

3.4 Factors influencing the fungal consortia in wine cellars

The data on density and fungal diversities were plotted against environmental variables (Tables 1, 2, 3), and regression analysis was applied to evaluate the strength of relationships.

Among the environmental conditions, elevation, age, reconstruction time of cellars, indoor ethanol concentration and number of chimneys influenced the diversity of mycota of the examined wine cellars to a certain degree ($p < 0.05$). Except for elevation, these parameters had no effect on the fungal diversity of the outdoor air ($p > 0.1$). Elevation had significant negative effect on the diversity of fungi, both outdoors ($R^2 = 0.801$) and indoors (air $R^2 = 0.787$; surface $R^2 = 0.779$). The level of relative humidity also influenced the diversity ($R^2 = 0.627$), which was significantly lower in the cellars at higher elevation; most probably the lower amount of water offered for fungal growth negatively affected the fungal diversity in these cellars. Indoor humidity is high in traditional or partially subterranean spaces, like wine cellars and dwellings, where it could pose problems if the presence of molds is not controlled (Cañas and Ocaña 2005; Malaktou et al. 2016).

The species diversity both on the cellar surfaces and in the indoor airspaces positively correlated with the age of cellars ($R^2 = 0.529$ and 0.870, respectively). The age after reconstruction—similarly to the age of the cellars—also positively correlated with the increase in fungal diversity both on surfaces ($R^2 = 0.659$) and in airspaces ($R^2 = 0.742$), indicating the rapid regeneration of characteristic mycobiota

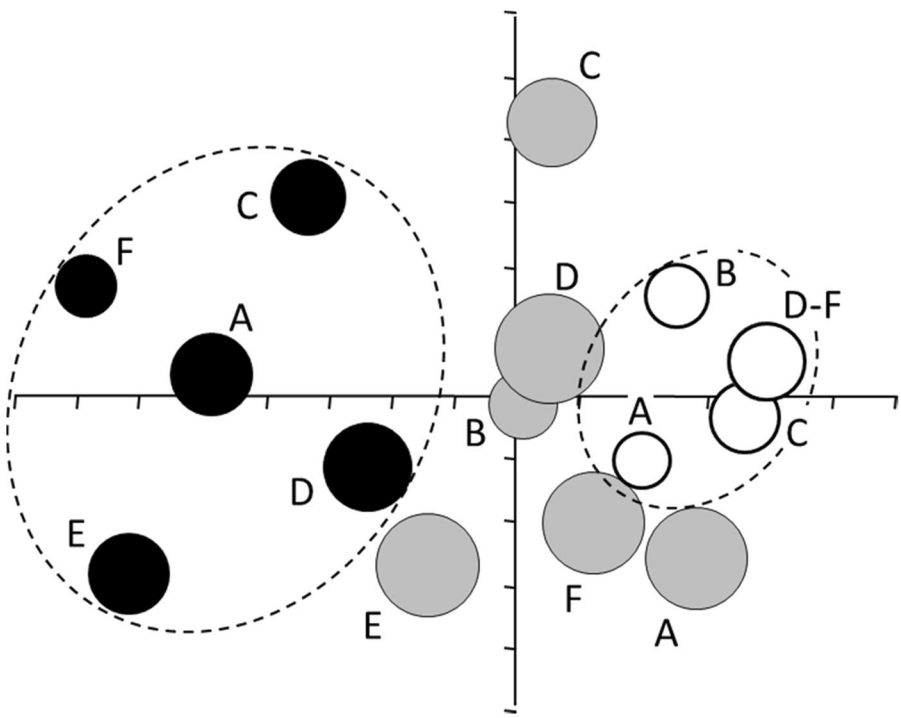


Fig. 5 Nonlinear map of sampling points. The size of circles is proportional to the number of fungal species collected in the sampling sites. The samples collected in either outdoor or indoor airspaces are filled with white and gray while those of surfaces with black. The capital letters A–F mark wine cellars (see Table 1)

in cellars. Regarding the diversity of the fungi growing on the cellar surface, elevation had about tenfold stronger effect than age, but was comparable with reconstruction time. The modern wine store was excluded from this analysis, since its unique features were not comparable with the subterranean cellars.

Since *Zasmidium* cover has a cultural role in traditional wine making and also attracts tourists, the importance of such studies should be emphasized. The reconstruction of the cellars included the removal of *Zasmidium* cover from the walls. This process decreased both the diversity ($p < 0.05$) and the density ($p < 0.001$) of airborne and surface fungi in the indoor airspace. Nevertheless, the resident fungal consortia recovered and seem to be stable; according to the trend calculated of the regression equation, constant members present in the area (*Cladosporium*, *Epicoccum*, *Penicillium*, *Zasmidium* and yeasts) rapidly start to colonize the subterranean surfaces. The old fungal cover can contribute to the species richness of the surfaces in the cellars sustaining favorable condition for microbiota. *Zasmidium* has a large surface area of mycelia but low spore production. We suppose that this *Zasmidium* cover acts as an air filter, trapping spores of other fungi. A fraction of the trapped fungal species may survive in the *Zasmidium* colonies as competitors or mycoparasites (unpublished observations of the authors). Some of these fungi can release large numbers of spores, which become airborne and may trigger allergy and asthma (Day and Ellis 2001). However, no medical cases were documented in the Tokaj region and no experience gave either rise to belief about the health hazard caused by fungal cover on cellar surfaces.

Ethanol can promote the growth of not only *Zasmidium* but also of other fungal species that have abundant spore production. The concentration of fungi in the air was positively correlated ($p < 0.005$) with the airborne concentration of ethanol; nevertheless, the latter poorly influenced ($p = 0.347$) the diversity of mycota of cellars. Our results did not confirm the hypothesis that the higher ethanol level decreases airborne spore concentration by intensifying the growth of the non-sporulating but spore-trapping *Zasmidium* on the walls. However, further studies are needed to find out whether the removal of the *Zasmidium* cover is beneficial or not, moreover, how a homogeneous *Zasmidium* cover can be created without sporulating 'partner species'.

Subterranean cellars ventilate through a door and chimneys; however, the number of chimneys varied markedly by the cellars studied. The number of chimneys affected the diversity of fungi positively in the indoor air ($R^2 = 0.68$) but not outdoors. Especially the number of sporadic species increased in the air of the cellars. Interestingly, the effect was negative on the diversity of surface mycota ($R^2 = 0.71$). It is thought that air currents may transport spores from the outdoor environment through doors, adding new components to the mycota of the indoor air, but these fungi were not able to establish colonies on the walls due to their low competitive ability against the resident mycota. More chimneys may reduce the relative humidity indoors by a better ventilation and make conditions unfavorable for fungal growth on the surfaces.

4 Conclusions

There were marked differences in the airborne concentration and composition of fungi in the examined wine cellars. Apparently, two factors have major effects on the fungal population: concentration of airborne ethanol (as nutrient and as an agent promoting the competitiveness of certain species) and ventilation (number of chimneys, temperature and air pressure). The number of spore-forming species generated high diversity of indoor fungi and differences between the cellars' fungal compositions; however, their dominant species proved to be the same.

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