FUNGI ISOLATED AND QUANTIFIED FROM BAT GUANO AND AIR IN HARMANECKÁ AND DRINY CAVES (SLOVAKIA)

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Abstract: This study is the first mycological evaluation of bat guano and the air around it in Harmanecká and Driny Caves in Slovakia. These caves are the most important underground localities of bats in Slovakia. Samples were collected in July 2014 and cultivated for fungi. Harmanecká Cave had seven species of filamentous fungi and one yeast-like fungus isolated from bat guano, compared to six species of filamentous fungi in guano from Driny Cave. Air samples from Harmanecká Cave had twelve species of fungi, compared to nine species from Driny Cave. Fungal density was higher in guano from Driny Cave (4720.1 CFU/g guano) than from Harmanecká Cave (3498.3 CFU/g). The pattern was reversed with fungi from the air. Fungal density in air from Harmanecká Cave (211.3 CFU/m^3) was higher than that from Driny Cave (175.7 CFU/m^3). *Penicillium granulatum* was the most frequently isolated fungal species, except in the guano of Driny Cave, where *Mucor hiemalis* was most common. Bat guano is a very good substrate for the development and survival of fungi in the caves, and it can be a reservoir of fungi harmful for bats. However, air samples from both caves contained more species of fungi than the bat guano, because the majority of fungi are transferred to underground ecosystems with air bioaerosols from the external environment.

INTRODUCTION

Cave ecosystems and other underground habitats are characterized by low temperatures and low availability of nutrients (Ogórek et al., 2013). Bat guano is rich in carbon, nitrogen, and vital minerals, so it is used in agriculture for fertilization of plants (Shetty et al., 2013). Bat guano, plant debris, animal carcasses, and other organic debris are the most important substrates for fungi inside caves (Nieves-Rivera et al., 2009).

Fungal conidia or spores are commonly found in caves and other underground environments. These elements enter mainly with air currents from the external environment (Pusz et al., 2014; Ogórek et al., 2014a, 2014b, 2014c). They can also be carried into caves by water, by animals such as bats and arthropods, or by humans visiting underground spaces (Mulec, 2008; Chelius et al., 2009; Vanderwolf et al., 2013; Griffin et al., 2014). The cave mycobiota are very important for underground ecology, because the fungi present are decomposers or parasites and probably constitute the major food source for other organisms (Sustr et al., 2005; Walochnik and Mulec, 2009; Bastian et al., 2010). Evidence for microbial activity in a cave includes spots on the cave surfaces, unusual coloration of speleothems, precipitates, corrosion residues, structural changes, and the presence of biofilms (Barton, 2006).

Bats are mammals that lead nocturnal lives and play important roles in the ecosystem such as plant pollination, seed dissemination, forest regeneration, and insect control,

so their health and factors that influence it should be of high concern. Investigating fungi in guano is appealing, because guano is found in the immediate neighborhood of bat roosts and is likely to be highly exposed to bat pathogens. Bat pathogens probably won't grow on guano, but can land on it. Moreover, sampling of guano is non-invasive, in contrast to direct examination of bats, and may be conducted at a time when bats are absent from their hibernacula (Mulec, 2008; Nováková, 2009).

Harmanecká and Driny Caves rank among the most important underground localities for bats in Slovakia. The dominant species in Harmanecká Cave are the greater mouse-eared bat (*Myotis myotis*) and the lesser mouse-eared bat (*M. blythii*), with 1000 to 1500 hibernating individuals. The dominant species in Driny Cave is the lesser horseshoe bat (*Rhinolophus hipposideros*), with 100 to 150 individuals (Lehotská and Lehotský, 2009).

Our research focused on two goals, the mycological analysis of the species composition of the fungi found in

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the guano of bats and airborne fungi around it in the Harmanecká and Driny Caves and the quantification of their concentrations.

MATERIALS AND METHODS

Harmanecká Cave is located in the Harmanec Valley to the northwest of Banská Bystrica city, in the southern part of the Great Fatra Mountains. Geographic coordinates of the cave are 8°81′39″ N, 19°04′01″ E. Its entrance is situated on the northern side of Kotolnica Hill at 821 m a.s.l. Its length is 3123 m, and the length of the tourist path is 1020 m. The cave was discovered in 1932 by Bacúrik, and it was opened to the public in 1950. During World War II, the cave served as a shelter for local people (Bella et al., 2001; Lehotská et al., 2011). The air temperature in the cave is between 5.8 and 6.4 °C, and the relative humidity is between 94 and 97% (Bella et al., 2001). Driny Cave is located in the Smolenice Karst in the Lesser Carpathian Mountains, south-west from Smolenice, in the Trnava district and near the recreation resort Jahodník. Geographic coordinates of the cave are 48°50′04″ N, 17°40′20″ E. Its entrance is situated on the western slope of Driny Hill at 399 m a.s.l. and its length is 680 m. The cave was discovered in 1929 by Vajsábel and Banič, and it was opened to the public in 1935 with provisional electric lighting for 175 meters. Now the length of the tourist path is 410 m (Bella et al., 2001; Lehotská et al., 2011). The air temperature in the cave is between 7.1 and 7.8 °C, and the relative humidity is between 92 and 97% (Bella et al., 2001). In 2014, Harmanecká Cave was visited by 17,425 people, and Driny Cave was visited by 31,859 people.

The samples were taken on July 24, 2014 from Harmanecká Cave and on July 25, 2014 from Driny Cave, before tourists arrived. Bat guano, not fresh, was found only in a single location in Harmanecká Cave, in the Entrance Dome in front of an airlock door, and also only in a single location in Driny Cave, about 140 m into the cave in the Slovak Speleological Society Hall (Fig. 1). The guano samples were collected using sterile forceps and placed into sterile sampling bags (three samples per cave, about 8 g of guano per sample). The samples were transported in cool conditions (ca. 7 °C) to the laboratory and were stored there in the cold room. Mycological evaluation of the samples was carried out within three days from the date of sampling. The air samples were collected using an Air Ideal 3P sampler and Potato Dextrose Agar (PDA) medium. It was programmed for air sample volumes of 50 L, 100 L, and 150 L. Measurements in several locations around each guano pile were performed in three replicates for each volume. The sampler was positioned about 1.5 m from the bat guano.

Potato Dextrose Agar (PDA, Biocorp) was used for the isolation of fungi from the guano and from the air and for the identification of some species. Czapek-Dox Agar (1.2%
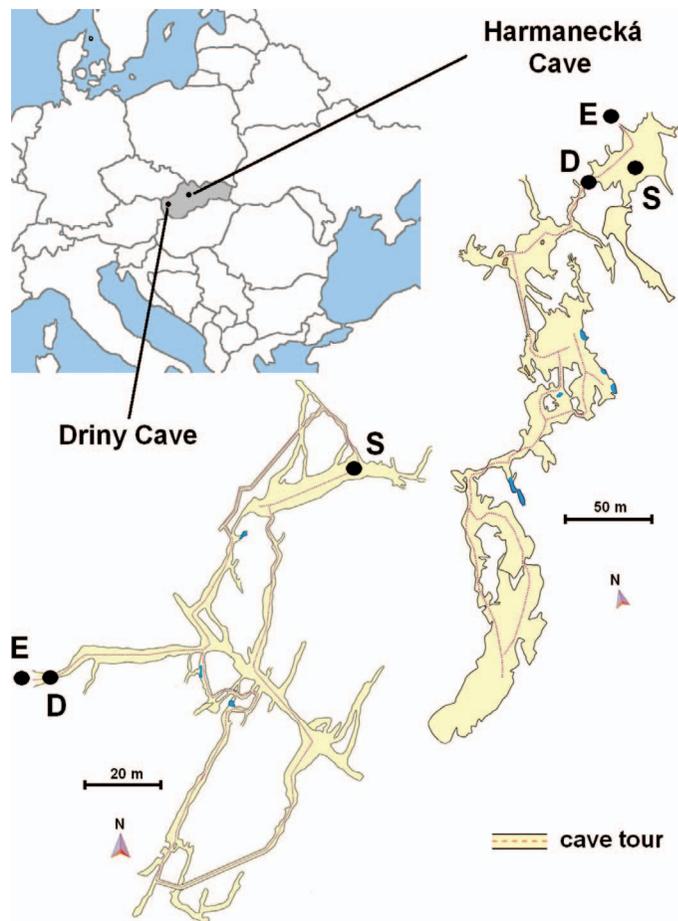

Figure 1. Geographic location of the caves and map of the tourist routes: E – entrance and exit of the cave; D – airlock door; S – sampling locations (Harmanecká Cave, before the airlock door in the Entrance Dome; Driny Cave, about 140 m into the cave, in the Slovak Speleological Society Hall).

agar, Biocorp) and Malt Extract Agar (MEA, Biocorp) were used for the identification of species belonging to the *Penicillium* and *Aspergillus* genera. Sabouraud Agar (4% dextrose, 2% agar, 1% peptone, A&A Biotechnology) medium was used for identification of yeast-like fungi.

One gram of guano (in three replicates) taken from each sample was shaken for 20 minutes in a 250-mL Erlenmeyer flask containing 9 mL of sterile distilled water. After shaking, samples were spread-plated using serial dilution onto PDA. Incubation was carried out at 15, 20, and 25 °C for 4 to 14 days in the dark. After incubation, the number of colony-forming units (CFU) per 1 g of guano was calculated as averages from the replicates at all incubation temperatures.

Similarly, the incubation of cultures from the air samples on PDA was carried out at 15, 20, and 25 °C for 4 to 14 days in the dark. After incubation, the number of CFU per m^3 of air was calculated as averages from the replicates at all incubation temperatures.

After incubation, the fungal colonies grown were counted and identified. The species identification was

Table 1. Filamentous and yeast-like fungi cultured from the bat guano and the air around it in the Harmanecká and Driny Caves. A + indicates that the species was found.

Fungi Species	Harmanecká		Driny	
	Guano	Air	Guano	Air
Absidia glauca Hagem	+	+		
Alternaria alternata (Fr.) Keissl.				+
Aspergillus foetidus Thom & Raper	+	+	+	
Aspergillus fumigatus Fresen.			+	+
Botrytis cinerea Pers. ex. Fr.		+		
Cladosporium herbarum (Pers.) Link		+		+
Epicoccum nigrum Link		+		+
Gliocladium roseum Bainier		+		
Mucor hiemalis Wehmer	+	+	+	+
Penicillium chrysogenum Thom	+	+	+	+
Penicillium granulatum Rainier	+	+	+	+
Penicillium lanosocoeruleum Thom		+		
Penicillium roseopurpureum Dierckx	+			
Penicillium urticae Bainier		+		+
Phoma fimeti Brunaud				+
Rhizopus stolonifer (Ehrenb.) Vuill.	+	+	+	
Rhodotorula glutinis (Fresen.) F.C. Harrison	+			
$\sum$ species	8	12	6	9

performed using macro- and microscopic observations, namely the morphology of hyphae, conidia, or spores and the colonies that had grown on culture media. The filamentous fungi were identified using diagnostic keys and descriptions by Pitt and Hocking (2009) and Watanabe (2010). The yeast-like fungi were identified using the diagnostic key and descriptions by Kurtzman and Fell (1998).

Results

The bat guano and the air around it in Harmanecká Cave contained more species of fungi than the samples from Driny Cave. Seven species of filamentous fungi and one of the yeast-like group of fungi were isolated from the guano of Harmanecká Cave and six species of filamentous fungi from Driny Cave. Air samples from Harmanecká Cave grew twelve species of filamentous fungi, and there were nine species from Driny Cave air. *Penicillium roseopurpureum* and *Rhodotorula glutinis* were isolated only from the guano sampled, whereas *Alternaria alternata*, *Botrytis cinerea*, *Cladosporium herbarum*, *Epicoccum nigrum*, *Gliocladium roseum*, *Penicillium lanosocoeruleum*, *Penicillium urticae*, and *Phoma fimeti* were found exclusively in the samples of air (Table 1).

The largest group of filamentous fungi in both caves constituted fungi of the genus *Penicillium*, whereas the yeast-like fungi were cultured only from guano sampled from Harmanecká Cave. The concentration of all fungi isolated from bat guano was 3498.3 colony-forming units per 1 g of guano from Harmanecká Cave and 4720.1 per 1 g of guano from Driny Cave. Air samples respectively grew 211.3 and 175.7 CFU per 1 m^3 of air (Tables 2 and 3).

Of the colonies cultured from guano, 75% from Harmanecká Cave were *Penicillium granulatum* and 54% from Driny Cave were *Mucor hiemalis* (Figs. 2 and 3). Fungal species such as *Absidia glauca*, *P. roseopurpureum* and *R. glutinis* were isolated only from the guano collected in Harmanecká Cave and *Aspergillus fumigatus* only from Driny Cave (Table 1).

The fungus most frequently cultured from the air was *P. granulatum* (Fig. 4) in both caves, and it constituted approximately 45% of fungi (Table 3, Figs. 2 and 3). *A. alternate*, *A. fumigatus* and *P. fimeti* were present only in the air of Driny Cave, whereas *A. glauca*, *Aspergillus foetidus*, *B. cinerea*, *G. roseum*, *P. lanosocoeruleum*, and *Rhizopus stolonifer* were found exclusively in the air of Harmanecká Cave (Table 1).

The air samples in both the caves contain both quantitatively and qualitatively more propagules of fungi than the bat guano. *P. granulatum* was the most frequently isolated fungus with the exception of the guano collected in Driny Cave, where *M. hiemalis* was the most common (Fig. 2 and 3).

Discussion

The most important factors affecting the survival of fungi in the environment are temperature and humidity. However, underground environments are characterized by a very specific, stable microclimate; therefore the most important factors that determine occurrence of fungi in

Table 2. Mean concentrations of filamentous and yeast-like fungi cultured from the bat guano collected in the Harmanecká and Driny Caves

| Cave | Fungi Species | Guano (CFU/g) | | Total |
| | | Individual Species | | |
		Range of Values	Mean ± SD[a]	
Harmanecká				3498.3
	Absidia glauca	71.0 – 185.0	133.2 ± 44.3	
	Aspergillus foetidus	45.0 – 135.0	94.7 ± 34.4	
	Mucor hiemalis	133.0 – 297.0	225.8 ± 56.1	
	Penicillium chrysogenum	103.0 – 244.0	180.5 ± 56.1	
	Penicillium granulatum	1521.0 – 3810.0	2644.0 ± 879.3	
	Penicillium roseopurpureum	42.0 – 115.0	79.8 ± 26.8	
	Rhizopus stolonifer	60.0 – 181.0	120.3 ± 44.8	
	Rhodotorula glutinis	2.0 – 40.0	20.0 ± 14.2	
Driny				4720.1
	Aspergillus foetidus	14.0 – 40.0	25.0 ± 10.1	
	Aspergillus fumigatus	21.0 – 74.0	50.3 ± 20.9	
	Mucor hiemalis	1500.0 – 3800.0	2550.1 ± 869.7	
	Penicillium chrysogenum	146.0 – 339.0	249.4 ± 78.2	
	Penicillium granulatum	1026.0 – 2679.0	1825.0 ± 598.0	
	Rhizopus stolonifer	8.0 – 34.0	20.3 ± 9.6	

[a] SD = Standard Deviation.

them are airflow, the conditions prevailing in the neighboring external environment, such as the season, local flora, the geographical location, and especially the availability of organic matter (Ogórek et al. 2013, 2014a, 2014b; Pusz et al. 2014; Vanderwolf et al. 2013). In our study, the most species of fungi were isolated from Harmanecká Cave. The situation is probably because the samples were taken in Harmanecká Cave between the entrance and the air-lock door and in Driny Cave behind the door, so the samples in Harmanecká Cave were directly exposed to airborne fungi from the surrounding external environment.

The traditional assessment method for fungi based on the calculation of colony-forming units and microscopic analysis, which is more common and cheaper than molecular biological methods, gives the ability to identify colonies to the species level, and a huge reference database is available for the proper identification of strains (Pasanen 2001; Rastogi and Sani, 2011). However, several disadvantages of CFU analysis are also apparent in comparison to molecular methods. CFU analysis does not allow us to detect non-culturable fungi, and also it can overlook fungal species that are not easily culturable. Furthermore, it might underrepresent those fungal types that grow slowly because they are overtaken by faster growing colonies (MacNeil et al., 1995; Wu et al., 2000; Macher, 2001; Pasanen, 2001). In addition, the type of culture medium and temperature of incubation have an influence on the in vitro growth rate of fungi isolated from the environment, their species composition, and their concentrations (Kaufman et al., 1963; Marshall et al., 1998; Meletiadis et al., 2001; Ogórek et al.,

2011a, 2011b). A variety of molecular methods based on direct isolation and analysis of nucleic acids or proteins from environmental samples could be a very good alternative to standard culture techniques for in-depth characterization of environmental microbial communities (Rastogi and Sani, 2011).

Littman (1947) reported that Sabouraud Agar medium is the most suitable for isolation of a large spectrum of fungal species from the environment. According to Ogórek et al. (2011a, 2011b), Potato Dextrose Agar medium demonstrates comparable efficacy. Therefore we used this medium for isolation of fungi from the guano and the air samples. Fungal response to temperature is quite varied. Active growth will usually be associated with a limited range of temperatures. However, many fungi remain alive for extended periods at temperatures unsuitable for growth (Smith, 1988). Generally, most fungi grow well at room temperature ranging from 20 to 25 °C, but, for example, the optimal temperatures for growth of *Geomyces destructans* (now *Pseudogymnoascus destructans*) is between 12.5 and 15.8 °C, and the upper critical temperature for growth is between 19.0 and 19.8 °C (Kaufman et al., 1963; Verant et al., 2012). This fungus causes white-nose syndrome in bats (Gargas et al., 2009; Minnis and Lindner, 2013). This is a widespread, epizootic disease affecting hibernating bats, and this disease is associated, inter alia, with an unprecedented bat mortality in the United States and Canada (Blehert et al., 2009). Therefore, we took into account incubation at different temperatures to obtain a wide spectrum of species of fungi.

Table 3. Mean concentrations of filamentous fungi cultured from the air around the bat guano collected in the Harmanecká and Driny Caves.

Cave	Fungi Species	Air (CFU/m^3)		Total
		Individual Species		
		Range of Values	Mean ± SD[a]	
Harmanecká				211.3
	Absidia glauca	4.0 – 10.0	6.7 ± 2.2	
	Aspergillus foetidus	1.0 – 3.0	2.1 ± 0.8	
	Botrytis cinerea	7.0 – 16.0	11.8 ± 3.0	
	Cladosporium herbarum	8.0 – 19.0	13.1 ± 3.6	
	Epicoccum nigrum	0.0 – 3.0	1.8 ± 1.1	
	Gliocladium roseum	1.0 – 3.0	2.1 ± 0.9	
	Mucor hiemalis	7.0 – 33.0	20.1 ± 9.5	
	Penicillium chrysogenum	9.0 – 26.0	18.3 ± 6.2	
	Penicillium granulatum	49.0 – 139.0	96.2 ± 33.4	
	Penicillium lanosocoeruleum	5.0 – 12.0	8.9 ± 2.1	
	Penicillium urticae	7.0 – 17.0	11.5 ± 3.5	
	Rhizopus stolonifer	9.0 – 27.0	18.7 ± 6.5	
Driny				175.7
	Alternaria alternata	1.0 – 3.0	1.9 ± 0.9	
	Aspergillus fumigatus	4.0 – 10.0	6.8 ± 2.0	
	Cladosporium herbarum	7.0 – 13.0	10.1 ± 1.9	
	Epicoccum nigrum	2.0 – 9.0	5.2 ± 2.7	
	Mucor hiemalis	29.0 – 88.0	59.1 ± 23.1	
	Penicillium chrysogenum	7.0 – 12.0	8.9 ± 1.9	
	Penicillium granulatum	44.0 – 115.0	82.2 ± 28.7	
	Penicillium urticae	0.0 – 3.0	1.3 ± 1.3	
	Phoma fimeti	0.0 – 1.0	0.2 ± 0.4	

[a] SD = Standard Deviation.

Bat guano is one of the most important energy inputs for caves in temperate climate zones for survival and development of fungi (Poulson and Lavoie, 2000). Our results agree with those previously described, because mostly the same species of fungi were isolated from both the guano and the air samples, especially *Penicillium granulatum*, which predominated (Fig. 4, Table 2).

We isolated more fungal species from the air than from the guano. This agrees with other reports that the most species of fungi were isolated from the air samples in underground sites (Ogórek et al., 2013, 2014a; Pusz et al., 2014). Fungi are transferred to mines, adits, and caves by airflow from the external environment, so the majority of fungal species are in the air. The overall mean concentrations of CFU found in the caves in the present study, 175.7 and 211.3 CFU/m^3, were similar to, or lower than, those observed by other studies inside underground enclosures or caves. For example, Ogórek et al. (2014c) isolated from 245.5 to 1040.3 CFU of fungi in 1 m^3 of air from the underground Rzeczka complex, and Pusz et al. (2014) collected from 92 to 259 CFU from the underground Osówka complex, while Ogórek et al. (2013) isolated a maximum of only 232 CFU from

Niedźwiedzia Cave. The concentration of airborne fungi in the caves Harmanecká and Driny did not exceed official limits and norms for a health risk to tourists. According to the World Health Organization, air is not too contaminated unless it contains more than 1500 CFU of a mixture of species of fungi in 1 m^3 of air (World Health Organization, 1990).

According to Nováková (2009), the largest group of filamentous fungi isolated from bat guano constituted fungi of the genus *Penicillium*. We obtained similar results. In addition, we isolated filamentous fungi, such as *Absidia glauca*, *Aspergillus* spp., *Mucor hiemalis*, and *Rhizopus stolonifer* and one species from the yeast-like group, *Rhodotorula glutinis*. According to other researchers, bat guano is also a reservoir of fungi that may be dangerous for mammal health such as *Candida* spp., *Geomyces* spp., *Microsporum* spp. *Trichosporon* spp., and *Trichophyton* spp. (Nováková, 2009; Mulec et al., 2013). In our studies, we did not detect any of the above fungi in the bat guano. Especially important is the absence of detection of *Pseudogymnoascus destructans* (Gargas et al., 2009; Minnis and Lindner, 2013).
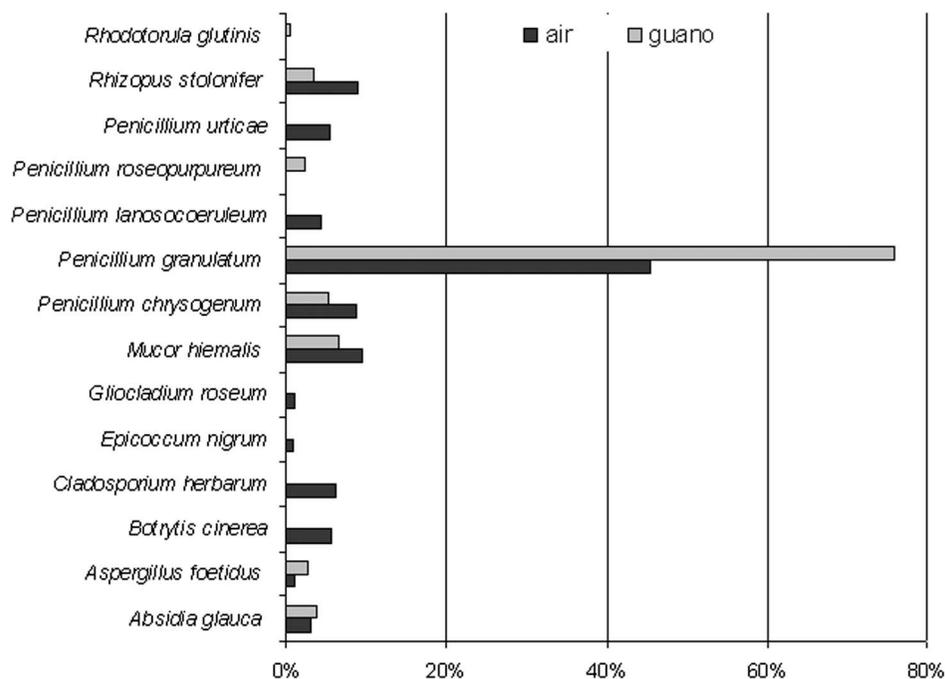
Figure 2. Relative percentages of colonies of filamentous fungi and yeast-like fungal species cultured from the bat guano and the air around it from Harmanecká Cave.

The most frequently isolated fungus from the guano and from the air collected in Harmanecká Cave and Driny Cave was *Penicillium granulatum*, with the exception of the guano collected in Driny Cave, where *Mucor hiemalis* was the most common. These species of fungi are common in many parts of the world. Their conidia and spores can be found in air, water, and soil and on animals and plants (Samson et al., 2011; Madden et al., 2012).

M. hiemalis may cause, for example, cutaneous and subcutaneous mucormycosis in humans belonging to the risk groups (Desai et al., 2013). *P. granulatum* may produce secondary metabolites, including mycotoxins, that are dangerous to animals and humans (Koteswara Rao et al., 2011). It can also cause allergic alveolitis, chronic respiratory symptoms, and sensitization by antigens from mold conidia (Qiu et al., 2014). Probably fungi such as
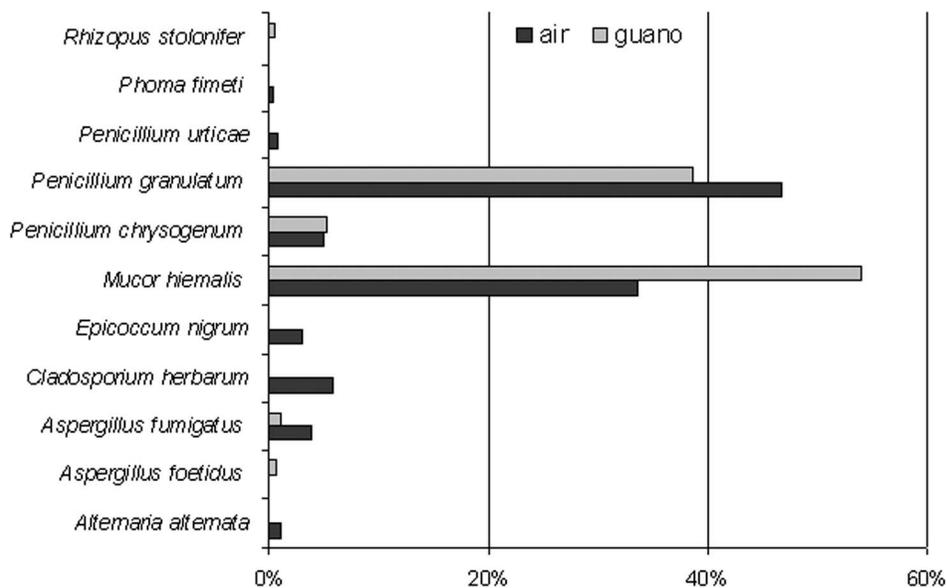

Figure 3. Relative percentages of colonies of filamentous fungal species cultured from the bat guano and the air around it in Driny Cave.
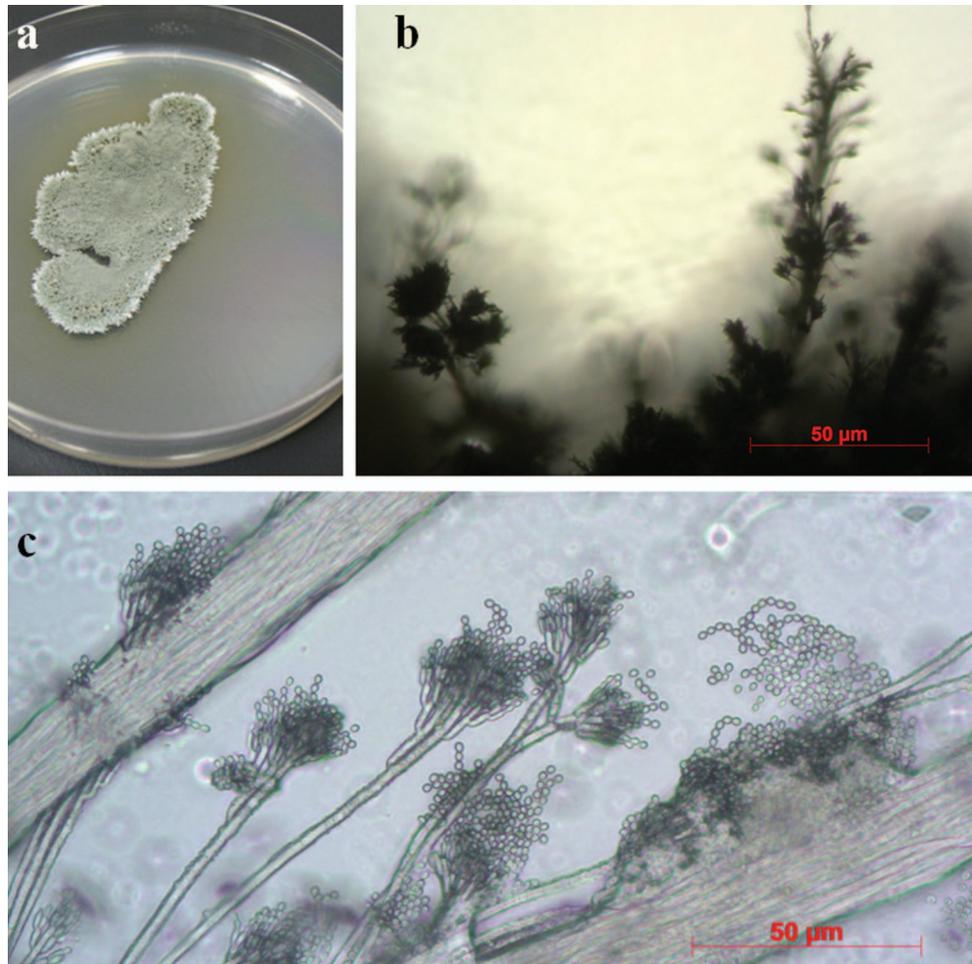
Figure 4. *Penicillium granulatum* **culture on Potato Dextrose Agar medium, 10-day-old culture at 25 °C: a) top view of a colony; b) branched conidiophores under the optical microscope; c) branched conidiophores, phialides, and conidia under the optical microscope.**

Penicillium spp. are also able to colonize membranes of hibernating bats. According to Johnson et al. (2013), fungi belonging to the *Penicillium* genus accounted for about 13% of all the strains isolated from hibernating bats. Nevertheless, the pathogenicity of species belonging to the *Penicillium* genus is controversial.

Conclusions

The results of the present study show that bat guano is a very good substrate for the development and survival of fungi inside caves, and it can also be a reservoir of fungi harmful to bats and humans. However, air samples from both caves contained more species of fungi than the bat guano, because the majority of fungi are transferred to underground ecosystems with bioaerosols from the external environment. Therefore, the external environment around an underground site and airflows are the major factors that affect the species composition and concentration of airborne fungi in indoor air of caves. The most frequently isolated species from the indoor and outdoor air and from the bat guano were fungi from the genus *Penicillium* (especially *P. granulatum*), except those isolated from guano of Driny Cave, where *Mucor hiemalis* was predominant. These fungi are common in many parts of the world. The observed concentrations and species composition of the fungi isolated from the air in both caves constitute no threat to the health of visiting tourists. We used only culture-based analysis of the fungal species, which did not allow us to detect non-culturable fungi. Therefore, in the near future, we are also going to use molecular techniques for in-depth characterization of environmental microbial communities in the case of underground mycobiota.

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