ORIGINAL ARTICLE

Annals of Agricultural and Environmental Medicine 2012, Vol 19, No 2, 279-284
www.aaem.pl

Effect of two aerosolization methods on the release of fungal propagules from a contaminated agar surface

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Górny RL, Ławniczek-Wałczyk A. Effect of two aerosolization methods on the release of fungal propagules from a contaminated agar surface. Ann Agric Environ Med. 2012; 19(2): 279-284.

Abstract

The effect of perpendicular and swirling aerozolization methods on the release of fungal fragments and spores from agar surface was studied. Three fungal species (*Aspergillus versicolor*, *Cladosporium cladosporioides*, *Penicillium chrysogenum*) were selected for the tests as they commonly occur indoors, create different hyphae structure when they grow on surfaces, and have different spore shapes, aerodynamic sizes, and formation mechanisms. As the tested surface, Petri dishes filled with malt extract agar, separately inoculated with fungal strains and cultivated to obtain an abundant and even growth were used. For the purpose of these experiments, a new aerosolization chamber was built in which HEPA-filtered air stream responsible for fungal propagule release was either perpendicularly directed towards the contaminated surface or set in swirling motion above it. The experiments were conducted at 2 air velocities, typical for outdoor environment (11.6 m/s) and ventilation ducts (29.1 m/s). Concentrations and size distributions of released fragments and spores were measured using an optical particle counter. The results showed that the propagule release depends on the direction (swirling motion was able to release up to 3.4×10^5 fragments and 3×10^5 spores from 1 cm^2 of contaminated surface, i.e. significantly more than the perpendicularly directed air stream), velocity (the higher the swirling air velocity applied, the higher the number of released propagules) of the air stream above the contaminated surface, and varied due to the taxonomical species origin (the higher number of particulates was released by *Aspergillus* colonies). Hence, the efficient control of both microbial fragments and spores, not only in the air, but also in their source should be an integral part of the quality control procedure.

Keywords

fungi, fragments, spores, agar surface, swirling release, air velocity

INTRODUCTION

Microbial fragments can be classified as large (i.e. greater than 1 μm), submicrometer (usually from 1 μm - 1 nm) and ultrafine (less than 1 nm) size particles. Due to their aerodynamic properties conditioned by physical features (such as e.g. diameters, density, etc.), they can remain airborne for a relatively long period of time (from several hours to several days). They derive from any intracellular or extracellular microbial structures and as such may be related to both hyphal and conidial elements of the colonies [1]. For fungi, a process of their creation is preceded by the formation of vacuoles following a reduction in available nutrients or environmental stress and leads to the separation of hyphae at septal junctions. In the case of fungal spores, their fragmentation may be due to the rupturing of multicellular conidia along septal wall junctions, or through osmotic pressure differences due to moisture differentials [2]. Some of these pieces preserve their viability and are capable of starting a new seat of growth. It is also possible that the fragments are parts of spores and fruiting bodies, or are formed through nucleation from secondary metabolites, such as microbial volatile organic compounds (MVOCs) [3].

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Received: 10 April 2012; accepted: 20 May 2012

Microbial fragments are common in both outdoor and indoor air; however, their presence, especially within submicrometer and ultrafine size ranges, has so far been overlooked when assessing microbial exposures. Fungal fragments are usually present in the atmosphere in 99% of days and their abundance has both diurnal and seasonal trends, i.e. for large fragments (>1 μm), the highest concentrations occurred between 14:00-17:00 [4, 5] in summer and fall and the lowest in late winter and early spring [6, 7, 8]. Large hyphal fragments have been shown to compose 6-56% of the total fungal particle counts in field samples based on microscopic sample analysis [2]. When atmospheric air was analyzed, the fragments showed a significant correlation with the number of total spores and many individual spore types [8]. For indoor air, an opposite relationship was observed. Several laboratory-based studies have reported that large quantities of submicrometer-sized fungal and actinomycetal fragments (ranging from 30 nm - 1 μm) are aerosolized from contaminated surfaces in much higher (300-500 times) concentrations together with intact spores [9, 10, 11, 12]. Nevertheless, the number of airborne fragments did not correlate with the corresponding spore counts, therefore fragment concentrations cannot be estimated based on spore data [9, 10, 11, 12, 13].

The most common mechanism of microbial particle aerosolization is dispersion by environmental airflow [9, 14, 15, 16, 17, 18, 19, 20]. Fragment bioaerosols are generated at relatively low velocities, which represent normal indoor

conditions (e.g. from 0.3 m/s - 7 m/s); however, no particle fragments are detected at 0.1 m/s [9, 18, 20]. Laboratory studies show that the specific parts of the fungal colony which undergo fragmentation are spores. During this process (e.g. for *Penicillium* spores), the number of submicrometer particles – which have a greater impact on health – were found to increase up to 400 times [19]. The abundant generation of spore bioaerosols usually requires higher air velocities, up to 30 m/s [9, 20]. Generally the increase of the air velocity above the microbiologically contaminated surfaces augments the emission rate of fungal propagules. However, in specific situations such as aerosolization from smooth surfaces, the release of fragments is not significantly affected by the air velocity. The different trend in the release of submicrometer propagules, compared to that of intact spores, indicates that the fragments are aerosolized through a different mechanism from that for spores. In the case of fungi, it can be assumed that the fragments are already liberated from the mycelium or spores before the air currents carry them away. Thus, all the fragments are aerosolized at low air velocity, and an increase in the velocity does not augment their release [3].

Environmental exposure to airborne microorganisms is still insufficiently investigated. Traditional analytical methods usually focus on quantitative and qualitative evaluation of microbial spores and/or vegetative cells. Such an approach neglects both the immunological reactivity of small fragments of their structural elements and emission strength of microbial colonies. The proper exposure assessment should contain not only comprehensive identification of microbial contamination but, if possible, also a credible measure of the emission strength of its source [3, 21]. Hence, the aim of this study was to test the effect of 2 (perpendicular and swirling) aerosolization methods on the release of fungal fragments and spores from a microbiologically contaminated agar surface.

MATERIALS AND METHODS

Three environmentally isolated fungal species (*Aspergillus versicolor, AV; Cladosporium cladosporioides*, *CC; Penicillium chrysogenum, PC*) were selected for the tests because they commonly occurred indoors, create different hyphae structure when they grow on surfaces, and have different spore shapes, aerodynamic sizes, and formation mechanisms [9, 17, 22]. As the tested surface, Petri dishes filled with malt extract agar (MEA; Merck KGaA, Darmstadt, Germany), separately inoculated with selected strains and cultivated were used to obtain abundant fungal growth. Examples of hyphae structure created by tested fungal species on MEA surface are shown in Fig. 1. The spore suspensions for MEA inoculation were prepared by washing fungal colonies from the agar plates using deionized and sterilized water (Direct-Q 3, Millipore Corporation, Billerica, MA, USA). The spore concentrations in the initial water suspensions were checked using a hemacytometer (Blaubrand®, Brand GmbH+CO KG, Wertheim, Germany). The agar plates were inoculated with 0.1 ml of fungal suspensions contained 10^6 spores/ml and incubated in separate chambers for 14 days at the temperature of 22°C and relative humidity of 97-99%. This humidity was achieved by placing a saturated K_2SO_4 (POCH SA, Gliwice, Poland) solution (150 g/l) [23] at the bottom of the incubation chamber. After incubation, 2 agar samples of each tested strain were used for testing the initial spore surface concentration. A 1 cm^2 piece of the contaminated agar was sterile cut and suspended by vortexing in 25 ml of deionized and sterilized water in a Falcon type test tube (Sarstedt, Nümbrecht, Germany). Their concentrations in the resulting suspension were examined using a hemacytometer (Blaubrand®) and for all tested species were between 10^6-10^7 spores/cm^2.

For the purpose of these experiments, a new aerosolization chamber was built in which the constant HEPA-filtered air stream responsible for the fungal propagule release was either perpendicularly directed towards the contaminated surface, or set in swirling motion above it (due to 3 jets mounted at 45° angle). Both an experimental setup and 2 tested configurations of the aerosolization chamber are schematically shown in Fig. 2. To prevent any contamination of the surrounding environment (which can occur when the air stream circulates through the tested system), a rubber gasket was mounted at the bottom of each tested chamber and the whole setup was placed inside a class II biosafety cabinet (Alpina-04 BIO, Alpina, Konin, Poland).

The study was designed to mimic the environmental worst-case scenario, in which the maximal number of immunologically reactive propagules can become airborne due to mechanical stress provoked by air movement. Hence, the experiments were carried out at 2 relatively high air velocities, i.e. 11.6 and 29.1 m/s, typical for the outdoor environment and ventilation ducts, respectively. Concentration and size distribution of released fragments and spores were measured using a Grimm optical particle counter (model 1.108, Grimm Aerosol Technik GmbH, Ainring, Germany). The duration of each experiment was 10 min as the substantial aerosolization is a short time process [9]. Before each test, the experimental system was purged by passing clean air through it. At the beginning of every experiment, the system was operated in the absence of test agar under the chamber until the particle level was zero, as measured by the optical particle counter. In the next step, a non-contaminated agar plate (incubated under the same conditions and time as the inoculated media) was placed in the aerosolization chamber to establish the background level for particles released from the test surface when exposed to air flow. These levels were negligibly low, up to 0.012% of the total released propagules. As fungal propagules have been shown to be aerosolized more easily when the air is dry [15, 24], all the experiments were carried out at room temperature (22±1°C) and low relative humidity (35±3%) controlled using the Omniport 20 portable handheld meter equipped with suitable sensing probes (E+E Elektronik GmbH, Engerwitzdorf, Austria). Each test was repeated 3 times.

All data were statistically elaborated with analysis of variance (ANOVA) followed by Scheffe's test, as well as t-test using Statistica (data analysis software system) version 7.1 – 2006 (StatSoft, Inc., Tulsa, OK, USA).

RESULTS AND DISCUSSION

To differentiate between fungal fragments and spores, the optical particulate size distribution was measured for each tested fungal species. The specific step plots for all tested fungal propagules released from agar colonies are shown

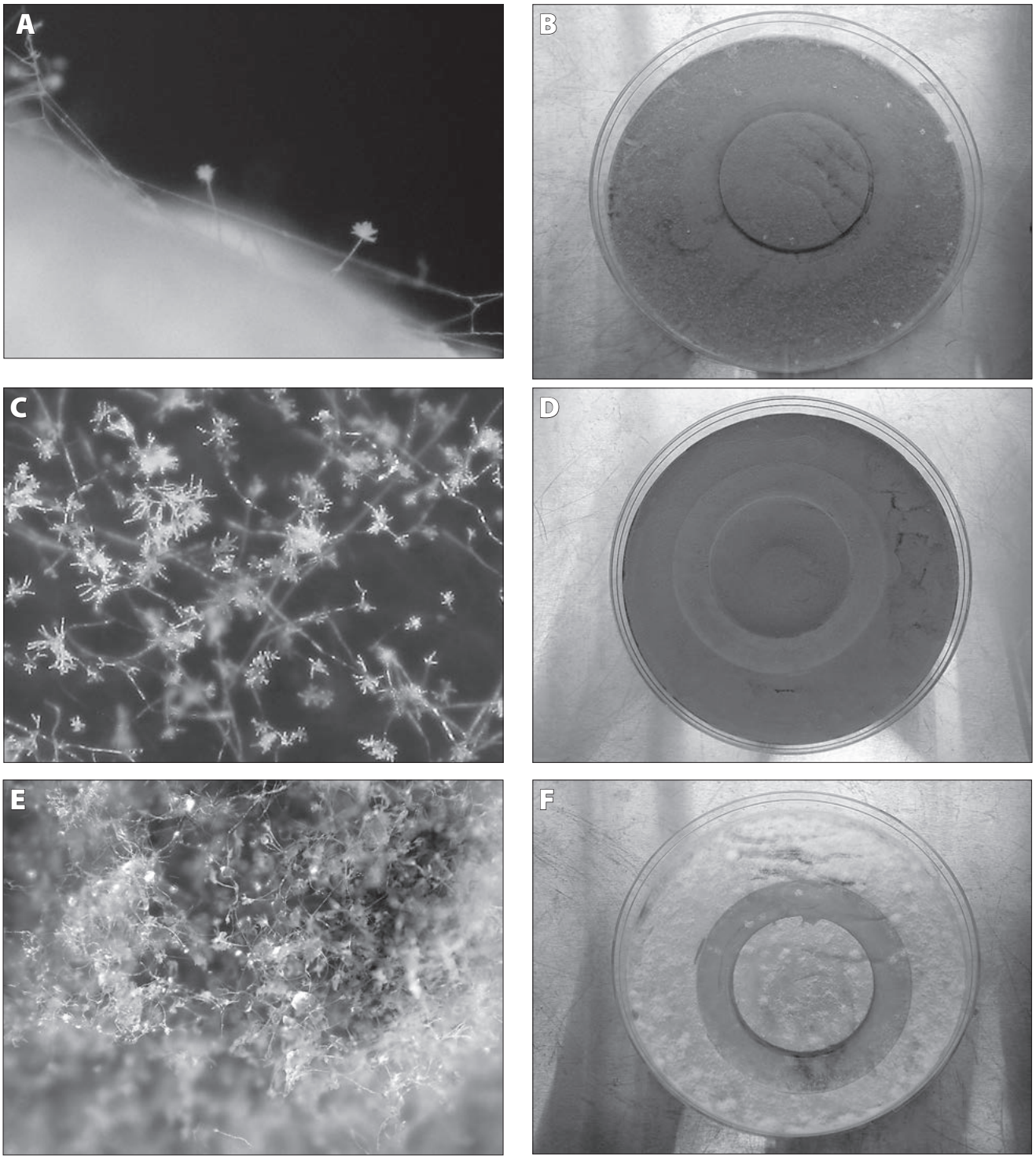

Figure 1. Spatial (stereomicroscopic views at magnification: A – 100×, C – 120×, d E – 80×, and surface (B, D, and F (all after aerosolization experiments) structures of *Aspergillus versicolor* (A, B), *Cladosporium cladosporioides* (C, D), and *Penicillium chrysogenum* (E, F) colonies

in Fig. 3. Taking into account the physical sizes of spores and their respective aerodynamic equivalents [9], the size distributions of tested strains showed that the peak in the number of released particulates corresponding to the spore size was always visible between 1.6-2 μm. Therefore, the particle size of 1.6 μm was selected as the lower counting limit separating fungal spores from their fragments.

The results show that the release of fungal propagules depends on the direction and velocity of the air stream above the contaminated surface, and varied due to taxonomical species origin. The air stream perpendicularly directed towards the agar was able to aerosolize during 10-min experiments up to 2.1×10^4 fragments and up to 4.6×10^4 spores from 1 cm^2 of the contaminated surface, whereas the swirling motion of the air was able to release significantly more propagules ($p<0.05$), i.e. up to 5.2×10^5 fragments and 5.3×10^5 spores from the same area (Fig. 4). There were significant differences between the numbers of propagules released into the air by the 3 tested fungal species (ANOVA: $p<0.05$). On average, significantly more fragments and spores

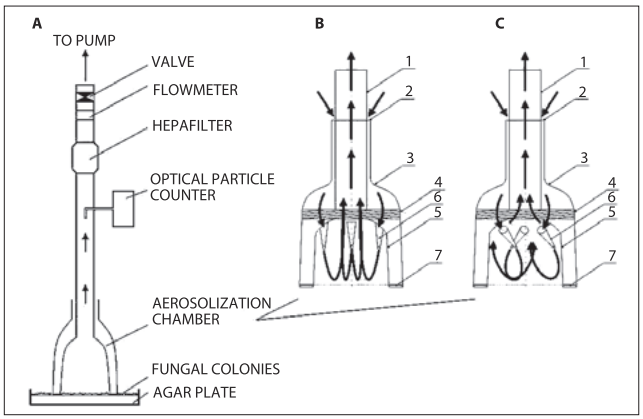

Figure 2. Schematic views of experimental setup (A) and a new aerosolization chamber in two tested (perpendicular – B and swirling – C) configurations. The arrows indicate direction of air stream circulation, algebraic notation: 1 – outlet (to pump), 2 – inlet, 3 – outer chamber, 4 – filter, 5 – inner chamber, 6 – jet, 7 – rubber gasket (which has direct contact with contaminated surface below the chamber)

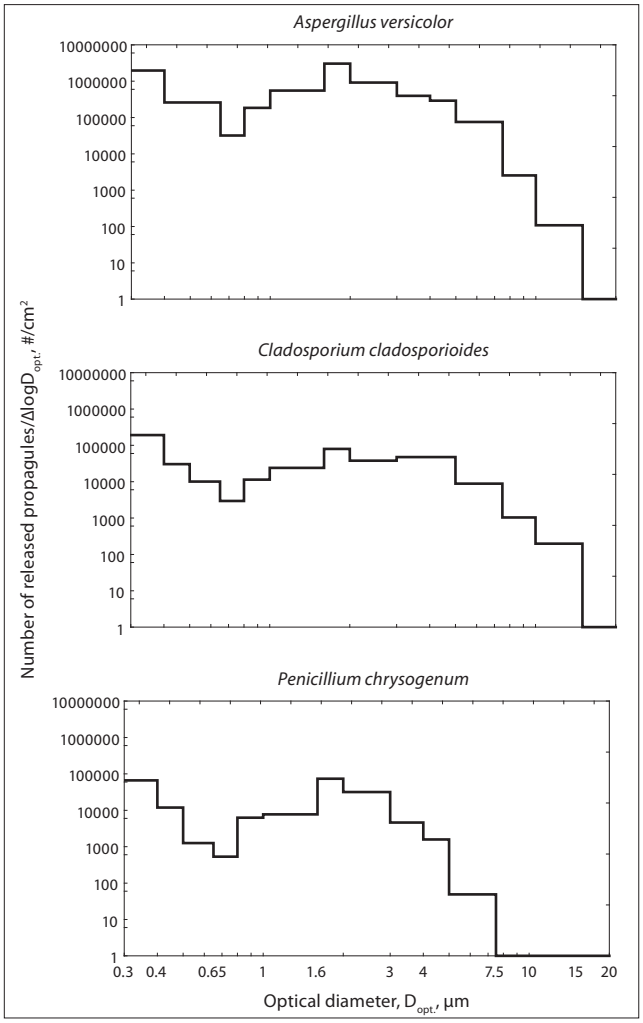

Figure 3. Optical size distributions of fungal propagules released from agar surface

were released by *AV* colonies (3.4×10^5 and 3.1×10^5) compared to *CC* (3×10^4 and 2.6×10^4) and *PC* (1.3×10^4 and 8.3×10^3) when swirling air movements were applied at the air velocity of 29.1 m s^{-1} (Scheffe test: $p<0.05$). The same trend was observed for the perpendicularly directed air stream; however, the numbers of aerosolized propagules were always significantly lower (1.5×10^4 and 3.3×10^4, 4.1×10^3 and 7×10^3 as well as 6×10^3 and 6.5×10^3 for fragments and spores of *AV*, *CC* as well as *PC*, respectively; $p<0.05$). Irrespective of the taxonomical origin of the tested species and aerosolization methods, an increase of air velocity above the contaminated surface caused a significant increase in the number of released propagules (t-test: $p<0.001$ and $p<0.01$ for *AV* fragments and spores, respectively; $p<0.01$ for *PC* in both cases; $p<0.01$ for *CC* fragments only) (Figure 4).

To date, the study on aerosolization of fungal propagules from contaminated surfaces has been limited to a few laboratory chamber experiments [9, 17, 18, 19, 21, 25, 26, 27] and field studies [28, 29, 30]. Except the experiments carried out by Kanaani *et al.* [19], in the rest of these studies the aerosolization processes were achieved by hitting fungal colonies with clean air perpendicularly directed at them. Kanaani *et al.* also tested this method to release fungal propagules from agar plates individually inoculated with *Aspergillus*, *Penicillium* and *Cladosporium* strains, but supplemented these analyses with additional experiments in which the continuous HEPA-filtered air stream of constant velocity was directed over the contaminated agar surface at an angle of 60°. In terms of the number of released propagules as a function of air velocity and direction of the air stream above the mycologically contaminated agar surface, the numbers of both fragments and spores released from MEA surface in this study was higher than those reported in the above-mentioned laboratory chamber experiments [9, 17, 18, 19, 25, 26, 27]; however, the release methods, design and construction of aerosolization chambers as well as cultivation conditions of tested fungal species described in previous studies were different than those used here.

As mentioned above, the observed differences between fungal species in both fragment and spore releases can be also explained by the differences in the structure of fungal colonies growing on the contaminated surface. In Fig. 1, a few examples of the surface (obtained using a light microscope Eclipse E200, Nikon, Tokyo, Japan) and spatial (documented with stereomicroscope; SteREO Discovery.V12, Carl Zeiss, Göttingen, Germany) structure of the tested fungal colonies are presented. Briefly, *AV* colonies have longer and thicker conidiophores (up to 700 µm long, 4-7 µm in diameter) than the colonies of *CC* and *PC* (up to 350 µm with 2-6 µm and up to 500 µm with 2.5-4 µm, respectively) [22, 31, 32, 33, 34]. Hence, elongated *Aspergillus* colony parts (stipes, metulas, and phialides) are much more susceptible to both desiccation stress (because of the larger exposed area) and mechanical forces (provoked by air turbulences) than the respective *Cladosporium* and *Penicillium* structures [9, 17]. Moreover, microscopic observations revealed that *AV* has much longer spore chains (up to 60-80 single spores in chains) than similar structural elements derived from *CC* and *PC* (usually from a few to several spores in chains). Also, differences in the shape of spores and in the characteristics of their biological connections probably play an important role in the release process. The *AV* conidia are 2-3.5 µm in diameter; most of them are globose to subglobose, with finely to distinctly roughened walls. As such, they have a smaller joint area between 2 subsequent spores in the chain than the larger, ellipsoidal and smooth-walled *CC* and *PC* conidia (3-11×2-5 µm and 3-4×2.8-3.8 µm, respectively) [22, 31, 34]. Moreover, the septum between 2 adjacent dry type *AV* conidia is differentiated into 3 lamellae, the central one of which gelatinizes and is soluble in water, thus assuring

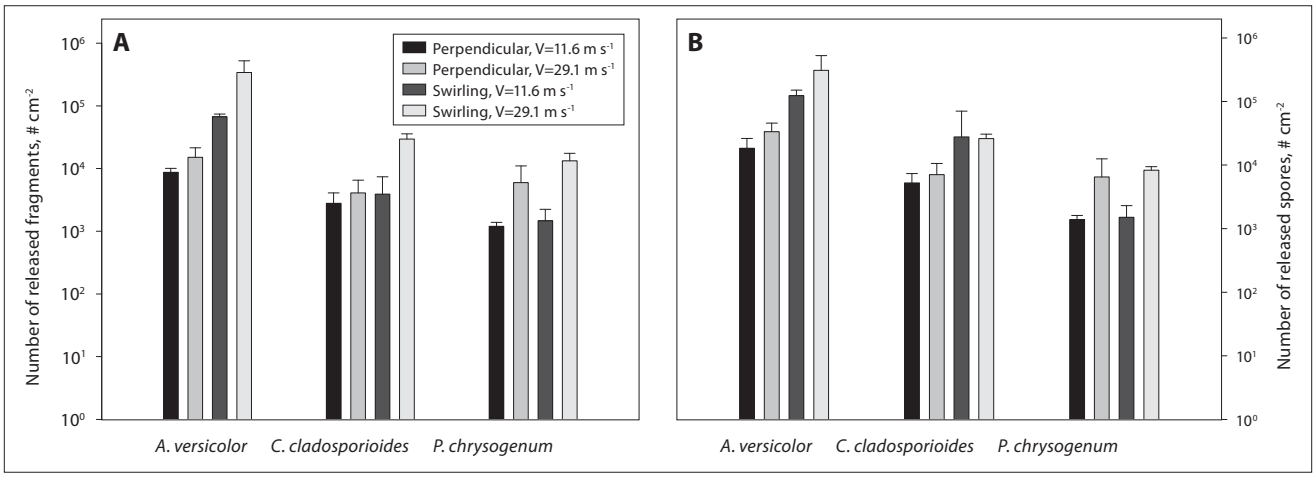

Figure 4. Number of fungal propagules (A – fragments, B – spores) released from agar surface at 2 air velocities during 10-min experiments (error bars indicate standard deviation of 3 repeats)

separation of the conidia [35]. The air stream passing between the spores in a chain may desiccate this type of a joint and break such fragile connections. Furthermore, the mechanisms of spore formation (understood here as the formation of asexual reproductive structures of phialidic conidiogenesis) can play an important role in their release. In the case of *AV*, the oldest spores are located at the end of the spore chain and may be more readily released than the youngest spores having much stronger connections with the hyphae structure. In contrast, the oldest *CC* spores are closer to the whole hyphae and thus closer to the surface because the formation process is based on sequential budding at the ends of hyphae and spore chains. At such a location, a turbulent air flow may not be able to liberate the matured *CC* spores to the same extend as it can with *AV* spores [17]. Moreover, *CC* conidium production is considerably higher under moist than dry conditions and the air stream passing over the growing colonies - by definition - does not support the release of these propagules [31].

Stereomicroscopic observations revealed that fungal mycelium rises vertically, often building a mesh-like structure on the contaminated surface. In this instance, the higher the air turbulences above the colony spatial structure, the greater the effectiveness of the fungal propagule release process. However, the colonies of filamentous fungi create quite frequently not only abundant aerial mycelium but also a dense mycelium structure which clings firmly to the surface. In such cases, the air stream must act as a 'crowbar', which prises open this compact structural net making possible the release of microbiological material trapped under it. In the situation in which the air current is directed at the contaminated surface at a certain angle, fungal propagules are released in much greater numbers than the air stream perpendicularly facing such a surface. When the air vertically approaches the surface covered with fungal colonies, it can close the mesh-like structure instead of opening it, resulting in the imprisonment of both fragments and spores. Moreover, the technical solution, in which the air stream is directed at the contaminated surface at a certain angle, but from one side only of the fungal colony (as tested by Kanaani *et al.* [19]) is not fully effective, as the particulates can be solely liberated from a certain surface-limited 'open' area. A novel technical solution proposed in this study removes these restrictions. A swirling air stream above the microbiologically contaminated surface is able to open the mycelium structure from each of the possible sites (not just the one), thus resulting in a maximization of fungal propagule emission.

The formation of reproductive structures of fungal colonies is a time dependent process, and the numbers of particles released from contaminated surfaces increases with incubation time. As shown in laboratory experiments in which 6-month-old cultures were compared with ones 1-month-old, by reduction of the adhesion forces during incubation (when dryness of the surface constantly increases), the numbers of aerosolized fragments were always significantly higher. This trend was also observed in many cases in the spore size range. The release of particulate (1→3)-β-D-glucan also increased with the age of the fungal culture [12]. To eliminate any influence of the time factor, in the present study all fungal colonies were cultivated for the same period of time and in the same environmental conditions and resulted in abundant growth of their agar colonies. Hence, the release processes described above became more objective and reflected the individual features of the 3 tested fungal strains.

CONCLUSIONS

Swirling motion of the air releases significantly more propagules than the air stream perpendicularly directed towards the microbiologically contaminated surface. Proper assessment of indoor exposure to microbial contaminants should take into account all propagules, which have immunological reactivity and the ability to become airborne. Hence, efficient control of both microbial fragments and spores, not only in the air but also in their source, should be an integral part of the quality control procedure.

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