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Biomechanics of conidial dispersal in the toxic mold *Stachybotrys chartarum*

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Abstract

Conidial dispersal in *Stachybotrys chartarum* in response to low-velocity airflow was studied using a microflow apparatus. The maximum rate of spore release occurred during the first 5 min of airflow, followed by a dramatic reduction in dispersal that left more than 99% of the conidia attached to their conidiophores. Micromanipulation of undisturbed colonies showed that micronewton (μN) forces were needed to dislodge spore clusters from their supporting conidiophores. Calculations show that airspeeds that normally prevail in the indoor environment disturb colonies with forces that are 1,000-fold lower, in the nanonewton (nN) range. Low-velocity airflow does not, therefore, cause sufficient disturbance to disperse a large proportion of the conidia of *S. chartarum*.

Keywords

allergen; conidiophore; digital video analysis; micromanipulation; mycotoxin; satratoxin; spore

1. Introduction

Conidia of *Stachybotrys chartarum* carry mycotoxins and potentially-allergenic proteins (Hossain et al., 2004). Some studies have associated the proliferation of this fungus in water-damaged buildings with adverse effects upon human health (Dearborn et al., 2002), but the clinical significance of this fungus is exceedingly controversial (Douwes and Pearce, 2003; Miller et al., 2003; Portnoy et al., 2005). The conidia of *S. chartarum* are relatively large (7–12 μm long \times 4–6 μm wide) and are produced in sticky heads of multiple spores at the tips of short conidiophores. This distinctive morphology contrasts with the appearance of other common indoor molds. For example, species of *Aspergillus* and *Penicillium* produce heads of thousands of tiny, spherical, dry conidia. The morphology of *S. chartarum* conidia is an important consideration for researchers investigating putative mold-related illnesses because (i) the sticky clusters of spores appear poorly adapted for dispersal in air, and (ii) the individual conidia are too big for inhalation deep into the lung (Kuhn and Ghannoum 2003). The second issue is complicated by the recent discovery of tiny, highly-respirable particulates from colonies of *S. chartarum* (Brasel et al., 2005). The present study was designed to assess conidial behavior in *S. chartarum* under low-speed airflow regimes that are characteristic of the indoor environment.

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2. Materials and methods

2.1. Cultures

S. chartarum (Ehrenberg) Hughes strain 58–17 (ATCC 201211) was kindly supplied by Dr. Steven Vesper (U.S. Environmental Protection Agency, Cincinnati, Ohio). This strain produces macrocyclic trichothecenes (Jarvis et al., 1998). Cultures of *Aspergillus niger*, *Cladosporium sphaerospermum*, and *Penicillium chrysogenum* were obtained from contaminated buildings in Ohio. *A. niger* was isolated from an air sample; *C. sphaerospermum* and *P. chrysogenum* were cultured from colonies growing on gypsum wallboard. Fungi were identified by comparison of their ITS sequences with GenBank data (accession nos. **EF105365**, **EF105366**, **EF105367**, **EF105368**). The primer pair ITS5 and ITS4 (White et al., 1990) was used for PCR and sequencing of *S. chartarum*; sample dilution was necessary to prevent interference with PCR caused by the high concentration of pigments associated with conidia. For the other species, the primer pair ITS1F (Gardes and Bruns 1993) and ITS4 was used. For experiments, cultures were grown on rectangular chips (10 × 7 mm) of gypsum wallboard (ProRoc® Regular, BPB America Inc., Tampa, FL) consisting of the face paper surface backed with a 2–3 mm thick mineral layer. After sterilization, the chips were inoculated by brief contact, paper side down, with the surface of fungal colonies sporulating on potato dextrose agar (PDA). The chips were then placed, gypsum side down, on the surface of water agar in Petri dishes. Each chip was hydrated with 100 µL of sterile distilled water delivered by pipet. The dishes were then sealed with Parafilm and incubated at room temperature.

2.2. Spore yield on gypsum wallboard

To estimate the total number of spores produced on the gypsum wallboard substrate, wallboard chips supporting 3-week-old colonies were submerged in 1 mL aliquots of sterile distilled water in microfuge tubes. The spores were dispersed by aggressive vortexing for 1 min. Conidia mixed readily with the water and showed no collection at the air-water interface after vortexing. The mean number of spores per cm² of wallboard surface was based upon 10 replicate counts using a standard hemocytometer slide.

2.3. Microflow apparatus for studying spore dispersal

The cuvette-based microflow apparatus is, in essence, a miniature wind tunnel (Fig. 1). The design is comparable to the apparatus used by Zoberi (1961) and Pady et al. (1969), though ours allows continuous microscopic observation of conidiophores during airflow. Standard methacrylate 10 × 10 mm cuvettes were adapted by machining a rectangular port (8 × 20 mm) in one side and by gluing a plastic cap to the open end. Holes were then drilled into both ends of the cuvette and connectors were glued to these openings for attachment of PVC tubing (1/8 ID × 1/16 wall). The internal volume of the modified cuvettes was 4.5 mL. For experiments, samples were inserted into the cuvette through the rectangular port, and this was closed with a 9 × 22 mm #1 coverslip (Electron Microscopy Supplies, Washington, PA) and sealed around the edges using thin strips of adhesive tape. Airflow was controlled using two different methods: negative pressure (sucking air through the cuvette) and positive pressure (blowing air through the cuvette). For the negative pressure method, one of the cuvette openings was connected to a portable IAQ sample pump (Environmental Monitoring Systems [EMS], Charleston, SC) that provided reproducible volumetric airflow between 2 and 10 L min⁻¹, corresponding to airspeeds between 0.3 and 1.6 m s⁻¹ over the samples (e.g., 2 L min⁻¹ airflow exchanges volume of 4.5 mL every 0.14 s, creating airspeed of 0.32 m s⁻¹ along the 43 mm internal length of the cuvette). These airspeeds correspond to the typical low velocity airflow within buildings that has been studied by other investigators (e.g., Górný et al., 2002; Kildesø et al., 2003). The effects of the much higher airspeeds that are measured in ventilation ducts (Krauter et al., 2005) were not studied. Spores released from the samples were captured on Micro5 Microcell cassettes (EMS) or Cyclex-D impactor cassettes (EMS) that were connected

between the cuvette outflow and pump inlet. For the positive pressure method, air was delivered to the cuvette inflow from a compressed air cylinder and spores were captured on Cyclex-D impactor cassettes connected to the cuvette outflow. Airflow from the cylinder reduced the relative humidity within the cuvette to 2–3% at 23 C (measured with a digital thermo-hygrometer; model RH 411, Omega; Stamford, CT). Spore counts were performed according to standard protocols (examination of 25% of trace with 10x and 40x objectives; Niemeier et al., 2006). For both types of impactor cassette, samples were collected for 5 min. Inlet and outlet pressures were controlled and monitored with flowmeters (Omega Engineering, Stamford, CT). For both methods of air delivery, the pressure drop through the apparatus (due primarily to air leakage through the adhesive tape closure of the cuvette) was less than 10%. The cuvette and outlet tubing were cleaned between runs by washing with 95% ethanol followed by scrupulous drying.

For video analysis of colonies, the cuvette was taped to the stage of an inverted microscope (Olympus IX-70) with the coverslip facing downward. Video images of colonies subjected to low speed airflow were captured with a digital video camera (Pixelfly QE P/N 270 XS; The Cooke Corporation, Romulus, MI) with 5 μ s shutter speed capability.

2.4. Scanning electron microscopy

Samples of wallboard supporting mold colonies were fixed in 2.5% gluteraldehyde and 1% formaldehyde in 0.05 M sodium cacodylate buffer (primary fixation), followed by 1% osmium (secondary fixation). Fixed samples were sputter coated with 20 nm of gold prior to imaging. Coverslips from the Cyclex-D impactor cassettes were mounted on metal stubs and coated with 45 nm of gold without fixation. Samples on wallboard and coverslip were viewed with a JEOL JSM-840A scanning electron microscope.

2.5. Micromanipulation of conidiophores

Data on displacement forces were obtained by manipulating conidiophores with microprobes attached to miniature strain gauges. First, micropipettes with 2.5 μ m tips were prepared from glass capillaries (OD 1.0 mm, ID 0.58 mm, w/fil; WPI, Sarasota, FL) using a horizontal micropipette puller (Sutter Instrument, Novato, CA). The tips of these pipettes were then sealed and polished by swift passage through a Bunsen flame, to produce smooth semi-ellipsoidal tips with diameters of 5–8 μ m. Straight-tipped micropipets were selected under a microscope and their wide ends were cut with a diamond knife to produce microprobes with a length of 5 mm. Finally, the microprobes were attached to a plastic peg glued to the end of a calibrated miniature strain gauge (type AE-801; Sensor One Technologies Corporation, Sausalito, CA). The instrument is shown in Fig. 2A. Strain gauges were prepared and calibrated according to the methods in MacDonald et al. (2002); a calibration curve for one of the strain gauges is shown in Fig. 2B. During the manipulation of the cultures, the strain gauges with attached probes were positioned with a motorized micromanipulator (MP-285; Sutter Instrument).

3. Results

3.1. Behavior of conidiophores of *S. chartarum* and other mold species at low airspeeds

Colonies of *S. chartarum* were battered by airspeeds of 0.3 to 1.6 m s⁻¹ within the microflow apparatus. Conidiophores showed considerable elasticity, moving backwards and forwards in the airstream, pivoting through angles of up to 45° (Fig. 3). Individual conidia were firmly fixed to one another within their conidial heads (clusters), and the heads showed strong attachment to their supporting conidiophores. Only in rare instances did we observe conidia separating from their conidiophores. There was no change in visible behavior of colonies of ages varying from 3 to 12 weeks after inoculation. Conidia of *S. chartarum* captured on sampling cassettes connected to the cuvette outflow are shown in Fig. 4.

Colonies of *A. niger*, *C. sphaerospermum*, and *P. chrysogenum* were also studied within the microflow apparatus. In all cases, the conidiophores pivoted in the manner described for *S. chartarum*, but there were differences in the ease with which the conidia separated from one another and became airborne. Video analysis showed only rare examples of spore release from conidial heads of *C. sphaerospermum*, but more frequent release of small numbers of conidia was observed from colonies of *A. niger* and *P. chrysogenum* at an airspeed of 1.6 m s^{-1} (data not shown).

3.2. Quantifying spore release at low airspeeds

Mean spore production on sterilized wallboard, measured after a 3-week incubation, is shown for each species in Table 1. The greatest spore yield was observed for *S. chartarum*: 1.93×10^6 spores cm^{-2} ($\pm 7.1 \times 10^4$ [s.e.m.], $n = 10$). Spore release from the gypsum board substrate was measured using the microflow apparatus. Initially, the effect of airspeed on spore release was tested on 5-week old colonies of *S. chartarum* (Table 2). In all of these experiments, spore release was assessed during a 5 min period of airflow from colonies that had been undisturbed during incubation. As we anticipated, the numbers of spores released showed a significant increase as airflow was increased from 4 L min^{-1} (0.6 m s^{-1}) to 10 L min^{-1} (1.6 m s^{-1}), but the number of spores released from their conidiophores remained very modest: a maximum of 0.025% of the total spore production was disseminated per min.

To determine whether the rates of spore release measured in these experiments were sustained for longer periods of time, we measured spore release from 3-week-old colonies at intervals of 5 min for 1 h of continuous airflow through the microflow apparatus. At the end of the airflow, the substrate was removed from the cuvette and the number of spores remaining on its surface was measured using the hemocytometer method detailed in section 2.2. This experiment showed that a cloud of spores was released in response to the initial disturbance caused by airflow, but that very few spores followed after this initial burst (Fig. 5A). Only 0.1–0.2% of the total spore production was released from the substrate during 1 h of airflow, and 87% of these mobile spores were liberated during the first 5 min of disturbance. The proportion of the spores that remained attached to the substrate after 1 h of airflow at a speed of 1.6 m s^{-1} exceeded 99.9%. For comparative purposes, we also studied patterns of spore release under conditions of continuous airflow for the other mold species (Fig. 5B). In every case, maximum conidial release occurred within the first few minutes of airflow, with the rate of release diminishing during the subsequent hour. The proportion of the spores released after 1 h of airflow was 0.8% for *C. sphaerospermum*, 1.1% for *A. niger*, and 1.8% for *P. chrysogenum*. While the percentages for the four mold species are very similar, the corresponding numbers of discharged spores show substantial differences. The release of 0.2% of the total conidial production of *S. chartarum* corresponds to approx. 2,000 conidia cm^{-2} ; 0.8% of the conidial production in *C. sphaerospermum* represents approx. 1,000 conidia cm^{-2} ; 1.1% of the conidial production in *A. niger* corresponds to 20,000 conidia cm^{-2} , and 1.8% of the conidial production of *P. chrysogenum* to 26,000 conidia cm^{-2} . When these differences are scaled up to larger areas of contaminated surface, it is clear that the dry-spored *A. niger* and *P. chrysogenum* will form far denser clouds of spores when exposed to the same airflow conditions as *S. chartarum*.

3.3. Micromanipulation experiments

Spore clusters of *S. chartarum* were manipulated using a microprobe attached to a miniature strain gauge. A few spores, and even whole conidial heads, were dislodged with the exertion of forces beneath the μN resolution of the strain gauge. Individual spores were also repelled and displaced by electrostatic charges distributed over the pipet tip. But the majority of the conidial heads resisted disruption, and separation of clusters and individual spores from conidiophores occurred only upon application of forces of a few μN by the pipet tip (Fig. 6). The mean force needed to disrupt these more tightly packed conidial heads was $4.6 \pm 1.0 \mu\text{N}$.

($n = 10$). Experimental manipulation of the other mold species showed that their conidia were more easily separated from one another by disturbances in the nN range. In common with *S. chartarum*, however, many spores remained attached to their conidiophores until μN forces were applied (data not shown).

4. Conclusions

4.1. Interpretation of airflow and micromanipulation experiments

In the four mold species examined in this study, spore release in response to low-velocity airflow occurred as an initial burst of hundreds or thousands of conidia per cm^2 of substrate, followed by the sustained ejection of small numbers of spores. Conidia of *S. chartarum* showed the greatest resistance to dispersion, with 99.8% of the spores remaining attached to the colony surface after 1 h of airflow at a speed of 1.6 m s^{-1} (data for 3-week-old colonies). It is possible that greater numbers of conidia would have been released from colonies older than 3 weeks, but Table 2 shows that a similarly minuscule proportion of the total spore production was dispersed after 5 weeks incubation (i.e., $< 0.1\%$ during first 5 min airflow up to a maximum velocity of 1.6 m s^{-1}), and 12-week-old colonies behaved in the same manner as younger ones in the microflow apparatus (section 3.1). The same conditions dispersed a larger proportion of the dry spores of *A. niger*, *C. sphaerospermum*, and *P. chrysogenum*, but the numbers of spores released by these fungi during 1h of airflow still represented less than 2% of the total spore production. The absence of colony microparticulates among the spores captured in our studies is probably a feature of the use of impactor cassettes rather than filters of defined pore size (Fig. 6). A study of Cyclax-D cassettes using PSL particles and NaCl crystals showed that capture efficiency fell sharply beneath a particle size of $1 \mu\text{m}$ (Grinshpun, 2001). Only 30% of $0.8 \mu\text{m}$ diameter particles were trapped, and the efficiency dropped below 20% for even smaller particles.

The forces acting upon the spore clusters of *S. chartarum* within the microflow apparatus are comparable to those acting upon an object that moves slowly through a liquid: viscosity is the dominant consideration. The viscous drag acting upon the spore is proportional to the velocity of the airflow (Figure 7). The estimated drag at 1.6 m s^{-1} (the fastest airspeed used in our experiments) is 8.1 nN (Figure 7B). The micromanipulation experiments demonstrated that the majority of conidial heads of *S. chartarum*, and individual conidia, resisted removal from their conidiophores until they were displaced by forces in the micronewton range. The small proportion of spores that were easily detached by micromanipulation (i.e., by nN forces) probably correspond to the sub-population of spores released upon initial exposure to airflow. There is, therefore, up to a three order-of-magnitude mismatch -- nN versus μN -- between the forces exerted against conidia by low-velocity airflow and the forces needed to separate the majority of them from their conidiophores.

Our data suggest that a small proportion of the conidia that develop in a colony in static air loosen their connection to their conidiophores. This sub-population of spores is easily dislodged by micromanipulation and is dispersed as a cloud upon initial exposure to airflow. Subsequently, a low rate of spore release is sustained indefinitely from the enormous reservoir of attached conidia. A similar pattern of spore release occurs in the other mold species examined in this study, but the sharp decline in dispersal following the initial burst was less pronounced in the dry-spored species (Fig. 5B). Sluggish release of conidia was documented in a number of earlier studies on spore liberation (Zoberi, 1961; Ingold, 1971), and, more recently, Górný et al. (2001) measured similar bursts of spore release from colonies of *Aspergillus versicolor*, *C. sphaerospermum*, and *Penicillium melinii* subjected to airflow and vibration.

In buildings, *S. chartarum* and other molds grow on flat surfaces of homogeneous chemical composition, and are buffered against high airspeeds and extremes in temperature and

humidity. Colonies growing on natural substrates, such as plant debris, are likely to be subjected to significantly greater physical disturbances than those growing in buildings. Invertebrates act as vectors for fungal dispersal in the natural environment (Ingold, 1971), and may prove particularly important in transporting the sticky conidia of *S. chartarum*. In addition to their resistance to release from the colony surface, the large conidia of this mold cannot remain airborne as long as those of other species. Stokes' law predicts a terminal velocity of 1.9 mm s^{-1} for *S. chartarum* (based on a radius of $3.6 \mu\text{m}$ for the equivalent sphere), versus 0.3 mm s^{-1} for *Aspergillus* (radius = $1.5 \mu\text{m}$; Gregory and Hendon, 1976; Deering et al., 2001). This means that *S. chartarum* spores will fall 1 m in still air in 9 min, compared with a settling time of 56 min for *Aspergillus*.

4.2. Implications for public health

Given continuing concerns about the significance of *S. chartarum* to public health, our data on dispersal throw some light upon the relationship between the proliferation of this mold on surfaces and resulting concentrations of airborne conidia. A number of authorities recommend that a mold-contaminated surface in a home covering an area of 10 ft^2 (0.93 m^2) should be cleaned by the homeowner without recourse to professional mold-remediation services (www.epa.gov). Larger areas may require professional remediation. Based upon the measured yield of $1.93 \times 10^6 \text{ spores cm}^{-2}$ (Table 1), more than 19 billion spores of *S. chartarum* could form on an area of 1 m^2 of confluent growth. According to our experiments, low speed airflow could eject 0.2% of these spores in 1 h, producing a cloud of 38 million potentially allergenic and toxic particles. If each of these spores carried 100 femtograms (10^{-13} g) of macrocyclic trichothecenes (published estimates range between 1 and 100 fg), the airborne toxin load would be around $4 \mu\text{g}$. We assume that the quantity of airborne mycotoxins increases in proportion to the area of mold growth in a particular indoor space, but it is difficult to quantify the extent of mold proliferation in situations where multiple discrete colonies are distributed over a wide area. Despite these uncertainties, the suggestion by Burge (1996) -- and supported by Kelman et al. (2004) -- that a person living in a highly contaminated environment could accumulate no more than a few nanograms of toxin in 24 h seems very reasonable. The significance of this level of mycotoxin exposure remains uncertain. The ejection of microparticulates from toxigenic colonies may boost the concentration of airborne mycotoxins beyond estimates based on conidial release (Brasel et al., 2005).

The present study shows that *S. chartarum* is poorly adapted for dispersal by airspeeds that prevail in the indoor environment. Even in homes that become heavily contaminated with *S. chartarum*, only modest numbers of conidia will become airborne unless contaminated surfaces are disturbed by high airspeeds or vibration. With this in mind, it is interesting that cases of putative mold-related illness may have been associated with HVAC systems that pulled air from contaminated basements, and with the use of electric fans that generate higher airspeeds than those in the rest of the living space (Montaña et al., 1997; Etzel et al., 1998). This issue should be considered when evaluating the use of high-capacity fans to dry homes after flooding.

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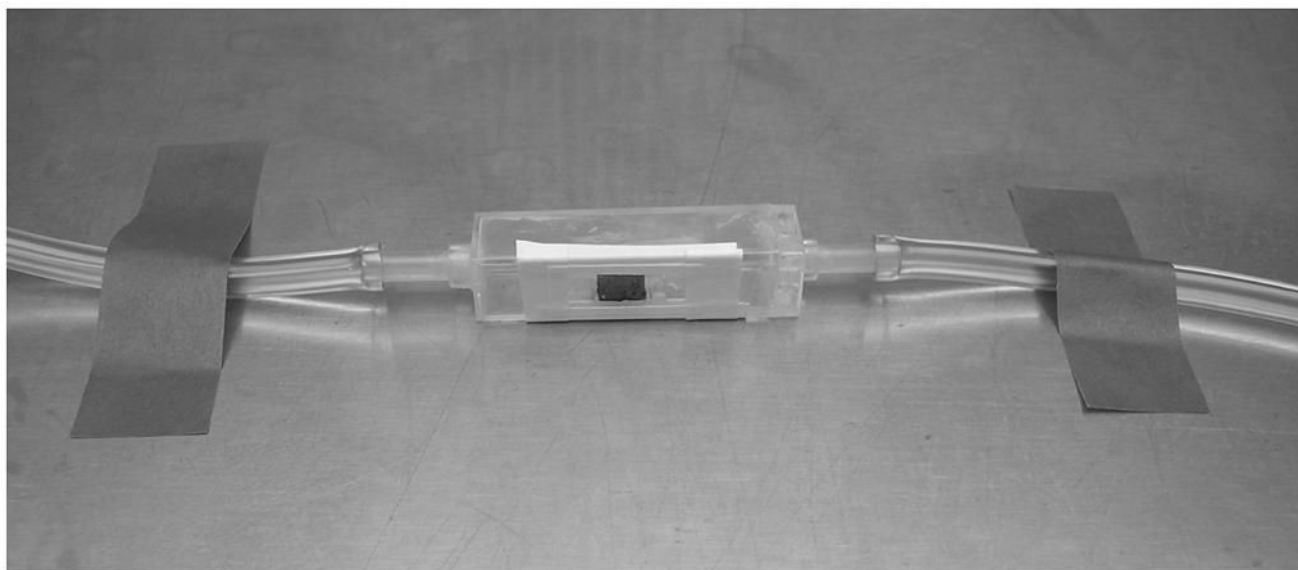


Figure 1.
Cuvette-based microflow apparatus.

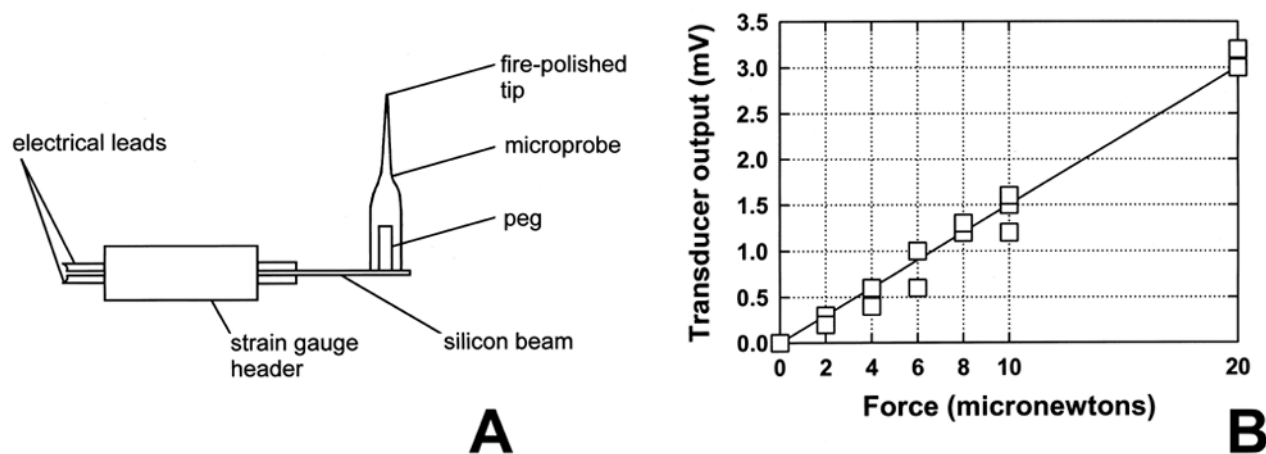


Figure 2. Apparatus used for micromanipulation experiments. A. Schematic of microprobe apparatus. B. Calibration curve showing relationship between applied force and strain gauge output.

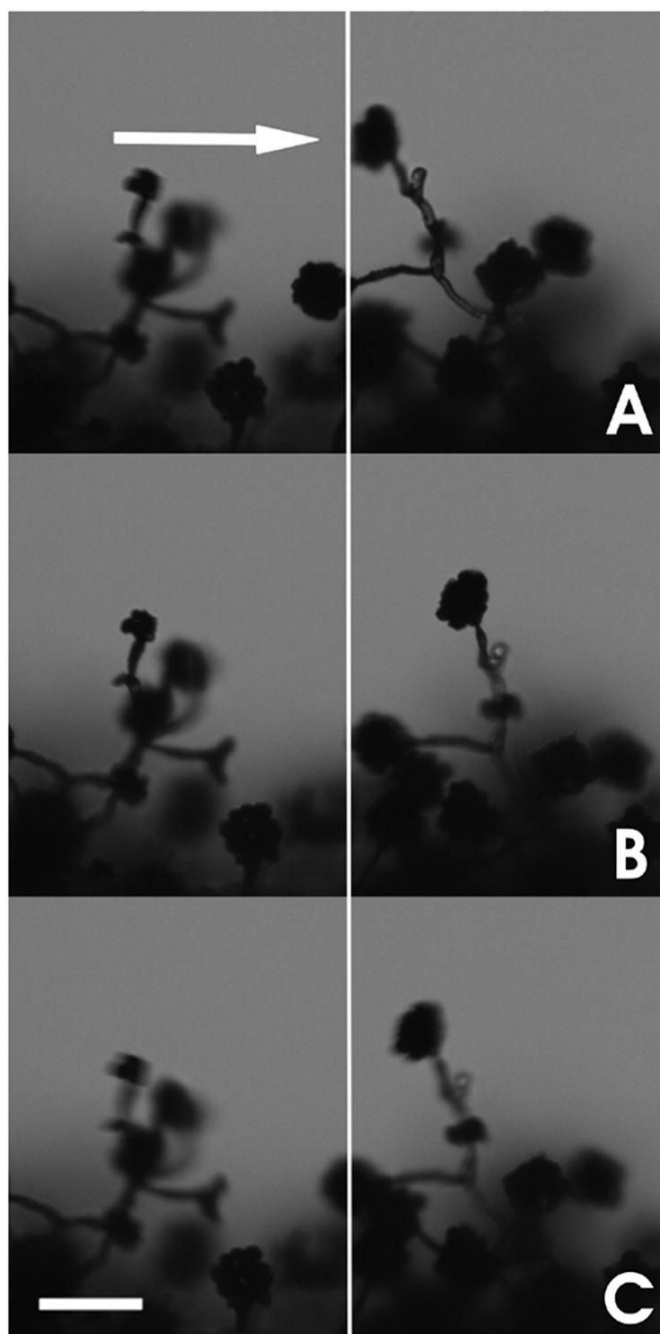


Figure 3. Motion of *S. chartarum* conidiophores subjected to low velocity airflow (1.6 m s^{-1}). A, B. Conidiophore is displaced through angle of 15° in direction of airflow (indicated by arrow). C. Conidiophore begins to rebound against prevailing airflow. Vertical reference line highlights conidiophore movement. Successive frames captured at 43.5 ms intervals from continuous video recording. Scale bar = $25 \mu\text{m}$.

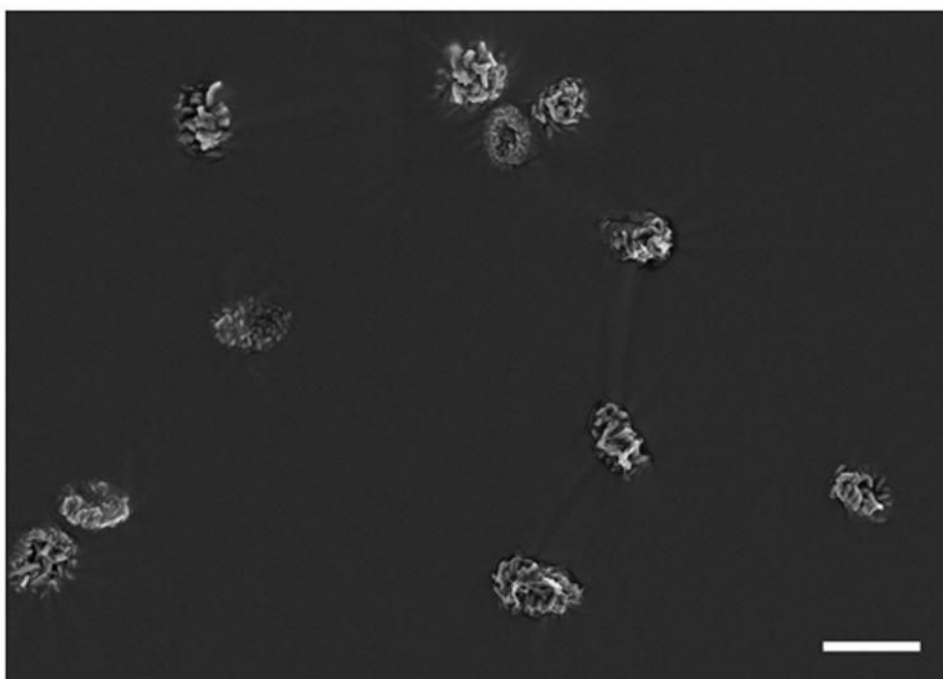


Figure 4. Scanning electron micrograph of individual conidia of *S. chartarum* captured on adhesive coverslip within sampling cassette. Note absence of microparticulates between spores. Scale bar = 10 μ m.

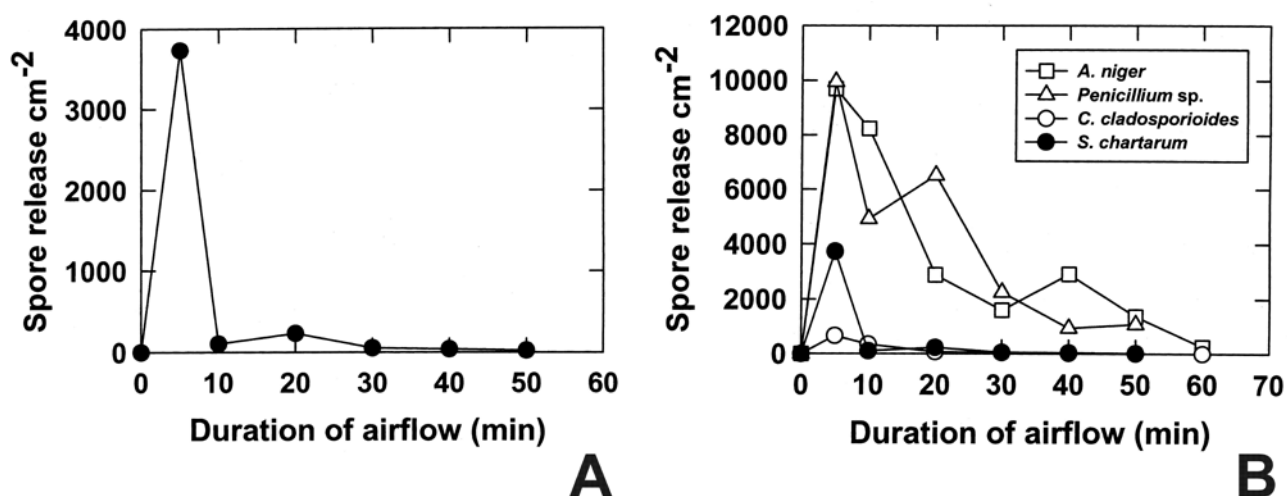


Figure 5.

Conidial release from wallboard samples at low airspeed (1.6 m s⁻¹). Airflow maintained by positive pressure method (air blown through the cuvette). A. Spore release in *S. chartarum*: initial burst followed by sustained period of low-frequency dispersion. B. Spore release in *S. chartarum* compared with three other mold species grown on the same substrate.

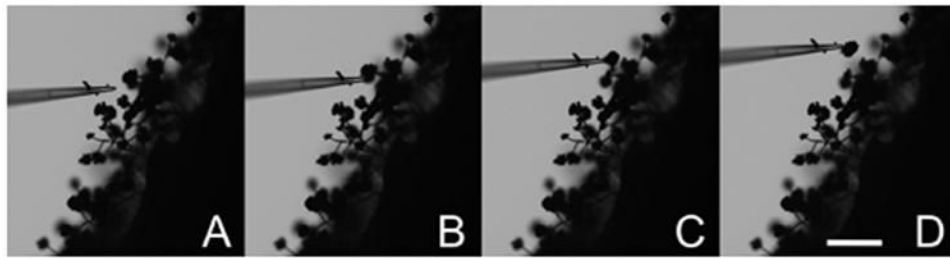


Figure 6.

Micromanipulation of 3-week-old sporulating colony of *S. chartarum* growing on gypsum wallboard. A–C. Successive frames are taken from a continuous video recording show displacement and disconnection of cluster of conidia from single conidiophore using glass microprobe. The microprobe was connected to a miniature strain gauge to record the forces applied at the microprobe tip (section 2.5). Scale bar = 50 μ m.

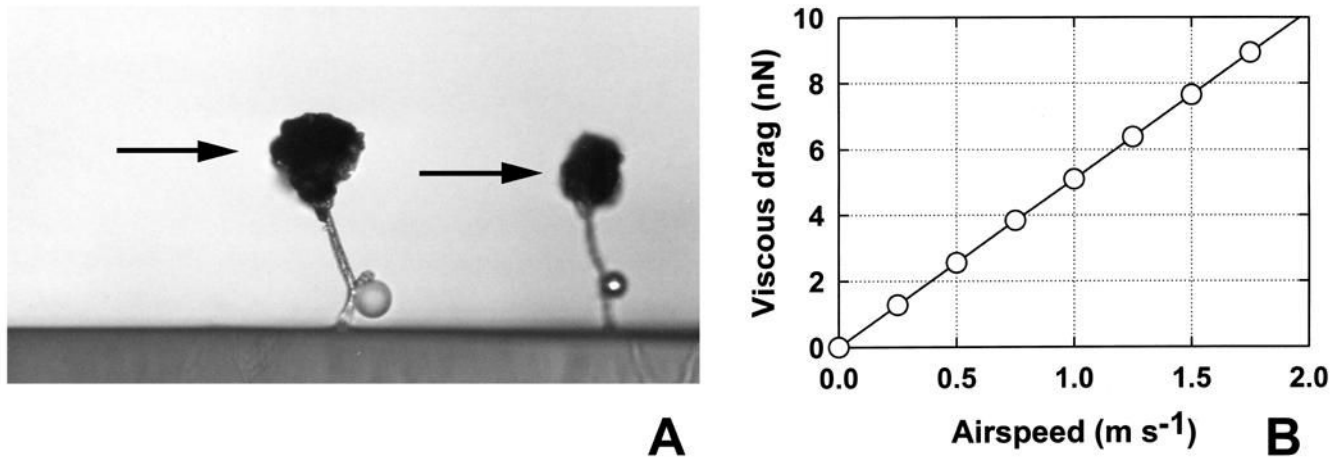


Figure 7.

Viscous drag acting on conidiophores of *S. chartarum*. A. The drag that displaces the conidiophores and conidial heads is estimated using the equation $F_{drag} = -6\pi a\eta v$, where, a = the radius of the spore cluster, η = viscosity of air = 1.8×10^{-5} N m⁻² s, and v = airspeed within the cuvette. B. Plot showing airspeed-dependent drag acting on a single conidial head with a radius of 15 μ m.

Table 1
Conidial production on hydrated face paper of gypsum wallboard after 3-week incubation

Species	Mean conidial production (spores cm ⁻²)
<i>S. chartarum</i>	1.93×10^6
<i>A. niger</i>	1.84×10^6
<i>C. sphaerospermum</i>	1.44×10^5
<i>Penicillium chrysogenum</i>	1.44×10^6

Table 2

Conidial release from 5-week old colonies of *S. chartarum* grown on gypsum wallboard. Airflow maintained by negative pressure method (air sucked through cuvette); conidial release sampled for 5 min.

Airflow and airspeed	Initial conidial release $\text{cm}^{-2} \text{min}^{-1}$ (mean \pm s.e.m.)	Percentage of total mean spore production
4 L min^{-1} , 0.6 m s^{-1}	121 \pm 89	0.006 %
6 L min^{-1} , 1.0 m s^{-1}	140 \pm 90	0.007 %
8 L min^{-1} , 1.3 m s^{-1}	307 \pm 56	0.016 %
10 L min^{-1} , 1.6 m s^{-1}	491 \pm 72	0.025 %