

The air spora of an orchid greenhouse

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Abstract The orchid collection of the ELTE Botanical Garden, Budapest, Hungary was monitored for airborne fungi using viable and non-viable air-sampling methods (Hirst-type and a 3-stage Andersen sampler) with three different culture media. A new culture method was also applied to identify fungal spores from Hirst-type samples. The aim of this study was to determine the diversity, human- and phytopathological potential of the air spora. To find out sources of airborne fungi, samples were collected from the air in an adjacent greenhouse and outdoors, and from necrotic plants. A total of 58 genera were found in the air samples. *Cladosporium* and *Penicillium* spp. were common members of the airborne biota. A high proportion (27.5%) of identified genera may be presented as a member of microbial consortium

associated with the orchids. Airborne fungi potentially pathogenic to humans were also detected. One species, *Zygosporium masonii*, was new to Hungary. Statistical analysis indicated that conditions of sampling had significant effects. The principal component analysis elucidated the three principal components representing 75.34% of the total variance; the clusters of variables were related to the three types of culture media. Relative abundance of small-sized spores was high, presumably because of the fungal species composition and accelerated sedimentation of large spores in still air. Apparently, in the studied orchid greenhouse, a specific mycobiota developed due to the climate and hosts (Orchideaceae) grown there.

Keywords Air spora · Biodiversity · Orchid · Greenhouse · Fungi

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1 Introduction

Concerning human health, fungi might have a potential risk as the causal agents of respiratory disorders and infections and as the producers of mycotoxins. Airborne fungi play an important role in allergic sensitization, the development of allergic rhinitis and asthma, as it has been demonstrated many times, especially in closed environments (hospitals, office buildings, etc. WHO 2009). The health risks generated by airborne fungi in greenhouses were discussed by Monsó et al. (2002) and Li and

LaMondia (2009). Attention was claimed for the potential human pathogenicity of airborne fungi in greenhouses with tropical climate by Rodolfi et al. (2003). Although monitoring of airborne fungi is a current scientific issue providing knowledge for human and plant pathology, aeromycological studies in greenhouses are scarce (Frinking and Scholte 1983; Keressies 1993a, b). Rodolfi et al. (2003) pointed out that greenhouse air received little attention, despite the fact that plants and humans (workers and visitors) may be exposed to high spore concentrations. Controlled greenhouse environments with warm and humid climate optimized for tropical plants offer suitable conditions for fungal growth. Presence of pathogens, their hosts and factors that permit proliferation, dispersal and germination may lead to disease development. Greenhouses play an important role in agricultural production and *ex situ* species conservation. Greenhouses are major tools for the preservation of orchid species. Some of them are endangered or extinct due to the habitat destruction (tropical deforestation) caused by human activity. The orchid family (Orchideaceae) is the largest family of flowering plants. Besides their genetic value, orchids also represent a high commercial value. Some orchid species are taken as medicines (Yuan et al. 2009), or used as spice (*Vanilla planifolia*) or decoration. Consequently, orchid collections need special attention. It is meaningful to study the air spora in greenhouses with conservation purpose, especially the orchid pathogens and symbionts, since these fungi may affect the success of orchid conservation. Although orchids-fungal biology has been largely explored (Yuan et al. 2009), the impact of air spora on orchids is less known. No studies were focused on the aerobiological quality in orchid greenhouses and their possible influence on the success of orchid conservation. Therefore, the aims of this study were to investigate the composition of airborne fungi and their effects on the plants in an orchid collection.

2 Materials and methods

2.1 Description of the greenhouse

The orchid collection (orchidarium) of the Botanical Garden of the Eötvös Loránd University (ELTE) is

located in the center of Budapest (Hungary) in a greenhouse built in 1984. The orchid collection contains about 380 botanical species and about 150 *Cattleya* hybrids, 60 *Paphiopedilum* hybrids, moreover *Vanda*, *Dendrobium* and *Zygopetalum* hybrids. The origins of the specimens are tropical and subtropical territories of the entire world (Central and South America, Asia, Australia, Africa etc.) and were obtained from other orchid collections, nature and sterile culture or via commerce. This collection is divided into a subtropical house and a tropical house according to the requirements of the plants. The length of a nave is 18.3 m, the width is 6 m, and the height is 6.3 m. The average temperature of the tropical part and the subtropical part is 22–25 and 15–18°C, respectively. Due to the large air-space, relative humidity decreases rapidly after the morning watering. The ventilation is necessary from spring to autumn. The top-windows of the naves automatically open when the house is too warm, but in summer, the back door is opened continuously (day and night). The supply of the plants with light during the winter period is suitable. From mid-March till mid-October, it is necessary to shade the collection with double green net.

In the tropical house part, there are stands (tables) along the glass walls, which are covered with pine-bark, and the pots are placed on that. In the middle of the house, there is a double stand covered with soil. There are trees (*Ficus* spp.) as well as different tropical plants and ferns to improve microclimate for orchids. Numerous orchid specimens are hanging down in the air-space, tied with a small piece of moss on tree barks and branches using thin copper wires.

In the subtropical house, the placement of stands is similar to that of the tropical house part, but the whole surface is covered with pine-bark, except from a 0.5 m² part, where *Paphiopedilum* species are planted out in orchid medium. Here, the hanging plants are placed in baskets or on barks.

The potting medium contains chopped pine-bark, dried sphagnum moss, perlite, cocos fiber and small quantities of nutrients. Heated, stagnant tap-water is used for watering.

2.2 Air samplings

The air was monitored with the objective of investigating fungal spectra in the tropical house with

orchid collection. Different methods were used to detect the presence of airborne fungi (Andersen- and Hirst-type spore traps and settling plates). Air samplers were operated at 150 cm a.g.l., placed on the stands.

The Andersen sampler (Andersen 1958; Lanzoni Co. Ltd., Bologna, Italy) is an extensively used device for viable sampling. Airflow provided by a vacuum pump is drawn through a succession of three stages, each perforated with 400 holes. The diameter of holes in the stages are decreasing progressively (1.20, 0.70 and 0.35 mm), and the air speed reaches successively higher intake velocities from stage to stage. Airborne particles are impacted inertially according to their size (>7 , 7 – 3.3 and <3.3 μm) onto culture media in Petri-plates, placed directly under the stages. Successively smaller particles are impacted at lower stages of the sampler. Sample collection was carried out at constant air stream of 28.3 L per minute. Three types of culture media [malt extract agar (MEA, Samson et al. 2000), Askew agar with Rose Bengal (RBA, Askew and Laing 1993) and *Rhizoctonia* selective media (KOA, Ko and Hora 1971)] have been applied. Sampling times were 10 min periods. Between samplings, the device was sterilized with ethanol (abs.). Samples were collected in duplicate and in two sites (facing *Epilobium* sp. and *Eulophia* sp.) on March 18, 2008. Colony-forming units per cubic meter of air sampled (CFU/ m^3) were calculated using the “positive hole correction” method (Feller 1950). During Andersen sampling, settling plates with MEA, RBA and KOA were also exposed (3, 1 and 3 plates, respectively). The plates with air samples were incubated in the dark for 3 days at 25°C. Pure cultures were made after counting from all morphologically different colonies. The colonies were transferred onto MEA and V8 (Samson et al. 2000) medium.

The portable Hirst-type spore trap (Hirst 1952; Lanzoni Co. Ltd., Italy) was used to collect air samples for direct microscopical investigations during the operation of the Andersen sampler. A microscope slide was thinly smeared with petroleum jelly as an adhesive to catch the particles from the instreaming air suctioned into the device by a vacuum pump (10 L of air is aspirated in every minute, 45 min, facing *E. nocturnum*). Reference air sample was collected outdoors, at the entrance of the greenhouse. Additional reference samples were collected in the

subtropical house (Dec. 14. 2007. 1300–Dec. 15. 2007. 1000, Dec. 17. 2007. 1400–Dec. 18. 2007. 0900) as well as at different locations in the tropical house (facing *Angrecum sesquipedale* Thouars, Dec. 11. 2007. 1400–Dec. 12. 2007. 0800; *Eulophia krebsii* [Rchb. f.] Bolus, Dec. 12. 2007. 1400–Dec. 13. 2007. 0800; *Coelogyne* sp., Dec. 18. 2007. 1600–Dec. 19. 2007. 0900; *Epidendrum nocturnum*, Jan. 25. 2008. 1300–Jan. 26. 2008. 1900; Jan 29. 2008. 1000–0000; Feb. 04. 2008. 2000–Feb. 05. 2008. 0400; Feb. 06. 2008. 1000–2200). The 100% of the area of the air samples were scanned to provide a full picture of the air spora.

To increase recovery, a combined method of viable and Hirst-type method (viable Hirst-type method) was also developed to culture selected spore types. Using a portable Hirst-type trap, air samples were collected onto the microscopic slides coated by petroleum jelly (10 L/min, 10 min) during the operation of the Andersen sampler. A block of water agar (20 g L^{-1} , $20 \times 15 \times 3.5$ mm) was cut and mounted over the total area of the air sample, which was incubated at 25°C for 12 h. Germinating spores penetrated onto the media. After incubation, the agar block was turned upside down. Germinating monospores were selected under the 800 $\times$ magnification of a light microscope (Olympus BX51) using a “New England” finder (S-7, Structure Probe, Inc., PA, USA) and transferred with a pin onto MEA and V8 medium and incubated for 3–14 days at 25°C.

2.3 Isolation of fungi from leaves

Samples were collected of orchid plants showing disease symptoms and carried into the laboratory. Isolation and identification of fungi were made by usual manner. The isolates were made of hyphal tips on potato malt agar (PMA, Atlas 1946).

2.4 Identification

The purified fungal strains (isolated from air samples and plants) were numbered and transferred to different media to initiate sporulation. KOA and SNA (Nirenberg 1976; Booth 1971) with a piece of sterilized filter paper were used to make easier isolation Basidiomycetes and *Fusarium* spp., respectively. Different media were used to identify *Aspergillus* species (MEA, CYA 25, CYA 37, CY 20S,

Klich 2002) and other isolates [V8, LCA (Miura and Kudo 1970), OA (Samson et al. 2000)]. Non-sporulating and doubtful isolates were identified by molecular methods.

2.5 Fungal genomic DNA extraction, PCR and ITS sequence analysis

For the purposes of molecular identification, mycelia of the fungal isolates grown in liquid YEG medium (5 gL⁻¹ glucose, 1 gL⁻¹ yeast extract, 5 gL⁻¹ KH₂PO₄ in distilled water) were ground into dust with a mortar and a pestle in liquid nitrogen. Extraction of total genomic DNA was carried out with the GenElute™ Plant Genomic DNA Miniprep Kit (Sigma–Aldrich) according to the protocol provided by the manufacturer. The internal transcribed spacer (ITS) region of the rRNA gene complex, incorporating ITS 1, the 5.8S rRNA gene and ITS 2, was amplified using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'TCCTCCGCTTA TTGATATGC-3') (White et al. 1990). All amplifications were performed in a MJ Mini™ Personal Thermal Cycler (Bio-Rad Laboratories) with the following temperature profile: 5 min initial denaturation at 94°C, followed by 35 cycles of 30 s denaturation at 94°C, 40 s primer annealing at 48°C, 1 min extension at 72°C and a final extension step of 3 min at 72°C. PCR products were subjected to purification and automatic sequencing with primer ITS4 at Beckman Coulter Genomics, Essex, England. The resulting sequences were deposited in the GenBank database of the National Center for Biotechnology Information (NCBI) under accession numbers HM052811–HM052828. Sequence analysis was carried out by BLASTN similarity search (Altschul et al. 1990) at the website of NCBI (<http://www.ncbi.nlm.nih.gov/blast/>).

2.6 Statistical analysis

Fisher's test (MANOVA) was applied to analyze the data of Andersen-type air samplings using statistical functions of Microsoft Office Excel 2003 (Microsoft, Redmondton, USA). Statistica5 program (StatSoft, Tusla, USA) was used for analysis of data by principal component analysis (PCA) and graphic presentation of result to disclose differences in methods of sampling.

3 Results

A total of 76 taxa (58 genera) were identified from the air of the orchidarium using non-viable and viable sampling methods (Tables 1 and 2, respectively, Fig. 1). *Cladosporium* and *Penicillium* spp. were common members of the airborne biota. In the samples collected by the viable and non-viable methods, 23 and 41 genera were found, respectively. Only 11.7% of the genera were detected by both Hirst- and Andersen methods. Considering all distinct, but unknown spore types on the Hirst-type samples, the total number of morphologically different spores was 101. The 21.8% of the greenhouse microfungi has been detected outdoors as well. Similar spectra (96.5%) of fungal species were found in the subtropical house and in the tropical house.

Orchid plants with disease symptoms were sampled and 19 fungal species, among them 14 pathogenic ones were isolated (Table 3). The phytopathogenic fungi causing leaf diseases (*Alternaria*, *Colletotrichum* and *Botrytis*) were associated with endobiotic *Fusarium* species, and antagonistic *Trichoderma* strains also were presented.

Data obtained by Hirst- and Andersen-type samplers are compiled in Table 1 and 2, respectively. The relative abundance of the taxa on different media, stages and sites is shown in Table 2 as well. A total of 3,923 colonies were isolated from 56 viable samples. The samplings detected a rather high concentration of fungi in the indoor ambient air (avg. 859 CFU/m³). Most colonies were found in air samples collected onto MEA, the concentrations detected were approximately 3 times higher than on the specific media (KOA, RBA). KOA appeared somewhat selective for *Cladosporium herbarum* and *Clonostachys rosea* f. *rosea*. The average of the total CFU counts was 1,544 on MEA. The 60.5, 23.2 and 16.3% of CFUs were counted on MEA, RBA and KOA, respectively. Results of MANOVA indicated that both culture media and stages had significantly different effects ($F_{\text{medium}} = 30.65 < F_{0.05} = 4.45$; $F_{\text{stage}} = 52.53 < F_{0.05} = 4.45$). There were no significant differences between the replications ($F = 2.27 < F_{0.1} = 3.03$). Frequency of airborne propagules slightly differed at the two sampling sites (*Epilobium* sp. and *Eulophia* sp. $F_{\text{species}} = 1.64 > F_{0.05} = 1.54$). There was a significant difference between the two sites of sampling ($F_{\text{site}} = 32.56 < F_{0.05} = 4.45$). When

Table 1 Concentration of the fungi in the air samples collected with Hirst-type spore trap in the orchid greenhouse (spores/m³/45 min)

	Tropical house	Outdoor
<i>Alternaria</i> sp. ^{ade}	10.6	19.6
<i>Aspergillus/Penicillium</i> ^{ae}	100.0	72.9
<i>Botrytis</i> -type ^{be}	2.1	0.0
<i>Cladosporium</i> spp. ^{ade}	844.7	624.0
Diatrypaceae ^{cde}	14.9	0.0
<i>Epicoccum nigrum</i> Link ^{abde}	4.3	3.6
<i>Leptosphaeria rubicunda</i> Rehm ^c	10.6	0.0
<i>Nectria</i> sp.1 ^{be}	10.6	0.0
<i>Nectria</i> sp.2 ^{be}	6.4	0.0
<i>Phaeosphaeria minima</i> Shoemaker & C.E. Bab ^{c,de}	29.8	3.6
<i>Torula herbarum</i> (Pers.) Link ^{de}	6.4	1.8
<i>Trematosphaeria pertusa</i> (Pers.) Fuckel ^c	6.4	0.0
<i>Ustilago</i> sp. ^{de}	8.5	19.6
Hyphal fragments ^{de}	87.2	26.6
Unknown basidiospore sp.1 ^c	6.4	0.0
Unknown hyaline 3-septate sp. ^{de}	6.4	3.6

Other species: *Ascobolus* sp., *Anthostomella/Herpotrichiella*^c, *Arthrinium* sp.^b, *Aspergillus niger*-type^c, *Cercospora* sp., *Chaetomium* sp., *Coprinus* sp.^d, *Crassochaeta fusispora* (Sivan.) Réblová, *Colletotrichum*-type^{be}, *Diplocladiella scalaroides* G. Arnaud, *Diplodia* sp., *Delitsia* sp.^c, *Fusarium* sp.^b, *Ganoderma* sp.^{db}, *Helicosporium* sp., *Karstenula shepherdiae* (Peck) M.E. Barr, *Lasiosphaeria* sp., *Myxomycetes*^c, *Oidium* sp., *Oncopodiella* sp.^c, *Ophiobolus* sp., *Pandora* sp., *Paraphaeosphaeria michotii* (Westend.) O.E. Erikss., *Pleospora* sp.^{de}, *Puccinia* sp., *Rhizoctonia*-like hyphal fragments, *Sphaeropsis* sp.^c, *Sporidesmium* sp., *Stachybotrys* sp., *Stemphylium* sp., *Telephoraceae*, *Tilletiopsis*-type, *Triadelphia heterospora* Shearer & J.L. Crane^d, *Triadelphia uniseptata* (Berk. & Broome) P.M. Kirk^d, *Xylariaceae* sp.1^{de}, *Xylariaceae* sp. 2, unknown hyaline 1-septate sp., unknown brown nonseptate sp., unknown basidiospore sp. 2

a: reported as common allergens; b: orchid-associated fungus (reported in the present study or the literature (see text)); c: present only in the subtropical house; d: present also in the outdoor samples; e: present in the tropical house during the Andersen-type sampling

principal component analysis was applied to analyze the Andersen-type samplings, the eigenvalues of the three-first principal components represented 75.34% of the total variance (PC1: 34.14%, PC2: 28.26%, PC3: 12.93%). By plotting the PC scores, 3 distinct clusters were identified, which related primarily to the three types of culture media (PC1: KOA, PC2: MEA, PC3: RBA); only the samplings from the 3rd stage with RBA had no correlation with PC3; however, they tended to PC1 (Fig. 2).

In the air samples collected by the Andersen-sampler, *Cladosporium* species were recovered in the highest concentration. The most common species was *C. herbarum*. The majority of the total CFU (22.8%) was recorded for *C. herbarum* on KOA, while this value was 2.45 and 0.34% on RBA and MEA, respectively. Its spores, as well as those of *C. cladosporioides*, were found mostly in the fraction between 7–3.3 µm (2nd stage). Small-sized

C. sphaerospermum spores were also frequent; 62.7% of them were caught on the 3rd stage. For *Cladosporium* spp., indoor and outdoor spore counts were similar (outdoor concentration was 1.2 times higher than indoors). *Penicillium* species (mostly *P. chrysogenum* and *P. griseofulvum*) were also common, but not as much as *Cladosporium* spp. Minute spores of penicillia impacted mostly (57.0%) on the 3rd stage. Its outdoor airborne concentration was only 1.1 times higher than indoors. *Clonostachys rosea* f. *rosea* (syn. *Gliocladium roseum*, teleomorph *Bionectria ochroleuca*) was a common member of the airborne biota and has been repeatedly isolated from the plants as well. No fusaria have been found in any viable air sample (*Fusarium* sp., represented by its macroconidia, was recovered only in one instance using Hirst-type air sampler). *Fusarium* species, however, have been isolated frequently from necrotic lesions of orchid leaves. A high number of

Table 2 Fungi collected with viable air-sampling methods in the orchid greenhouse, their concentration and relative abundance on different media (MEA, RBA and KOA) and stages (1, 2 and 3) of the Andersen sampler

Taxa	Colony-forming units per cubic meter				Proportion (%) of the taxa							
	Sum	Avg	Max	SD	MEA	KOA	RBA	Stage 1	Stage 2	Stage 3	Site 1	Site 2
<i>Acremonium strictum</i> W. Gams ^{abeg}	46.24	1.32	35.653	6.06	0.00	0.00	100.00	7.69	76.92	15.38	15.38	84.62
<i>Alternaria alternata</i> (Fr.) Keissl. ^{adefg}	24.71	0.71	7.06	1.67	14.28	57.142	28.57	14.28	57.14	28.57	28.57	71.43
<i>Arhrium</i> state of <i>Apiospora montagnei</i> Sacc. ^{ad}	60.01	1.71	14.12	3.57	100.00	0.00	0.00	17.65	35.29	47.09	76.47	23.53
<i>Aspergillus niger</i> Tiegh. ^{ad}	7.06	0.20	3.53	0.83	50.00	0.00	50.00	0.00	0.00	100.00	50.00	50.00
<i>Aspergillus ochraceus</i> G. Wilh. ^a	3.53	0.10	3.53	0.60	0.00	100.00	0.00	0.00	0.00	100.00	0.00	100.00
<i>Cladosporium cladosporioides</i> (Fresen.) G.A. de Vries ^{adfg}	166.96	4.77	54.01	10.56	0.00	19.03	80.97	6.34	70.40	23.25	40.17	59.83
<i>Cladosporium herbarum</i> (Pers.) Link ^{abf}	597.27	17.06	125.67	28.33	4.73	81.10	14.18	16.55	55.97	27.48	41.90	58.09
<i>Cladosporium sphaerospermum</i> Penz. ^{abf}	285.93	8.17	72.36	17.09	3.70	4.94	91.36	4.94	34.94	60.12	37.28	62.71
<i>Clonostachys rosea</i> f. <i>rosea</i> (Link) Schroers, Samuels, Seifert & W. Gams ^{abeg}	78.36	2.24	46.59	8.54	0.00	100.00	0.00	4.50	27.03	68.49	90.99	9.01
<i>Colletoichium gloeosporioides</i> (Penz.) Penz. & Sacc. ^{acdfg}	38.83	1.11	10.59	2.94	100.00	0.00	0.00	0.00	54.54	45.45	54.54	45.45
<i>Penicillium</i> spp. ^{afg}	1049.12	29.97	254.51	56.63	79.93	9.51	10.56	7.04	35.91	57.04	33.45	66.55
<i>Torula herbarum</i> (Pers.) Link ^{ad}	3.53	0.10	3.53	0.60	0.00	0.00	100.00	0.00	100.00	0.00	100.00	0.00
<i>Trichoderma</i> sp. ^{ag}	3.53	0.10	3.53	0.60	0.00	0.00	100.00	0.00	100.00	0.00	0.00	100.00
<i>Pseudozyma</i> sp. ^{?ae}	130.96	3.74	35.65	8.47	0.00	0.00	100.00	5.39	64.96	29.65	38.00	61.99

Species detected with viable sampling techniques from the air of the orchid collection

Sampling sites 1 and 2: *Epilobium* sp. and *Eulophia* sp, respectively

Other species: *Aspergillus sydowii* (Bainier & Sartory) Thom & Church^{ae}, *Aureobasidium* sp.^a, *Bjerkandera adusta* (Willd.) P. Karst.^{ae}, *Cytosporella* sp.^a, *Cladosporium* sp.^a, *Fomitopsis pinicola* (Sw.) P. Karst.^{ae}, *Geotrichum* sp.^a, *Glomerella* sp.^{eg}, *Libertella* state of *Peroneutypa scoparia* (Schwein.) Carmarán & A.I. Romero^{ec}, *Myrothecium roridum* Tode^{ag}, *Phoma*-like sp.^{ab}, *Scopulariopsis chartarum* (G. Sm.) F.J. Morton & G. Sm.^a, unknown Basidiomycetes^c, *Xylaria* sp. Fl. Edin^{cdg}, *Zygosporium masonii* S. Hughes^{ac}

a: detected with Andersen sampler; b: detected with sedimentation method; c: detected with viable-Hirst method; d: detected also with Hirst-type spore trap (at genus level); e: identified by ITS sequences; f: reported as common allergens; g: orchid-associated fungus (reported in the present study or in the literature (see text)); h: mainly *Penicillium chrysogenum* Thom^{be} and *Penicillium griseofulvum* Dierckx^e



Fig. 1 Air sampling and airborne microorganisms in the tropical greenhouse of the orchid collection of ELTE Botanical Garden, Budapest, Hungary. a: orchids and plant-holding tree branches in the greenhouse, b: Andersen-sampler, c: portable Hirst-type spore trap (located 150 cm a.g.l. during operation), d-e: *Xylaria* sp., f-g: *Phoma*-like sp., h-i, m: Basidiomycetes

sp., j, n, o: *Libertella* state of *Peroneutypa scoparia*, k: *Crassochaeta fusispora*, l: *Karstenula shepherdiae*, p: *Nectria* sp. 1, q: unknown basidiospore sp.1, r: *Phaeosphaeria minima*, s: *Oscillatoria/Lyngbia*-like sp., t: *Zygosporium masonii*. Bar 20 μ m, except in n and t where the Bar is 40 μ m

colonies (MEA: 93.9%, RBA: 86.0%, KOA: 79.9%), those of non-sporulating and slow-growing fungi, have not been quantified at the genus or species level, since their colony morphology did not allow their differentiation in the time of subculturing. Some non-sporulating colonies belonged to the Basidiomycetes according to the characteristics of their hyphae. Several slow-growing fungi were also subcultured and identified, and they are listed in the Appendix of the Table 2.

In the Hirst-type samples, *Nectria* sp.1 was one of the most common fungi (ascospores subhyaline, ellipsoidal, striate, two-celled, $10\text{--}11 \times 4\text{--}5.5 \mu\text{m}$, Fig. 1p). Such spores have not been found in the samples collected outdoors. It is interesting to note that *Nectria* sp.1 ascospores had higher concentration in the air samples collected in the evenings (with a distinct peak at 20 h). This result suggests that such spores have a diurnal variation in the greenhouse. *Botrytis cinerea* was repeatedly isolated from

Table 3 Fungi isolated from orchid species

Fungal species	Orchid taxa
<i>Alternaria alternata</i> (Fr.) Keissl ^a	<i>Cymbidium traceyanum</i> , <i>Dendrobium aberrans</i> , <i>D. delicatum</i>
<i>Aspergillus amstelodami</i> Thom & Church	<i>D. aberrans</i>
<i>Botrytis cinerea</i> Pers	<i>Coelogyne hybrid</i> , <i>Oecoclades sandersiana</i>
<i>Cladosporium sphaerospermum</i> Penz.	<i>Bulbophyllum mastersianum</i> , <i>O. sandersiana</i>
<i>Clonostachys rosea</i> f. <i>rosea</i> (Link) Schroers, Samuels, Seifert & W. Gamsa	<i>Cattleya</i> sp., <i>D. delicatum</i> , <i>Phalaenopsis</i> sp.
<i>Clonostachys</i> sp. ^a	<i>Phalaenopsis</i> sp.
<i>Colletotrichum gloeosporioides</i> (Penz.) Penz. & Sacc.a	<i>Cattleya</i> sp., <i>Gongora</i> sp., <i>Paphiopedilum</i> 'Cygnet'
<i>Fusarium equiseti</i> (Corda) Sacc ^a	<i>B. mastersianum</i>
<i>F. oxysporum</i> Schltdl.	<i>Brassolaelia hybrid</i> , <i>B. mastersianum</i> , <i>Gongora flaveola</i> , <i>Liparis distans</i> , <i>Paphiopedilum</i> 'Calypso', <i>Peristeria elata</i> , <i>O. sandersiana</i> , <i>V. planifolia</i> .
<i>F. solani</i> (Mart.) Sacc.	<i>Paphiopedilum spicerianum</i>
<i>F. verticillioides</i> (Sacc.) Nirenberg	<i>D. delicatum</i> , <i>Paphiopedilum</i> sp.
<i>Gliocladium virens</i> J.H. Mill., Giddens & A.A. Foster	<i>B. hybrid</i> , <i>C. hybrid</i> , <i>Coelogyne</i> sp., <i>G. flaveola</i> , <i>Gongora</i> sp., <i>L. distans</i> , <i>Paphiopedilum</i> 'Calypso', <i>P. spicerianum</i> , <i>Paphiopedilum</i> sp., <i>V. planifolia</i> , <i>Maxillaria</i> sp.
<i>Mucor plumbeus</i> Bonord.	<i>B. mastersianum</i> , <i>L. distans</i> , <i>Maxillaria</i> sp.
<i>Myrothecium roridum</i> Tode ^a	<i>Bulbophyllum</i> 'Burfordiense', <i>Coelogyne</i> sp.
<i>Phoma cucurbitacearum</i> (Fr.) Sacc ^a	<i>Cymbidium</i> sp.
<i>Trichoderma atroviride</i> P. Karst.	<i>C. traceyanum</i> , <i>Cymbidium</i> sp., <i>D. aberrans</i> ,
<i>T. harzianum</i> Rifai	<i>D. delicatum</i> , <i>Cattleya</i> sp., <i>C. hybrid</i> , <i>Coelogyne</i> sp., <i>O. sandersiana</i> , <i>Paphiopedilum</i> 'Calypso', <i>Peristeria elata</i> , <i>Phalaenopsis</i> sp., <i>V. planifolia</i>
<i>T. minutisporum</i> Bissett	<i>Phalaenopsis</i> sp.
<i>Ulocladium chartarum</i> (Preuss) E.G. Simmons	<i>D. delicatum</i>

^a Identified by ITS sequences

necrotic plant tissues, and its conidia were present in the air samples collected by the Hirst-type device (by viable samplings, the fungus has not been detected). *Alternaria* species were found in both air samples and plant tissues. Their spore concentrations were higher outdoors than indoors (2.9 times); and consequently, their CFU counts in the greenhouse were higher (2.9 times) near the door than deeper inside the building. *Ustilago* sp. were found in higher concentration outdoors than indoors (3.5 times). Hirst-type air samples were rich in different forms of minute, hyaline basidiospores. By viable methods, some fungi belonging to Basidiomycetes were isolated (one of them was identified as *Fomitopsis pinicola*). Xylariaceous fungi were detected by viable and non-viable Hirst methods, but not with Andersen-type device or settling plates. *Zygosporium masonii* detected by the Andersen sampler and the viable Hirst method is reported here for the first time from Hungary (Fig. 1t). A common 3-septate, brown spore type

(11–13 × 3–3.5 µm) was identified as *Phaeosphaeria minima*; its concentration was 5.4 times higher indoors than outdoors (Fig. 1r). Culturings from Hirst-trap samples recovered an anamorphic fungus, resembling *Libertella* spp. in having non-septate, hyaline, hook-like, filamentous conidia (12–25 × 1–1.2 µm, Fig. 1o). ITS analysis confirmed that this fungus was the *Libertella* state of *Peroneutypa scoparia*.

The presence of airborne particles of non-fungal origin like orchid seeds was characteristic elements of the bioaerosol in orchid collections. Spontaneous growth of orchid seedlings (*Peristeria elata* Hook.) by airborne seeds was observed. Pteridophyta spores were also present in the air samples; however, the pollen grains have been scarcely found and belonged to the outdoor tree species (*Quercus*, *Betula*, Cupressaceae/*Taxus*, *Fraxinus* spp.). The total pollen concentration outdoors was three times higher than in the building (70.2 and 216.8 pollen grains/m³/45 min,

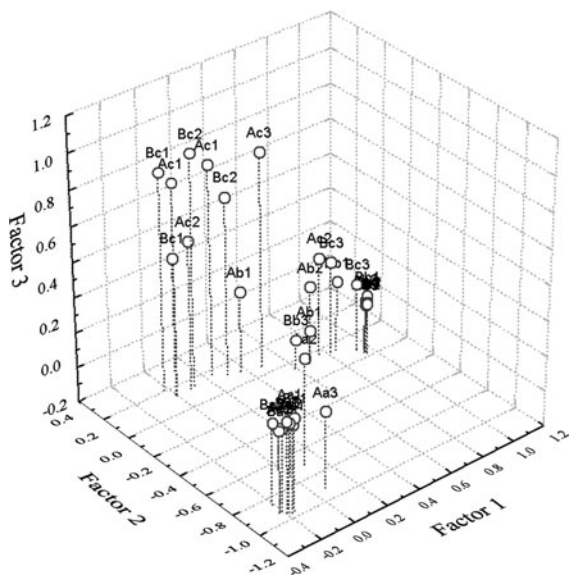


Fig. 2 Principal component analysis of the data obtained by Andersen-sampler at two different sampling sites (A: *Epilobium* sp. [site 1] and B: *Eulophia* sp. [site 2]), with different culture media (a: MEA b: KOA and c: RBA) and stages (1: 1.20 mm, 2: 0.70 mm, 3: 0.35 mm) in the orchid greenhouse. Eigenvalues were 12.29, 10.18 and 4.66; variance proportion 34.14, 28.26 and 12.94 percents for the first, second and third axes, respectively, and thus, this figure explains 75.34% of the variability in the data

indoor and outdoor, respectively). Airborne algae were represented by fragmented hormogonia of *Oscillatoria/Lyngbia*-like sp. (Cyanobacteria; 6.9 particles/m³, Fig. 1s) and by diatoms (*Aulacoseira* sp.).

4 Discussion

To our knowledge, this study is the first to examine the air spora of an orchid collection. This work was induced by the necessity of orchid conservation in the greenhouses of the ELTE Botanical Garden. Information about the health status of the conserved plants and the presence of airborne fungi on orchids was provided via different sampling techniques. The feasibility of the applied sampling methods to detect airborne fungi was also tested. It was showed that aerobiology, combined with the examination of plants, is an effective mean to control air quality of greenhouses with orchid collections. Because aerobiology offers new methods to study orchid-associated fungi in greenhouses, it may be of a great

importance to further elucidate the ecology of this special habitat, thus developing new strategies for plant protection/conservation. As it was proposed in an earlier study (Magyar et al. 2009), a combination of viable and non-viable methods and a wide variety of selective isolation techniques are necessary to see a wider spectrum of air spora. By non-viable air sampling, more fungal genera (41) were recovered than by viable ones (23). Several members of the air spora could be distinguished or even identified on the basis of spore morphology. Advantages and disadvantages of non-viable samplings were discussed earlier (Magyar et al. 2009). It seems that viable sampling methods (such as Andersen sampler) also have some limitations, especially in environments having high fungal diversity and high spore concentration. In the viable samplings, the common practice is the selection of colonies from the air sample for further subculturing and identification. The selection is based on the morphological characters of the colonies in the air samples. To obtain pure cultures, subculturing should be timed before overgrowing, and strong sporulating colonies are present, consequently as early as possible. This method may lead to a loss in the species number, especially when several non-distinguishable colonies are present in the time of selection. (Alternatively, all colonies of the air sample could be subcultured, but it is applicable only in low-concentration samples). As our results showed, the detection of species—even that of the common ones—largely depends on the culture media used (e.g. *Pseudozyma* sp., were common fungus according to the samplings on KOA, while it was not detected on any other culture media). The different methods applied in this study completed each other, especially viable and non-viable ones, where there was a low overlap between the identified genera (11.7%). Despite our efforts to reveal the full fungal spectrum indoors using a wide scale of sampling strategies, we suppose that further species have remained unexplored. The number of unexplored taxa could be estimated by the number of unknown, but distinct taxa, which was 101 in non-viable samples. In viable samples, however, a similar estimation can not be performed. Here, some species may remain unexplored by overlooking their minute colonies before subculturing, having no cultural characteristics to separate them from other species. According to Currah et al. (1997), many fungi found

in orchids do not sporulate in pure culture or do not grow on artificial media. Similar problems complicated the study of the airborne fungi of the orchid greenhouse. Notwithstanding our efforts to induce sporulation in vitro, morphology-based identification was successful only in a low proportion of the fungal species (representing 6–20% of the colony counts). Applying different selective media (as has been shown by MANOVA and PCA) and viable-Hirst method, these problems could be diminished (Magyar et al. 2009). It was demonstrated that by viable-Hirst method, even common fungi exhibiting low competitive ability and growth rate on artificial media could be detected and identified. The present technique could provide a deeper insight into the airborne spore composition, as well as another viable-Hirst method published earlier (Magyar et al. 2006). The disadvantage of this method is the difficulty of the manual monospore isolation.

PCR-based approaches to identify orchid endophytes are also limited, since such methods need specific PCR primers to preferentially amplify certain groups of fungi. Using more than one set of primers and varying amplification conditions may help reveal additional taxa (Bayman and Tupac Otero 2006). Several fungal species important from the point of view of orchid conservation may be remaining unexplored. In future studies, therefore, specific primers should be used; thus, targeted species (important for orchid conservation) may be detected and quantified in air samples.

The fungi found in the orchid greenhouse may have been introduced by imported plants, substrates or ventilation. *Cladosporium* species were predominant amongst airborne fungi both inside and outside of the building (having higher concentration outdoors), being a common sooty mold on plant leaves. Rodolfi et al. (2003) reported that *C. cladosporioides* dominated a tropical greenhouse. *Penicillium* species are also common in soil and decaying plant materials. A high percentage (27.5%) of the genera identified in the present study may be associated with the orchids, according to our isolations from the plants and literature data (Taubenhaus 1920; Currah et al. 1997; Bayman and Tupac Otero 2006). A single fungus can behave as endophyte, mycorrhizal symbiont, pathogen or saprotroph. Orchid mycorrhizal fungi play important roles both for seed germination and for plant survival. Some fungi recovered from necrotic

orchid tissues were also present in air samples, many of them are known as pathogens or endophytes of orchids (Taubenhaus 1920; Currah et al. 1997; Bayman and Tupac Otero 2006). Common plant pathogens like *Botrytis*, *Fusarium* spp., *Myrothecium roridum* and *Colletotrichum gloeosporioides* often cause epidemics in greenhouses.

Botrytis cinerea, the causative agent of gray mold, is common worldwide and responsible for major losses of ornamental hosts in greenhouses (Friedrich et al. 2005). Although this fungus attacked two specimens in the studied period, its conidia were present in low airborne concentration; nevertheless, plant protection should be focused on this species before higher levels of conidia would be detected in the air. *Colletotrichum gloeosporioides* was found in different orchid species, especially *Cattleya* spp. This fungus is a well-known plant pathogen; however, it was frequently isolated as an endophyte from the roots of tropical orchids (Richardson et al. 1993). *Myrothecium roridum* often causes infections on many hosts under glasshouse conditions (Fitton and Holliday 1970), and it can also act as a mycoparasite (Vajna 1987).

One of the most interesting groups of orchid-associated fungi is *Fusarium* and its teleomorphs (Hypocreaceae). It is known that *F. oxysporum* is a pathogen of the vanilla orchid (*Vanilla planifolia*) hampering severely the cultivation of the crops (Divakaran et al. 2008). Fusaria can induce disease symptoms in many other orchid species as well, but on the other hand, their beneficial role played in stimulation of orchid seed germination has also been known for 100 years (Bernard 1909). In our study, *Fusarium* species were the most frequently cultured fungi from orchid leaves, with *F. oxysporum* being the predominant species. Other fusaria (*F. equiseti*, *F. solani* and *F. verticillioides*) were also found. Despite its low concentration in the air, *Trichoderma* spp. were found to be frequent on diseased plants. Such fungi are frequent soilborne fungi, but sometimes penetrate roots and live as an opportunistic, avirulent plant symbiont (Harman et al. 2004). In a greenhouse studied by Li and LaMondia (2009), *Trichoderma* sp. was the most dominant fungus in the air samples, but it was introduced into the greenhouse as a biocontrol agent. Lower airborne concentrations were also found in another tropical greenhouse (Rodolfi et al. 2003). In a *Trichoderma*-infected

mushroom-growing house, very low *Trichoderma* counts were found in the air samples (unpublished study of the authors). Thus, surprisingly, neither *Fusarium* nor *Trichoderma* species have been cultured from the air samples with expected frequency, and it was hypothesized that their conidia have not been liberated during the sampling period.

Two ascospore types belonging to the pyrenomycetous genera *Nectria* (*Nectria* sp.1 and 2) were common in our air samples. It is also observed that the concentration of *Nectria* sp.1. peaked at the evenings. Such diurnal variation may be probably related to the light and watering conditions (Walkey and Harvey 1966). There are seven *Nectria* spp. recognized as pathogens living in the roots, pseudobulbs and branches of orchids (Taubenhaus 1920). Further studies are needed to find out whether these nectriaceous fungi belong to any of the orchid pathogenic species endangering the orchid collection directly (or indirectly as teleomorphs of some *Acremonium*, *Clonostachys*, *Fusarium* and *Myrothecium* spp.).

Clonostachys rosea f. *rosea* is a common saprotroph and mycoparasite with a worldwide distribution. It was confirmed in a recent publication that this fungus could be developed into an effective biocontrol agent with its characteristics of induction of systemic resistance in plants towards pathogenic fungi (Jensen et al. 2007). Rodolfi et al. (2003) pointed out the role of mycoparasitic fungi in greenhouses. According to this, we suppose that this fungus due to its high frequency may play an important role in regulating fungal growth in the orchid greenhouse. This fungus may also be a beneficial member of the air spora for improving acclimatization, vigor and growth of micropropagated orchid plantlets (Yuan et al. 2009).

Xylaria ascospores were common in the air samples, and cultures were obtained using the viable-Hirst method. Cultures of these fungi were darkly pigmented and gave raise to finger-like branched stromata (Fig. 1d). *Xylaria* species are saprotrophs often colonizing dead branches of forest trees. But also, it is the most common genus isolated from roots of orchids collected in Costa Rica and Puerto Rico (Richardson et al. 1993; Bayman and Tupac Otero 2006). It was demonstrated that representatives of Xylariaceae living as endophytes have beneficial effects to hosts in vitro. It was hypothesized that endophytic xylariaceous fungi primarily

serve as litter decomposers once the host is undergoing senescence (Yuan et al. 2009).

Zygosporium masonii, reported from dead leaves of various plant species (e.g. *Cocos*, *Ficus*, *Gossypium*, *Musa*, etc.), from soils in Australia, Africa, South Asia, Venezuela (Ellis 1971), and from indoor air samples collected in North America (Li and Yang 2004), was found in the tropical greenhouse. Another *Zygosporium* species was also reported by Rodolfi et al. (2003) from a tropical greenhouse, Pavia (Italy). Since *Z. masonii* has not been reported previously from Hungary, it is thought that this species might have been introduced by some tropical plant species or substrates.

Although all orchids have an obligate relationship with mycorrhizal symbionts, and most orchid mycorrhizal fungi are classified in the form-genus *Rhizoctonia* (Tupac Otero et al. 2002), these fungi have not been detected by any viable air sampling method (Hirst-type air sampling scarcely detected some *Rhizoctonia*-like hyphal fragments). Some of the unknown, hyaline basidiospores presumably had indoor origin, and they are thought to belong to the polyporous fungi colonizing plant-holding tree branches, or the teleomorphs of orchid-associated *Rhizoctonia*-like fungi.

Oscillatoria-like cyanobacteria were frequent in our Hirst-type air samples. According to Tsavkelova et al. (2001), epiphytic *Oscillatoria* formed a biofilm on the rhizoplane of orchids in a greenhouse. This organism is capable of nitrogen fixation, and it was suggested that it has some special affinity for the root surface, since it was not isolated from the soil collected near the plants. The associations of cyanobacteria and plants as well as the aerobiology of cyanobacteria are still poorly studied. Therefore, the questions how the cyanobacteria were aerosolized and how the deposited propagules affect the health status of orchids and greenhouse workers need further research.

Rodolfi et al. (2003) collected 42 genera and 72 species in a tropical greenhouse in Italy with a viable sampling (gravity settling plate) method. The 18.2% of the taxa were found in our tropical greenhouse as well, including the predominant fungus *C. cladosporioides* and other species (*B. cinerea*, *C. herbarum*, *C. sphaerospermum*, *Clonostachys rosea*, *Colletotrichum gloeosporioides*, *Fusarium oxysporum*, *F. solani*). Some common taxa of the Italian study

have not been detected in our samples (e.g. *Conidiobolus* spp.). Our results are also comparable with the study of Li and LaMondia (2009) where a non-viable sampling technique was used. Some of the predominant taxa in their samples (*Aspergillus*/*Penicillium*-like, *Cladosporium*) were common in our samples as well, while others (*Botrytis*, *Trichoderma*, yeast-like fungi and hyphal fragments) have been found to be relatively rare.

Spore dispersal process in greenhouses is complex (Frinking and Scholte 1983; Frinking et al. 1987) where activity of man has a major influence (Hausbeck and Pennypacker 1991). Indoor concentration of fungal species is also depending on the substrates available in the indoor environment as well as on ventilation (Blomquist and Andersson 1994). Due to the natural ventilation of the greenhouse investigated, there could be an exchange of air between greenhouse and outdoor environment, depending on the outdoor climate (Wang et al. 1999). Because of this, fungal spores can enter and leave the greenhouse (Zadoks 1967; Schepers 1984, Frinking 1991; Kerssies 1993a). In the present study, less than the quarter of the greenhouse microfungal taxa has been detected in the outdoors samples. There was also some difference in the spore concentrations between the sampling sites. Lower concentration inside the building, higher concentration near the door and the highest concentration outside of the building suggested an outdoor source for some fungi.

Minute, penetrator-type spores (e.g. *Cladosporium*, *Clonostachys*, *Penicillium*, see Gregory 1961) were common in the air samples, while large-sized or elongated ones (impactors) were less frequent. The total concentration of small spores (collected by the 3rd stage of the Andersen sampler) was 13 times higher than that of the larger ones. *Penicillium* spp., Basidiomycetes and *C. sphaerospermum* were common minute-spored fungi caught on the 3rd stage, while *Alternaria* was the most common large-spored one from the 1st stage. Morphometrical data of spores collected by the Hirst-type samplers confirmed that penetrators dominated the air of the orchidarium. Because our samplings have been performed under windless conditions, sedimentation of large airborne spores should have been accelerated. This is probably why the relative abundance of penetrator type spores increased. The prevalence of small-spored species may also have an influence on this result.

Gloiosporae (fungi having slimy spores) represented 60.3% of the genera. It was shown in previous studies that these fungi could be dispersed by their splash-borne conidia (Jarvis 1962; Hislop 1969; Sutton et al. 1978; Sutton 1982; Rajasab and Ramalingam 1989; Rossi et al. 2000). The technology required for the maintenance of orchid species, as high relative humidity and spraying water, may be responsible for the dispersal of such fungi in indoor environments.

Some allergenic and other human pathogenic fungi were recognized in our air samples. It was demonstrated by Monsó et al. (2002) that the fungi associated with the respiratory complaints of greenhouse workers were *Alternaria* spp., *Aspergillus* spp., *C. herbarum* and *Penicillium* spp. Such genera were predominant in the air of the orchid greenhouse. The concentration of other fungi, recognized as causal agents of health problems in humans, e.g. *Stachybotrys* sp., was low, representing a minimal health risk for greenhouse workers and visitors. As Monsó et al. (2002) pointed out, the 25% of greenhouse workers suffers from occupational asthma caused by fungi and pollen grains. Our findings are in agreement with the previous results showing that the concentration of airborne fungi in greenhouses can be high. The availability of colonizable materials (soil, plants and plant holding tree branches) and the favorable microclimate (high relative humidity and temperature, watering) may be responsible for high fungal counts observed in the air of the orchid greenhouse. As it has been done to the other indoor environments like offices, housings and hospitals, attention should also be paid to the air quality in greenhouses, to provide healthy environment for humans and plants therein (Rodolfi et al. 2003). The quantitative aerobiological data of greenhouse fungi can provide important information to evaluate occupational health hazards as well.

It is even more important to focus on orchid collections, because many species of orchids are considered rare and endangered; thus, an urgent need arises to conserve them. In orchid greenhouses, specific mycobiota may develop according to different factors, like climate, hosts and substrates (Illyés et al. 2009). Since there is a strong impact of fungi on orchid performance and response to stress (Brundrett 2007), it is necessary to study the aerobiology of symbionts, pathogens and endophytes. It seems that

the studied orchid greenhouse has a special air spora composition, possibly as an effect of climate, substrates and host plants (Orchideaceae) grown there. Such air spora contains many orchid-associated fungi; thus, it may contribute to the development of a suitable environment for orchid conservation in the greenhouse. Therefore, aerobiological knowledge supplied by combined methods may provide this information to perform *ex situ* orchid conservation successfully.

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