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Fungal spore content of the atmosphere of the Cave of Nerja (southern Spain): Diversity and origin

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ABSTRACT

Fungal spores are of great interest in aerobiology and allergy due to their high incidence in both outdoor and indoor environments and their widely recognized ability to cause respiratory diseases and other pathologies. In this work, we study the spore content of the atmosphere of the Cave of Nerja, a karstic cavity and an important tourist attraction situated on the eastern coast of Malaga (southern Spain), which receives more than half a million visitors every year. This study was carried out over an uninterrupted period of 4 years (2002–2005) with the aid of two Hirst-type volumetric pollen traps (Lanzoni VPPS 2000) situated in different halls of the cave. In the atmosphere of the Cave of Nerja, 72 different spore types were detected during the studied period and daily mean concentrations of up to 282,195 spores/m^3 were reached. Thirty-five of the spore types detected are included within Ascomycota and Basidiomycota (19 and 16 types, respectively). Of the remaining spore types, 32 were categorized within the group of so-called imperfect fungi, while Oomycota and Myxomycota were represented by 2 and 3 spore types, respectively. *Aspergillus*/*Penicillium* was the most abundant spore type with a yearly mean percentage that represented 50% of the total, followed by *Cladosporium*. Finally, the origin of the fungal spores found inside the cave is discussed on the basis of the indoor/outdoor concentrations and the seasonal behaviour observed.

1. Introduction

Large karstic caves have always aroused the curiosity of both scientists and the general public, so many have become tourist attractions visited by large numbers of people each year. Moreover, during recent years, the study of fungal allergens associated with allergies has become a matter of great importance (Kurup et al., 2002). Many indoor studies have been carried out in Spain and throughout the world in different buildings, such as hospitals (Sautour et al., 2009), homes (Basilico et al., 2007; Crawford et al., 2009), offices (Baxter et al., 2005; Law et al., 2001), schools (Meklin et al., 2003; Scheff et al., 2000), factories (Awad et al., 2010), farms (Miao et al., 2010), stables (Beck et al., 2007), markets (Arya and Arya, 2007), museums (Camuffo et al., 1999; Niesler et al., 2010), wineries (Li and LaMondia, 2010), churches (Aira et al., 2007), etc. However, after an exhaustive search of the literature, there seem to be few works that address the study of biological airborne particles inside natural cavities. Although some references were found such as Bastian et al. (2010), Borda and Borda (2006), Borda et al. (2009), Groth et al. (1999), Jurado et al. (2009), Koilraj et al. (1999), and Mulec et al. (2002), most studies used viable methods and counting of colony forming units (CFUs), usually over a short period of time, which means that the results of our study at Nerja, which involved a non-viable uninterrupted sampling over 4 years, cannot be compared with similar studies.

The aerobiological study was carried out inside the Cave of Nerja, situated near the village of the same name, southern Spain. This coastal town, (21 m above sea level), is located 65 km east of the capital of the province, Malaga, on the border with the province of Granada.

The surrounding area of the Cave of Nerja is situated in the thermo-Mediterranean belt (Rivas-Martínez, 1981) and is characterized by mild frost-free winters. The rainfall is unevenly distributed throughout the year, the highest levels occurring in early autumn and late winter–early spring. This leads to a prolonged drought that causes a large water deficit in the soil from June to October, which is further accentuated by the high temperatures reached during the summer.

The cave was formed by the dissolution of the calcium–magnesium carbonate of the dolomitic rocks that comprise the mountain range of Almijara. It is situated 158 m above sea level and 800 m from it. It is thought that the genesis of the cave started in the Pliocene although the greatest lithogenic activity took place during the Middle Pliocene (Carrasco, 1993). Although the Cave of Nerja was re-discovered in 1959, evidence (archaeological remains and examples of rock art) indicate that it was inhabited by members of an uninterrupted succession of prehistoric cultures from the beginning of the Upper Palaeolithic (20,000 B.C.) to the Bronze Age (1800 B.C.) (Sanchidrian, 1994). Nowadays, the surroundings of the cave have been developed into a tourist complex that receives over 500,000 visitors a year. This natural

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cave in which one of the most noteworthy features is the huge size of its halls with a total volume of more than 250,000 m^3 and a topographic development of 4823 m, is divided into two zones: the low galleries, which can be visited by tourists and the general public (representing a third of the total), and the high galleries and new galleries which, for the moment, cannot be visited (Carrasco et al., 1998). The low galleries (zone in which our study was carried out) have a major axis 250 m in length and consist of a succession of big halls and small diverticula separated by substantial speleothem deposits. Nowadays, the cave is accessible from an artificial opening dug through a holocenic plug of sedimentary origin.

The inner temperature in the Nerja Cave remains more or less constant throughout the year (18–19 °C), while the relative humidity is high, varying between 78 and 100%, the maximum values being recorded in summer. This phenomenon is due to a seasonal lag between the rainwater entering the rock and its dripping out, as a consequence of its slow circulation through the marble that forms the ceiling of the cave (Liñán et al., 2002).

Several works carried out on the concentration of radon in the atmosphere of the cave have revealed that it is relatively well ventilated. In autumn and winter, when the outside temperature is lower than inside the cave, the air flow is better and the air is replaced in 1 day, while in spring and summer the same process lasts up to 5 days (Cañete, 1997; Dueñas et al., 1993).

The aim of this study was to analyse the airborne spore content in a frequently visited natural cave, to assess the incidence of the same in its atmosphere and the variations that occur throughout the year, a topic to which we have found no references in the literature. In addition to the impact that the spores of these fungi have on human health, another factor to be considered is their importance as causal agents of geological changes (Burford et al., 2003). Fungi are involved in the precipitation of calcium carbonate (Northup and Lavoie, 2001), as well as in the degradation of limestone materials. Many of them produce acid compounds and pigments that cause corrosion and stain substrates (Bastian et al., 2010; Šimonovičová et al., 2004).

2. Materials and methods

2.1. Type and location of the samplers

The sampling was carried out with the aid of two non-viable Hirst-type volumetric spore traps (Hirst, 1952), a seven-day recorder (model VPPS 2000) manufactured by Lanzoni, in which the vacuum pump was regulated at a constant suction flow of 10 l/min. For this study, the peaks and the weather vanes were removed from the spore traps, since they were in a sheltered and fixed position, with the air inlet facing the corridor through which visitors pass.

The first sampler was screwed to the floor of the so-called "Hall of the Manger", the first chamber after the stairs of the main entrance, near the hall entrance and the exit, 1.5 m from the cement pathway along which the visitors pass. This chamber is a vaulted corridor, which is 20 m long, 10 m wide and 5 m high.

The second sampler was situated in another hall, the so-called "Hall of the Cataclysm", further from the entrance and exit. This is a huge chamber with a large central column 32 m in height, situated in the lowest point of the cave and at the end of the visited area (Carrasco et al., 1998). In this case the sampler was also screwed to the floor but at a height of about 3 m above the pathway.

2.2. Sampling treatment and spore counting

The data obtained from the sampler in the "Hall of the Manger" were used for both the qualitative and quantitative study of the fungal spores inside the cave. The sampling was carried out from 1 January 2002 to 31 December 2005, uninterruptedly, except for the period of 24–25 July, from 4 to 6 November 2002 and from 2 to 5 January 2005 because of a failure in the electrical power supply. The second sensor, located in the "Hall of the Cataclysm", was operational from 1 April 2003 to 31 December 2005. Silicone fluid (silicone dissolved in carbon tetrachloride at a concentration of 2%) was used as adhesive substance, and glycerine jelly as mounting medium.

As regards the spore counts a longitudinal sweep at 1000× was made because of the large number of small fungal spores that would otherwise go undetected and a second sweep was performed at 400×, which allows a wider microscopic field, reducing the error when extrapolating the total number of spores counted to the whole sampling. Finally, the data obtained were expressed as the number of spores per cubic metre of air (daily mean concentration).

In this paper we have followed the classification proposed in the IX edition of Ainsworth and Bisby's Dictionary of Fungi (Kirk et al., 2001). However, although there is a tendency to ignore the category "imperfect" or "mitosporic" in favour of including such fungi in previously existing groups, we prefer to use this artificial group in order to facilitate the grouping of the spores on the basis of their sexual or asexual origin.

2.3. Comparative study

After the qualitative and quantitative study, we proceeded to compare the results obtained by the two samplers installed inside the cave with the aim of analysing the heterogeneity of the spore spectrum in the atmosphere of the cave. Although both samplers operated uninterruptedly, for this study we only took the data corresponding to 1 day a week at a time interval of 8 days in order to avoid the bias that might arise from using the same day of the week. In this way, we took into account the daily mean concentrations of the overlapping sampling period for the years 2004–2005, in which the two samplers operated simultaneously.

The study is accompanied by a statistical analysis, using a Spearman's correlation test, searching for degrees of association between the concentrations of spores obtained by both samplers.

However, since the cave is not a totally isolated environment, we could observe the presence of pollen in the interior whose provenance cannot be other than outdoors. So, if we compare the outdoor/indoor concentrations of pollen, it is possible to estimate the percentage of biological particles that enter the cave due to the replacement of air. For this study we used the daily and monthly total pollen concentrations from a third sampler situated outside, near the entrance to the cave (Docampo et al., 2007), as well as the data obtained from the sampler located in the "Hall of the Manger" during the years of simultaneous sampling (2002 and 2003). Besides the pollen concentrations, we also compared the concentrations of two spore types (*Cladosporium* and *Alternaria*) with the levels reached by these types in the atmosphere outside the cave.

Finally, we analysed the monthly indices obtained for the three samplers (2 inside and 1 outside), setting a rate that gave a value of 1 to the level found outside (point of origin), while for the other two samplers the rates were obtained as a function of the first. Thus, the rates of monthly indices of the samplers in the "Hall of the Manger" and "Hall of the Cataclysm" were calculated by dividing their respective indices by the monthly index obtained outdoors. For this, the data of 2003 were taken, because this was the only year in which the samplings of the 3 spore traps coincided. No data are available for the first 3 months in the "Hall of the Cataclysm", because sampling did not start until April.

3. Results

3.1. Spore diversity

In the study carried out with the aid of the sampler located in the "Hall of the Manger" a total of 72 spore types were identified, 35 of which are included within Ascomycota and Basidiomycota (19 and 16 types, respectively). Of the remaining types, 32 were categorized within the group of so-called imperfect fungi, while Oomycota and Myxomycota were represented by 3 and 2 spore types, respectively. Table 1 shows a list of the types found and identified.

As regards the spore index for the different years, the highest percentage in all cases corresponded to the group of imperfect fungi with a mean value close to 68% of the annual total (Fig. 1). Although the number of spore types identified as belonging to Ascomycota was similar to Basidiomycota, the spore annual rate differed (2 and 21% respectively). The remaining groups never exceeded 0.15%.

Throughout the study period 2002–2005 a mean annual spore index of 856,033 spores were recorded, which represented a daily average concentration of 2345 spores/m^3, the higher levels being registered in 2003 with over a million spores (Fig. 2). However, the spore levels were not uniformly distributed in the air of the cave throughout the year, and considerable differences were detected between different days, ranging from 7 to 282,195 spores/m^3 of air, with more than 1000 spores/m^3 being recorded on 60% of the days (Fig. 3).

Table 1
Spore types identified at the "Sala del Belén", annual mean spore index and percentage reached by the different types studied. * Percentage lower than 0.01%. The values in parentheses show the number of spore types included in each category.

Ascomycota (18) Ph. fungi	Mean values index	%	Imperfect fungi (33)	Mean values index	%
Ascobolus	2	0.00*	*Alternaria*	1110	0.13
Chaetomium	146	0.02	*Arthrinium*	429	0.05
Delitschia	95	0.01	*Aspergillus/Penicillium*	430,032	50.24
Diatrypaceae	1305	0.15	*Asperisporium*	44	0.00*
Didymella	8339	0.97	*Basipetospora*	97	0.00*
Emericella	44	0.00*	*Beltrania*	2	0.00*
Helicogermslita	42	0.00*	*Bispora*	38	0.00*
Herpotrichia	1	0.00*	*Botrytis*	760	0.09
Keissleriella	49	0.00*	*Cercospora*	210	0.03
Leptosphaeria	5415	0.63	*Circinotrichium*	1	0.00*
Leptosphaerulina	31	0.00*	*Cladosporium*	146,227	17.08
Phaeosphaeria annulata	324	0.04	*Curvularia*	37	0.00*
Pleospora	655	0.78	*Diplodina*	17	0.00*
Sordaria	231	0.03	*Drechslera*	209	0.02
Sporormiella	10	0.00*	*Endophragmiella*	7	0.00*
Telochistaceae	5	0.00*	*Epicoccum*	72	0.00*
Venturia	734	0.09	*Fusariella*	1	0.00*
Xylariaceae	29	0.00*	*Fusarium*	63	0.00*
			Helicoma	9	0.00*
Basidiomycota (16)			*Helicomyces*	524	0.06
Ph. fungi			*Massarina*	33	0.00*
Agaricus	108,541	12.52	*Nigrospora*	94	0.01
Agrocybe	973	0.11	*Oidium*	124	0.01
Boletus	49,119	5.75	*Periconia*	120	0.01
Bovista	22	0.00*	*Phitomyces*	65	0.00*
Calvatia	99	0.01	*Polythrincium*	60	0.00*
Coprinus	5509	0.64	*Septonema*	12	0.00*
Cortinarius	251	0.03	*Septoria*	4267	0.50
Ganoderma	380	0.04	*Spegazzinia*	14	0.00*
Inocybe	18	0.00*	*Stachibotrys*	308	0.04
Puccinia	62	0.00*	*Stemphylium*	77	0.00*
Russula	233	0.03	*Torula*	418	0.05
Scleroderma	1	0.00*	*Tripospermum*	9	0.00*
Tilletia	16	0.00*			
Tomentella	5687	0.66	Oomycota (3)		
Uredospora[a]	97	0.01	Ph. chromista		
Ustilago	13,626	1.59	*Albugo*	94	0.01
			Peronospora	82	0.00*
Myxomycota (2)			*Plasmopara*	21	0.00*
Ph. protozoa					
Myxomycete[b]	1065	0.12			
Trichia	71	0.00*			

[a] Excluding *Puccinia*.
[b] Excluding *Trichia*.

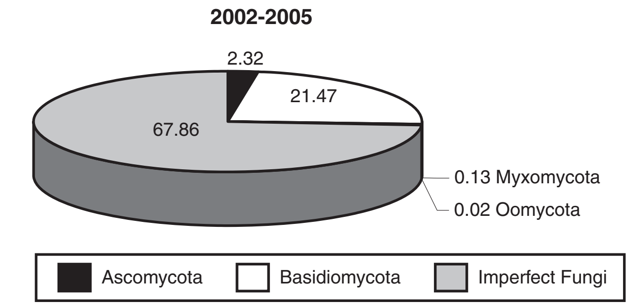

Fig. 1. Annual mean percentage of the annual index reached by the different spore groups during the period of 2002–2005.

With respect to the annual total spore distribution, three peaks were evident: a very intense peak during the summer and two more, although with considerably lower concentrations, in spring and autumn. However, the behaviour differed according to the taxonomic groups since the spore types belonging to imperfect fungi showed the highest levels during the summer, when the rest of the groups showed their minimum values, reaching their maximum concentrations in autumn (Fig. 4).

Aspergillus/Penicillium was the most abundant spore type throughout the monitoring time, representing, on average, 50% of the total spore counts (Table 1). This spore type was followed by *Cladosporium*, except in 2005, when the *Agaricus* type was in second position. In any case, the quantities of these spore types were less than half of those of *Aspergillus/Penicillium*. *Boletus* and *Ustilago* presented even lower rates on average, especially *Ustilago* whose annual spore index did not exceed 2.6% of the total in the best case. The remaining types identified did not reach 1% of the annual total, except *Didymella* in 2004, with 2% of the annual total. Fig. 3 shows the daily mean concentrations of the two more relevant spore types over the 4 years sampled.

3.2. Origin

In the "Hall of the Cataclysm", situated at the end of the cave, where the outside influence is lower, fewer spore types were registered, with only 45 types identified, and none differing from those found in the other hall. In this hall, the number of spores detected was just over 23% of the total recorded in the "Hall of the Manger", which is located at the beginning of the cave and, therefore, more susceptible to external influence (Table 2). Although the daily mean spore concentrations on 50% of the days were less than 200 spores/m^3, the most important spore types evenly matched in the two halls (Fig. 5).

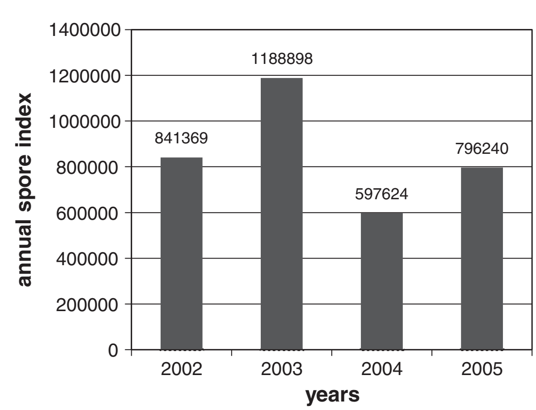

Fig. 2. Annual total spore index registered in the "Hall of the Manger" for the different years studied.

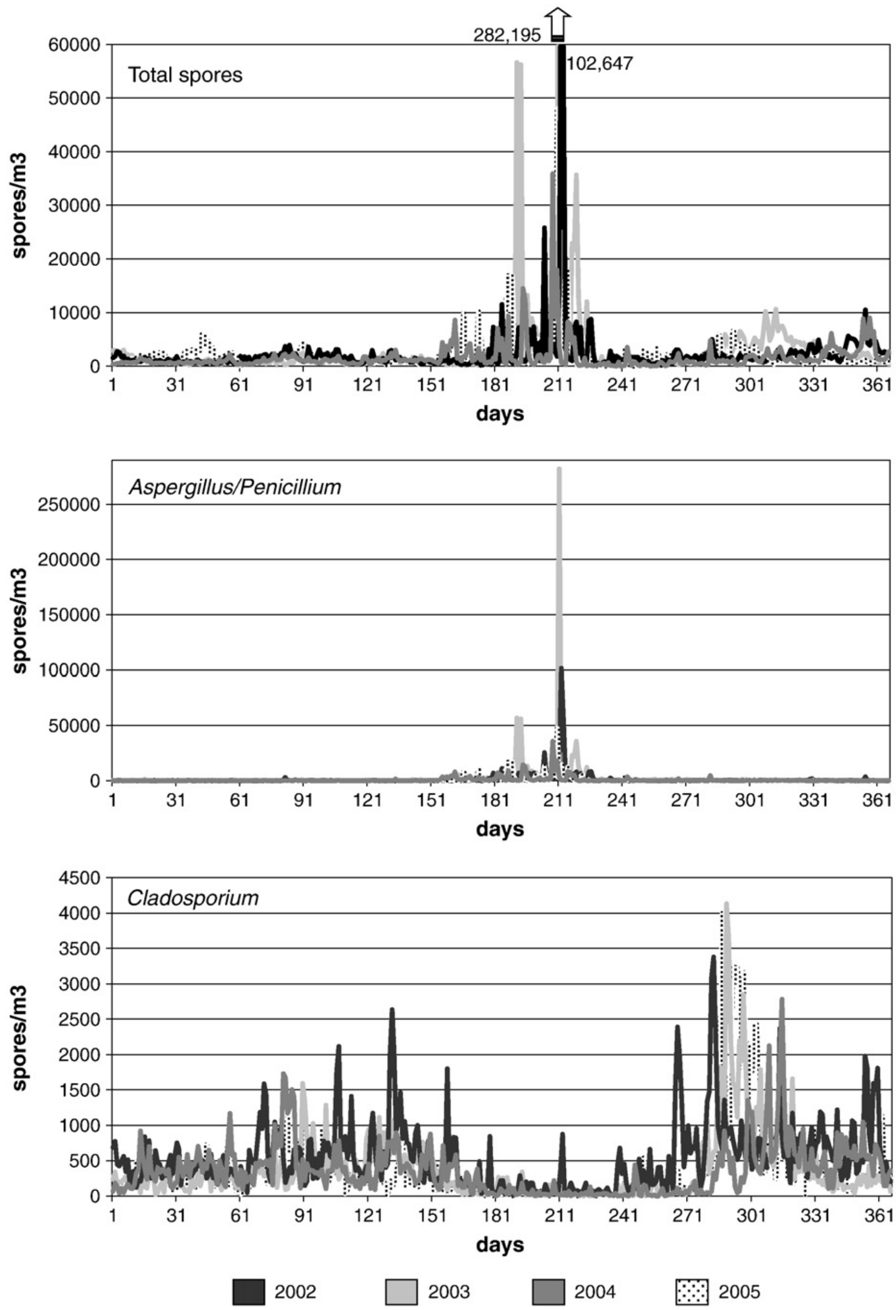

Fig. 3. Seasonal behaviour of the total daily mean spore concentrations and the two more relevant spore types during the different years studied.

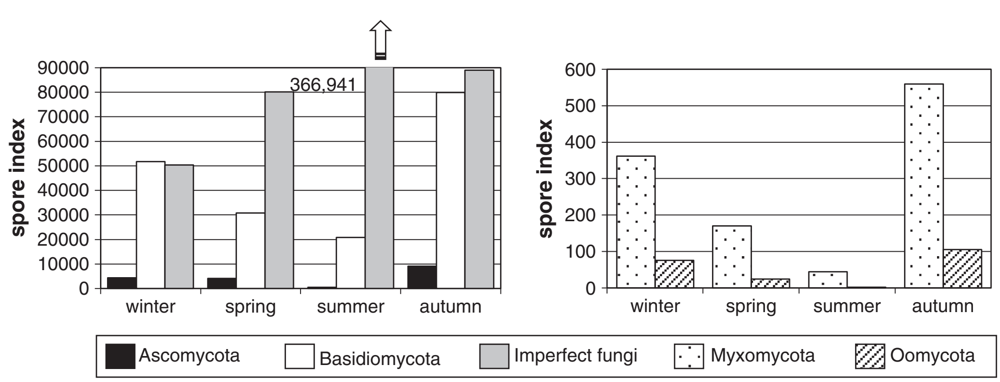

Fig. 4. Seasonal mean spore index reached by the different spore groups during the studied period.

Table 2
Spore types identified at the "Sala del Cataclismo" and annual percentage reached by the different types studied. * Percentage lower than 0.01%. The values in parentheses show the number of spore types included in each category.

Ascomycota (8) Ph. fungi	%	Imperfect fungi (22)	%
Chaetomium	0.02	*Alternaria*	0.09
Didymella	0.17	*Arthrinium*	0.04
Leptosphaeria	0.13	*Aspergillus/Penicillium*	65.06
Phaeosphaeria annulata	0.02	*Asperisporium*	0.00*
Pleospora	0.02	*Basipetospora*	0.31
Sordaria	0.00*	*Bispora*	0.07
Venturia	0.01	*Botrytis*	0.00*
Xylariaceae	0.00*	*Cercospora*	0.00*
		Cladosporium	13.08
Basidiomycota (11)		*Curvularia*	0.00*
Ph. fungi		*Drechslera*	0.00*
Agaricus	6.25	*Epicoccum*	0.01
Agrocybe	0.06	*Massarina*	0.00*
Boletus	3.79	*Oidium*	0.00*
Bovista	0.00*	*Periconia*	0.01
Coprinus	0.13	*Phitomyces*	0.02
Cortinarius	0.00*	*Polythrincium*	0.00*
Ganoderma	0.02	*Septoria*	0.00*
Puccinia	0.02	*Stachibotrys*	0.00*
Tomentella	0.08	*Stemphylium*	0.02
Uredospora[a]	0.00*	*Torula*	0.02
Ustilago	2.17	*Tripospermum*	0.00*
Myxomycota (2)		Oomycota (2)	
Ph. protozoa		Ph. chromista	
Myxomycete[b]	0.04	*Albugo*	0.00*
Trichia	0.03	*Peronospora*	0.00*

[a] Excluding *Puccinia*.
[b] Excluding *Trichia*.

After checking the heterogeneity of the atmosphere of the cave, attempts were made to ascertain the origin of the spores. For this, we compared the concentrations registered in the interior ("Hall of the Manger") for the spore types *Cladosporium* and *Alternaria*, with those found outdoors, and registered by a third sampler located in the surroundings of the cave (Docampo et al., 2007). The results showed a significant positive correlation in almost all cases (Fig. 6). It is noteworthy that, although the differences were not homogeneous, the annual spore index was higher outside. While in spring and summer the concentrations were significantly lower in the interior, in autumn and winter these differences were not only less pronounced, especially in the case of *Cladosporium*, but concentrations in the atmosphere of the cave even outnumbered the outside ones on 39 and 52 days in 2002 and 2003, respectively (Table 3).

This topic, which will be discussed in the next section along with other aspects, will help to elucidate the origin of the spores in the Cave of Nerja.

4. Discussion

A feature that characterizes fungal spores is their high frequency in both indoor and outdoor environments. About 100,000 species have been identified so far (Curtis et al., 2004), although the species list increases every year, and it is estimated that the total number could reach a million and a half (Hawksworth, 2001). During this study carried out in Nerja Cave, a total of 72 spore types were identified, with a total spore index in the studied period of 3,424,131, of which about 8% corresponded to spores that could not be identified.

If the results obtained in other studies carried out indoors are analysed, it can be seen that, although the number of types identified may differ, imperfect fungi always represent a high percentage of the spores recorded, as was the case in our analysis (Chakraborty et al., 2000; El-Morsy, 2006). Although we have found no study comparable in sampling time and methodology with the present, Koilraj et al. (1999) described a total of 42 species in a study published on several caves in India, of which the vast majority were imperfect fungi. In studies performed outdoors, which are more frequent, a higher number of airborne spores belonging to this group were also found (De Antoni et al., 2006; Díez et al., 2006; Mallo et al., 2010; Mitakakis and Guest, 2001; Morales et al., 2006; Sabariego et al., 2000).

As regards the seasonal distribution of spores, some differences from the trends observed in other studies emerged. While the curves of seasonal distribution of total spores in several Spanish cities such as Madrid (Díez et al., 2006), Seville (Morales, 2004) and Cartagena (Elvira, 2001) presented a bi-seasonal behaviour, with the presence of two peaks, one in autumn and another in spring, this did not occur in the Nerja Cave, where three peaks were evident, the highest in summer. So, the distribution of daily concentrations of spores throughout the year differs from those found elsewhere with similar weather conditions and, despite being situated in a Mediterranean climate area, the patterns in the Cave of Nerja showed a greater resemblance to those found in cities with a colder and more continental climate, such as Palencia (N Spain)

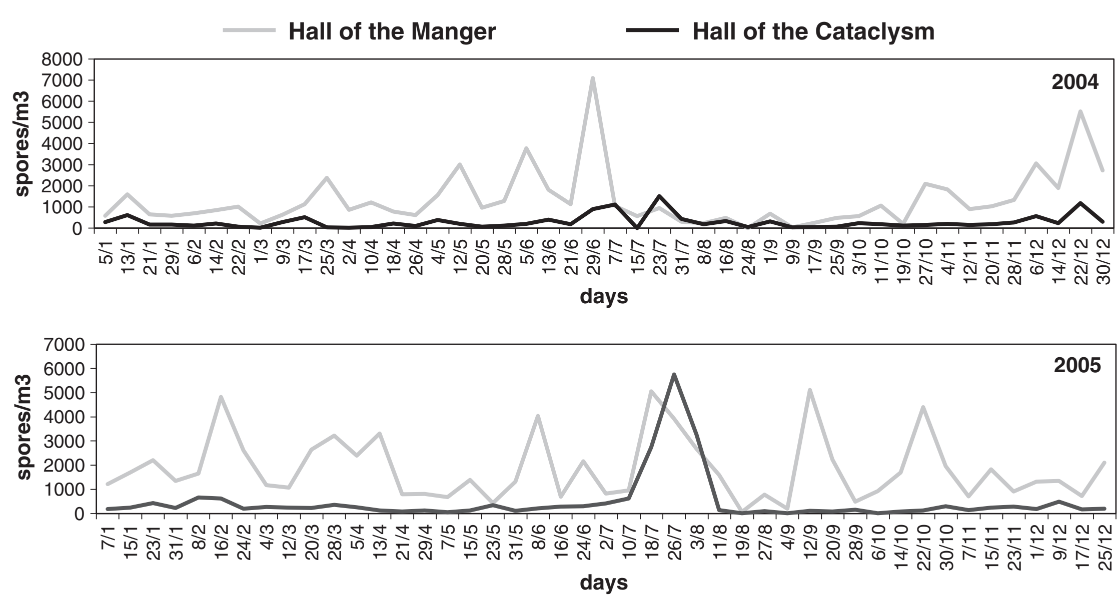

Fig. 5. Daily mean spore concentration distribution in "Hall of the Manger" and "Hall of the Cataclysm" during the matching days (2004–2005).

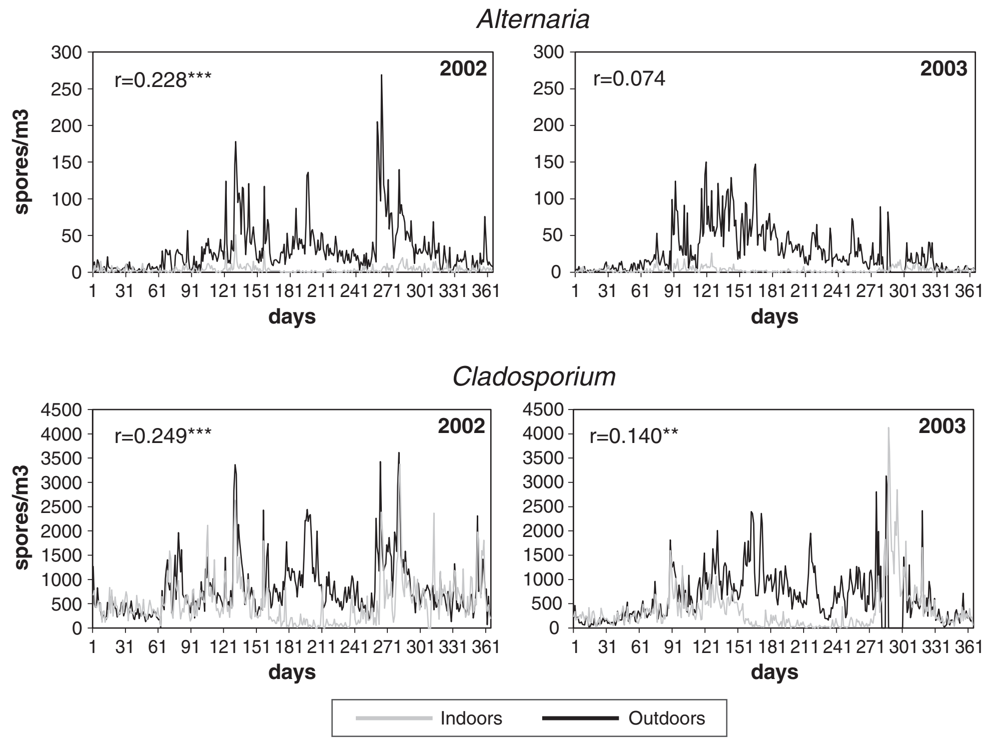

Fig. 6. *Alternaria* and *Cladosporium* daily mean spore distribution indoors ("Hall of the Manger") and outdoors. Years 2002 and 2003.

or other cities situated in northern Europe (Herrero et al., 1996; Spieksma FTHM, 1995), in which a high peak occurs during the summer, accompanied by other smaller ones in spring and autumn.

However, in our case, not all the spores showed the same seasonal pattern, and two different and opposite patterns were evident: while imperfect fungi reached their highest concentrations in summer, the other groups (Ascomycota, Basidiomycota, Myxomycota and Oomycota) presented their lowest levels in that season. These low levels registered by the second group in summer coincide with the results obtained in Cartagena, SE Spain, (Elvira, 2001) and Saudi Arabia (Al-Suwaine et al., 1999), where the high summer temperatures and drought periods may hinder the development of many fungi, which tend to present their highest values during autumn and spring, coinciding with the rainy seasons.

Despite the high number of spores detected, the annual percentage reached by over 91% of the different types identified did not exceed 1% of the annual index, *Aspergillus*/*Penicillium* type (50%), followed by *Cladosporium* (17%) were the most abundant spore types, especially in July and August, a period in which only 32 out of 70 spore types were detected. As regards the behaviour of these two most abundant spore types, many similarities have been found in the literature reviewed. Thus, while in studies carried out outdoors the most abundant spore type is *Cladosporium*, representing in many European cities 40 to 80% of the total spore numbers in the air (Spieksma FTHM, 1995), *Aspergillus*/*Penicillium* type is the most abundant indoors, as was the case in Nerja Cave. The same pattern has also been observed in many indoor environments such as factories, poultry farms, warehouses and hospitals in India and Egypt (Awad et al., 2010; Sharma et al., 2010), in several buildings in Kansas (Portnoy et al., 2001) as well as in comparative studies carried out indoors and outdoors (Awad, 2007; Nayar et al., 2007), among others.

Given the clear dominance of one spore type, we should perhaps ask ourselves whether the general behaviour discussed above for all the spores actually represents the behaviour of one or a few dominant types. For this purpose, *Aspergillus*/*Penicillium* type were removed from the total spore counts, which resulted in a completely different behaviour, whereby the dominant peaks appeared in spring and autumn instead of summer, and the levels found in summer greatly decreased. This resulting pattern resembles that found in many other cities as mentioned above. The particular behaviour of *Aspergillus*/*Penicillium* type in Nerja Cave has already been widely discussed by Docampo et al. (2010), human action being established as the cause of the high spore peaks detected inside the cave during the summer (end of July), particularly following the installation of a wooden stage for a festival of music and dance which, together with the high relative humidity reached in this season, favours the development of this kind of fungus.

Some types of spores commonly found in outdoor areas did not reach major indices in the cave. This is the case of *Alternaria* and *Botrytis*, the latter especially abundant in rural areas with agricultural crops, since it is a plant pathogen as *Oidium* and *Plasmopara* (Reineria et al., 1998), which accounted only for 0.13% of the total spore index in the case of *Alternaria*. The remaining types hardly reached 0.1% of the total. This very low presence of certain spore types indoors was already described by Emberlin et al. (1995) in a study carried out in London homes.

Caves are generally considered as extreme environments for the development of life due to the absence of light, which prevents the setting of plants and other photosynthetic organisms, which, ultimately, are the primary producers of organic matter. This limits nutrient sources and, therefore, the ability to colonize this environment (Burford et al., 2003). On the other hand, we have already mentioned how spore distribution patterns in outdoor environments reflect the behaviour of

Table 3
Mean percentage rates (2002–2003) obtained indoors/outdoors for *Cladosporium* and *Alternaria* spore types.

Spores inside "S. Belén" according to outdoor spores		
	Cladosporium type	*Alternaria* type
Winter	130.7	57.7
Spring	59.5	13.0
Summer	19.8	5.8
Autumn	115.5	51.2
Annual	80.3	30.5

the vast majority of the spores found in our study. This high similarity, together with the lack of macroscopic organic matter or litter in the cave, the fact that no mycelia or sporocarps of fungi were observed, and no colonies were detected after sampling several substrates at different points in the cave, led us question the view that the cave itself might be the source of the spores detected, especially in the case of basidiospores and many fungal spores from parasites of plants, suggesting, therefore, that their origin lies outdoors. This would be consistent with the comments of authors such as Sterling and Lewis (1998) and Shelton et al. (2002), in whose studies, the fungal spores found indoors mainly came from outside.

Moreover, in studies carried out in several caves, such as Altamira and Tito Bustillo in Northern Spain, and the Grotta dei Cervi in Porto Badisco, Italy, only bacteria and Actinomycetes were isolated from walls and other surfaces (Groth et al., 1999, 2001). Shapiro and Pringle (2010) found differences in analysis of the fungal content of several caves with diverse levels of human disturbance in USA, suggesting that many fungi found in caves were carried into from outside since no fungi were isolated from the sites in which the number of visitors is very low, a certain level of disturbance being necessary to carry spores into a cave. Another case is that related by Jurado et al. (2010) who studied a fungal outbreak inside Castañar de Ibor cave (Spain) showing that it was related to anthropic action.

To obtain more data to support this hypothesis, we looked for a possible relationship between an outdoor meteorological parameter, rainfall, and some spore types with a proven association between the rainy season and increases in their airborne concentrations, as is the case of many Basidiomycota and Ascomycota (Díez et al., 2006; Jones and Harrison, 2004; Van Osdol et al., 2004). For the Cave of Nerja, a significant positive correlation was established between rainfall and the concentration of *Didymella* spore type inside the cave, $r=0.455$ ($p\leq0.001$). Also positive and significant was the correlation established for *Agaricus* and *Boletus* types ($r=0.204$ and $r=0.202$, respectively; $p\leq0.001$). In principle, there would be no reason why this association should exist because the conditions inside the cave are not affected by rainfall.

As mentioned in the Results section, the relationship between the concentrations of the *Cladosporium* and *Alternaria* spore types indoors and outdoors was not constant throughout the year. In principle, if the spores come from outside, a lower or equal number of spores could be expected indoors, which did not always occur (Table 3). However, in this study it should be taken into account that this natural cavity is more or less isolated from outside, and only communicates with the exterior world through narrow openings. For that reason, we think it opportune to mention some details of interest about the cave. Firstly, the rate of air renovation inside the cave is more or less limited, depending on the season. The ventilation rates ranges from 0.2 m^3/s during the hot season to 2.8 m^3/s during autumn–winter. This is accompanied by variations in wind speed inside the cave, since during the summer the velocity decreases and air renewal take longer. Moreover, another key factor that modifies the environmental characteristics is human activity. The number of visitors that the Cave of Nerja receives can reach more than 5000 in 1 day. The number of visitors is particularly high during the summer, August being the month that showed the greatest number of visitors during the study period, with over 100,000 people. This produces an increase in CO_2 and relative humidity, the increase in humidity being estimated at between 2 and 3% due to the water vapour exhaled by visitors. To this, must be added the delay mentioned above in the process of rainwater infiltration, a phenomenon that is particularly noticeable in summer (Carrasco et al., 2002).

For all the above mentioned reasons, the main differences in the number of spores between the two environments (indoors/outdoors) during the summer can be explained by the higher relative humidity resulting from both hydrogeological characteristics and the influence of visitors, together with the lower air velocity and lower renewal rate registered in this season, which makes it more likely that spores found inside will be deposited since their transport and re-suspension is hindered. However, during the rest of the year, especially during autumn and winter, both air renewal and wind speed are higher. This, together with lower humidity levels, leads to a greater spore buoyancy and facilitates transport from outdoors, the levels obtained inside being more similar to those registered outside. This behaviour has also been described by Kulmala et al. (1999), who developed a model to predict changes in concentrations of particulate matter indoors, based on the influence of external conditions. In his study, low rates of ventilation caused major differences between indoor and outdoor concentrations, the levels registered inside diminishing. In contrast, an increase in ventilation meant that similar levels were obtained in both environments and that internal levels could even exceed the external ones, as occurred in our study.

In the case of *Alternaria*, although this spore type showed similar behaviour to *Cladosporium* as regards inside/outside levels in the different seasons of the year, it was present at much lower levels in the interior of the cave, never reaching the outdoors seasonal indexes. This may be due to the larger size of this spore type, which largely diminishes its buoyancy, hindering the transport and decreasing the time spent in the air. This behaviour of *Alternaria* spore type coincides with that observed in a study carried out in households (Emberlin et al., 1995).

The areas located near the entrance are strongly influenced by external environmental conditions, while the innermost part is hardly affected by the exterior features since the internal conditions of the cave, with higher humidity and few oscillations in temperature, predominate in this area (Northup and Lavoie, 2001). This could explain both the higher number of spore types and the percentages detected in the “Hall of the Manger”, close to the exterior, compared with those recorded in the “Hall of the Cataclysm”. This behaviour was also described by Koilraj et al. (1999) in several caves in India, and by Borda and Borda (2006) in caves in Rumania. In accordance with this hypothesis, a negative gradient could be established from the cave entrance to the “Hall of the Cataclysm” passing through the “Hall of the Manger”.

Another finding that supports the entry of particles from outdoors is the pollen grains recorded in the atmosphere of the cave. In the case of pollen, as with spores, the introduction rate was higher in autumn and winter, showing an average annual rate of 10% compared to those found outside (Fig. 7). This rate cannot be generalized. In fact, the rates calculated for the two spore types studied indoors and outdoors, *Alternaria* and *Cladosporium*, were different: 80% and 30%, respectively. If we compare the different rates obtained, an inverse relationship between particle size and indoor/outdoor percentage can be observed: the smaller the particle, the greater the percentage. In other studies in which the indoor/outdoor content of certain airborne spores was compared, a greater quantitative difference was also observed in the case of larger spores, possibly due to the decrease in buoyancy. Thus,

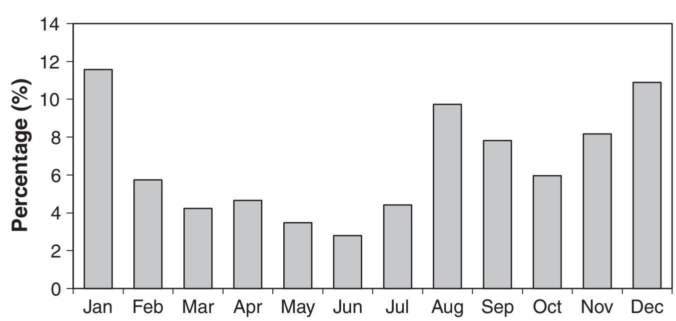

Fig. 7. Monthly percentages of pollen recorded inside the cave, referred to the total registered outdoors (average years 2002 and 2003).

authors such as Takahashi (1997) reported abundances for *Alternaria* and *Curvularia* of 313 and 10 CFU/m^3 respectively in exterior areas, while values hardly reached 80 and 3 CFU/m^3 indoors. For other smaller spores such as *Cladosporium*, the values were 469 and 535 CFU/m^3 outdoors/indoors, respectively.

Despite the external origin of the great part of the fungal spore types and the absence of fungal colonies, with the exception of *Aspergillus*/*Penicillium* spores commented, from the point of view of conservation of cultural heritage, it must be taken into account the effect that fungi, bacteria and other microorganisms could have in this karstic cave since they have been cited as responsible for the biodeterioration of surfaces of historic buildings, sculptures and even cave paintings (Bastian et al., 2010; González and Sáiz-Jiménez, 2004; Sáiz-Jiménez and González, 2007).

Finally, as regards the sampling method, many comparative studies between different spore-sampling devices can be found in the literature (Burge, 1990; Carvalho et al., 2008; Li and Lin, 1999; Morris et al., 2000; Pyrri and Kapsanaki-Gotsi, 2007; Spurgeon, 2003; Wang et al., 2001; Wüst et al., 2003). Although some authors consider Hirst-type samplers to be of little use for particles smaller than 5 μm (Spieksma FTHM, 1995), this type of sampler allows a continuous study, hour by hour, for a long period of time (4 years in this case), the results being available almost immediately after the sample collection (Baxter et al., 2005), unlike other types of spore traps, with time restriction records, and whose samples need to be incubated for several days.

5. Conclusions

In the atmosphere of the Cave of Nerja, 72 different spore types were registered during the studied period with daily mean concentrations of up to 282,195 spores/m^3. Although there are no standard indices related with the risk of exposure to spore concentrations (Fung and Hughson, 2003; Paugam et al., 1999), this high variety of spore types, together with the high levels detected of certain spore types described as allergens, such as *Aspergillus*/*Penicillium*, *Cladosporium*, *Boletus*, *Didymella* and *Ustilago* could represent a potential risk for visitors and the implementation of preventive measures is recommended, especially during the Festival of Dance and Music, when the levels of *Aspergillus*/*Penicillium* spores increases considerably. Moreover, fungal spores deposited on different surfaces of the cave may serve as nutrient for the development of other microorganisms, especially bacteria, which could affect the cave conservation.

The similar distribution patterns, the positive correlations shown between the concentrations of certain spore types inside and outside the Cave of Nerja, as well as the negative gradient established from the cave entrance to the “Hall of the Cataclysm” passing through the “Hall of the Manger”, lead us to think that most fungal spores detected inside the cave, excluding *Aspergillus*/*Penicillium* type, come from outside. The presence of pollen grains in the atmosphere of the cave constitutes another aspect that verifies the entrance of particles from outdoors.

Despite the limitations presented by the volumetric sampler used inside the cave, we think that it contributes to providing a more complete picture of the quantity and quality of the airborne biological particles present in the cave than other methods. Such methods would operate for a shorter period and could therefore underestimate the diversity and both the seasonal and intradiurnal distribution of the spores.

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