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Opinion Article

Natural folding of airborne fungal spores: a mechanism for dispersal and long-term survival?☆

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ABSTRACT

Analysis of numerous air samples has indicated that dormant, viable fungal spores are highly present, which suggests that aerial dispersion is important for fungi. Whereas the majority of the spores may travel only very short distances, there is indication that a notable number of them cover much longer distances. Harmomegathy is a terminology coined by Wodehouse (1935) describing the natural folding of pollen to accommodate controlled and reversible water loss. Here, we discuss evidence that this concept may also apply to airborne fungal spores that face similar challenges and have to survive periods of drought and low temperatures while retaining viability to germinate after deposition upon a suitable moist substrate. In fact, (air)dried conidia, appear collapsed, survive for much longer times compared to spores in liquid, that deteriorate in time. This indicates that for some types of fungal spores, true dormancy is reached in the desiccated state. For these airborne spores this might be regarded as a pre-adaptation that supports long-distance transport of viable cells through air. We state that spores are naturally folded during transport in air if the humidity is low enough. We hypothesize that this is a pre-adaptation supporting release, dispersal and survival of airborne spores. Moreover, the smaller size of dry naturally-folded spores may also be relevant, e.g. for the opportunistic pathogenic fungus *Aspergillus fumigatus* reduced spore size supports deposition within the alveoli in the lung.

1. Fungi and plants both travel the air

The air around us is filled with numerous macroscopic and microscopic organisms. For example, numerous seeds reach novel colonization opportunities by air. On the microscopic level, viruses, bacteria, pollen, fungi and animals use the air for transport. In addition, numerous inanimate particles are introduced into the air by natural processes and human activity.

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In general, particles are classified as coarse (2.5–10 μm), fine (0.1–2.5 μm)- and ultrafine (<100 nm) (Heal et al., 2012). At the molecular level, volatiles and other (bio)molecules originating from numerous sources are spread into the air.

Plants have developed wind-dispersed distribution of male gametes, known as pollen, as one of the main ways of fertilization (Mulcahy, 1979). Up to 10% of the angiosperms use wind dispersion and it appears to have developed at least 65 times during evolution from animal-pollinated ancestors (Friedman and Barrett, 2009). It has been less well-known that also within the fungal kingdom aerial dispersion, by spores, is important. The combined biomass of the possibly 1.5 million species of fungi is only surpassed by that of all plant species or the members of the domains Bacteria and Archaea (Bar-On et al., 2018). The ecological function of fungi is diverse and include degradation of biopolymers and mutualistic symbiosis with plants (Lutzoni et al., 2018). Fungal spores exhibit a dramatic variability in shape, mode of transport and stress-resistance for reproduction and distribution (Wyatt et al., 2013). Different types of spores are spread as aerosols, connected to water droplets and by other vectors including animals and travelling humans. Additionally, they are transported on and inside the products humans use as food and other purposes.

A very distinct adaptation of pollen grains was proposed including a programmed and controlled water loss according to preformed folding structures in the cell wall to slowdown the rate of dehydration (Katifori et al., 2010). This process is called harmomegathy (Wodehouse, 1935) and the changed shape and reduced weight and volume are thought to help increase the dispersal and survival of pollen in the air. Here, we discuss whether such a reversible reduction of cell volume accompanied with a change of the cell wall shape, is a broader concept also used by fungal spores, helping to disperse and to survive the conditions in air while retaining the capacity to germinate and establish a colony when conditions are favorable. Spores and pollen, both must survive the changing circumstances in the air, which are adverse including drought, high- or low temperature and UV-radiation. Both type of cells have a stage of dormancy, accumulate protective sugars and proteins and have a relatively thick cell wall and probably membranes in a gel-like phase during the dried state (Hoekstra, 2002; Van Leeuwen et al., 2008).

Fungal spore release is extensively reviewed by Money and Fischer (2009) stating that to enter the air, spores have to be lifted through the boundary layer of air around objects and enter turbulent air streams (this is also highlighted by McCartney and West, 2007). Some fungal species produce very high numbers of spores and the majority released may deposit close to the location of formation. Relatively small proportions of the spore population are released following an initial impact by airflow. These were typically 0.8–2.2% in the case of *Cladosporium*, *Penicillium* and *Aspergillus* and clearly less in the case of *Stachybotrys chartarum* that forms spores in slimy heads (Tucker et al., 2007). *Aspergillus fumigatus* and *A. versicolor* and *Penicillium chrysogenum* were subjected to airflow and showed release of single spores in the case of 53, 7 and 6% respectively of all particles released (Afanou et al., 2015).

Notably, spores of fungi are present in the air throughout the year in virtually every cubic meter of air collected. Many studies and reviews highlight their presence in outdoor, as well as indoor air (among them Mullins and Flannigan, 2011; Flannigan, 2011; Fradkin et al., 1987). From these and other studies it is clear that fungal genera as *Cladosporium*, *Penicillium* and *Aspergillus* are notably present in air samples, but many other species are indicated as well (see e.g. Qi et al., 2020).

Whether long distance dispersion of fungal spores widely occurs is very difficult to estimate (Golan and Pringle, 2017). Experiments have shown that released (asco)spores of *Fusarium*, *Mycosphaerella* and *Lobaria* travel between 40 and 400 m through air (Werth et al., 2006). Alternatively, there is indirect evidence that uredospores of coffee leaf rust or conidia of *Aspergillus sydowi* have travelled for thousands of kilometers (Bowden et al., 1971; Shinn et al., 2000). Spores of *Cladosporium* and *Sporobolomyces* are collected above the North Sea, hundreds of kilometers out of the coast (as reviewed by Gregory, 1978). Some fungal species as *Penicillium chrysogenum*, avid producers of airborne conidia, have a world-wide distribution (Henk et al., 2011). Other fungi including basidiomycetes, *Alternaria*, *Aureobasidium*, *Epicoccum* and many other species are less predominant, but still common in air, albeit more on a seasonal level (Li and Kendrick, 1995; Yamamoto et al., 2012; Fernández-Rodríguez, et al., 2014). In the light of the spread of plant diseases, fungi have different strategies for release of spores with varying resistance to aerial conditions (Oneto et al., 2020). For instance, some spores are released by night, during the beginning of the day or also during the day.

2. Airborne conidia become naturally folded upon maturation and drying at the air

Fungal spores can be found in many shapes and sizes (Wyatt et al., 2013), but for pollen (Fig. 1) and fungal spores both, a spherical shape, whether ornamented or not, is very common. Typically, in literature, the swollen rounded conidium shape, as it appears at or near 100% RH or in a watery suspension, is considered useful for taxonomic description (see Samson et al., 2019). In addition, fungal cultures grown in Petri-dishes with high humidity exhibit fully expanded spores on conidiophores. For example, the taxonomical description of *Aspergillus niger* conidia is: “mostly globose, irregularly roughened, 5.0 μm” (Domsch et al., 2007).

Fig. 2 shows a conidiophore, of the fungus *Talaromyces calidicanium*, studied with cryo-electron microscopy in which fungal structures are snap-frozen in liquid nitrogen. Some spores in the chains are folded (‘collapsed’ or ‘shrivelled’). Several studies have indicated that conidia collected via the air are in this state (Bekker et al., 2012; Afanou et al., 2014, 2015; Buskirk et al., 2014; Madsen et al., 2016). When these spore-forming structures are formed in open air on natural substrates and during an episode of desiccation, the dehydrated and folded spore might be a very common state of the cell. Fig. 3 shows mature spores collected directly on glass slides (by tapping) from cultures of *A. fumigatus* and *A. niger*. Dry spores were all folded and more darkly pigmented (especially *A. niger*), but in water immediately became swollen and more transparent. Conidia of the fungus *A. niger*, appear as dented wheel-shaped dormant conidia at 6–7 % RH (Morozova et al., 2001) and this occurs also in the case of pigmentation mutants (van Veluw et al., 2013). Conidia, both single and in chains, imaged with SEM from indoor environments all

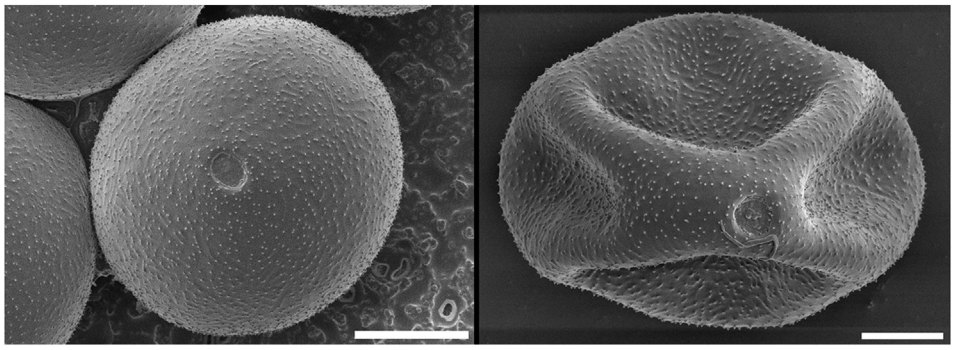

Fig. 1 – Examples of shrinkage of pollen of *Alnus glutinosa;* Bars are 5 (left) μm and 10 (right) and. The pollen samples were frozen in liquid nitrogen and cryo-transferred to a low-temperature scanning electron microscope (JEOL 6300F field emission SEM; Tokyo, Japan). The samples were freeze-etched for 3 min at −89 °C to enhance contrast and remove water vapor contamination, sputter coated with platinum, and subsequently analyzed at −190 °C with an accelerating voltage of 5 kV. Picture made by Adriaan van Aelst, WUR, Wageningen the Netherlands.

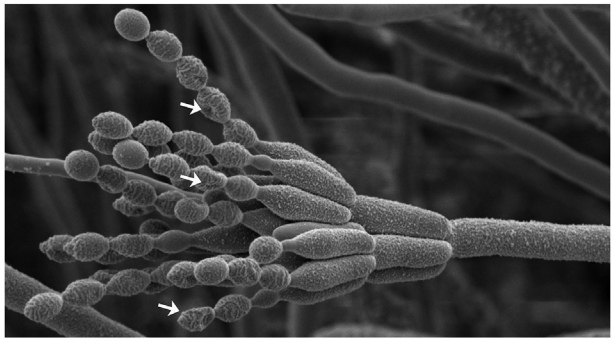

Fig. 2 – Cryo-SEM image of a conidiophore of a *Talaromyces calidicanius* (for the first time described as *Penicillium calidicanium*, Chen *et al.*, 2002) with newly formed conidia. Several conidia show a folded state, which is usually considered to be artefactual (see arrows). Conidia are approx. 4 μm long. The relevant parts of the fungal colony was selected using a stereo microscope, excised with a surgical blade as small agar (approx. 3 × 3 mm) blocks, and transferred to a copper cup for snap-freezing in nitrogen slush. Agar blocks were glued to the copper surface with frozen tissue medium (KP-Cryoblock, Klinipath, Duiven) Samples were examined in a JEOL 5600LV scanning electron microscope (JEOL, Tokyo, Japan) equipped with an Oxford CT1500 Cryostation for cryo-electron microscopy (cryo-SEM).

showed this folded state (Madsen *et al.*, 2016). The collapsed conidia show similarity in shape to collapsed pollen grains without apertures, such as *Aristolochia gigantean* and *Zea mays* (Katifori *et al.*, 2010). Fig. 4 shows naturally folded conidia of different *Aspergillus* species and *Neurospora*, which even suggest species dependent differences in folding, ranging from half-spherical, wheel- and box shaped conidia (Fig. 4 A–D, respectively). We propose to refer to these spores as 'naturally folded' rather than 'collapsed'. This state has never been considered as an adaptive strategy for fungi. Here, we challenge this view and hypothesize that fungal airborne conidia at low RH are naturally folded as an adaptation to dispersal and survival.

3. Folded dry spores have superb survival properties, but also need to survive rewetting

a. The dried state provides protection towards cells

Conidia of *A. niger* and *A. fumigatus* are naturally folded in ambient dry conditions and instantaneously regain their globose shape in liquid (Fig. 3). Profound desiccation of fungal conidia, e.g. by freeze-drying, silica gel or other methods, is widely used for long-term storage of cultures (Larena *et al.*, 2003; Morgan *et al.*, 2006). Lamarre *et al.* (2008) stored conidia from *A. fumigatus* originating from dried agar slants at 20 °C, for one year in glass beakers. The composition and integrity of mRNA pools inside the conidia as well as the germination characteristics remained mostly unchanged when compared to freshly harvested conidia. On the contrary, conidia in water at the same temperature showed around 30%–37% reduction in viability after 7–10 days, respectively (Valsecchi *et al.*, 2017). Furthermore, Wang *et al.* (2021) provided evidence that upon release from the conidiophore, time-dependent deterioration of conidia is halted including stoppage of ATP pool-depletion. Released spores were stored in a more dehydrated state, suggesting that this change is instrumental in prolonging the viability of conidia by dormancy.

During dehydration, the concentration of the cell constituents and molecules become higher. This may introduce damaging chemical reactions or changes in the composition of the membrane due to partitioning when unwanted cytoplasmic molecules dissolve in the membrane (Golovina *et al.*, 1998). Further, dehydration leads to a gel-like state of the (plasma) membrane. Dehydration also leads to protein aggregate formation that inactivates their functioning. These aspects of dehydration have to be negotiated with, by compatible solutes (Wyatt *et al.*, 2013) or by protein- and membrane protection

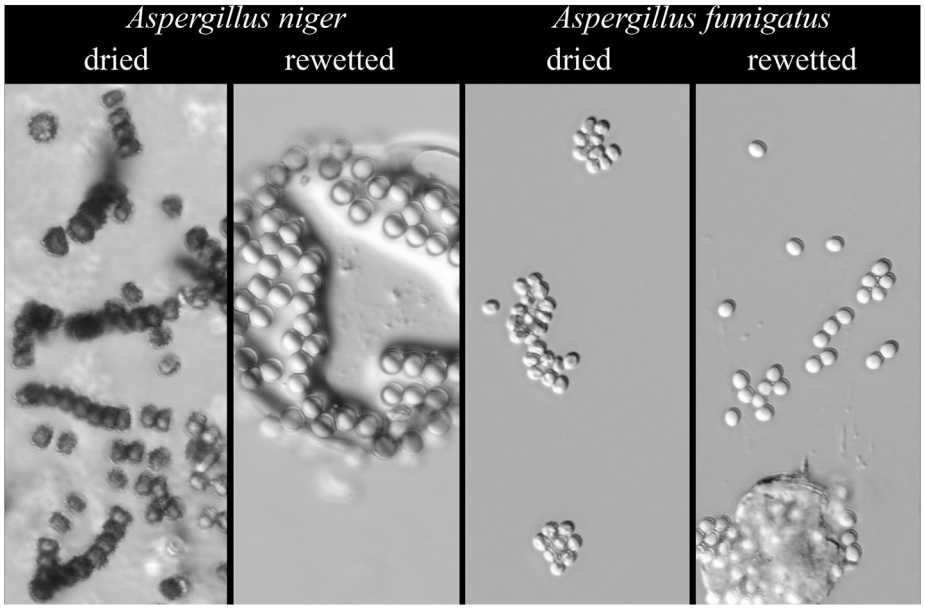

Fig. 3 – Folded conidia of *Aspergillus niger* and *Aspergillus fumigatus* in dry conditions and as rewetted and unfolded conidia in suspension as observed at the same magnification.

due to small heat shock proteins which are described in both pollen and fungi (Wolkers *et al.*, 2001; Welker *et al.*, 2010; van Leeuwen *et al.*, 2016) or by other, unknown mechanisms. Freshly harvested conidia of *A. niger* contain high transcript numbers of several proteins including sequences with similarity to a LEA-like protein, a protein involved in protection of pollen against drying (van Leeuwen *et al.*, 2013; Wolkers *et al.*, 2001). In addition, transcripts with similarity to genes encoding for hydrophilins as dehydrins and hsp9 were present in high numbers. Taken together, these observations suggest that a dried state of conidia provides long-term survival and may be a natural characteristic of dormant fungal conidia.

b. Rewetting of the cells might be a critical stage for survival; imbibitional damage

Sporangiospores of the Zygomycete *Rhizopus oligosporus* stored on dried rice before use during production of tempe (Thanh *et al.*, 2005, 2007) showed only partial germination during application. Interestingly, resuscitating these spores in rich (malt extract) medium at elevated temperature resulted in much higher germination (Thanh *et al.*, 2007). Dried fungal spores and plant pollen are at risk when suspended in cold liquid (Crow *et al.*, 1989). This is studied in the case of insect pathogenic fungi, where dried spores are used as agent for biological control (Faria *et al.*, 2009). The rationale is that the plasma membrane is in the dried gel-like state and when rewetted at too low temperature (15 °C or lower) becomes leaky due to transition to the semi-liquid crystalline state (Crowe *et al.*, 1989). This effect is not occurring in the case of warmer liquid or incubation at high RH. These observations suggest that returning to the liquid state is a similar important challenge for survival as effective protection during drying. It could be that a process of water acquisition is occurring here. The conditions of formation of spores also have an effect on the amount of water that is taken up or is adhered to the spores as was seen described by van Laarhoven *et al.* (2017). Conidia produced by *P. rubens* grown at low water activity (a_w) attracted markedly more water from humid air.

4. Consequences of natural folding for release, dispersion and pathogenicity of fungal spores

a. Release of spores

In an extensive study on *Cladosporium* conidia in the air in Britain it was found that very high daily mean concentrations (6000–32000 conidia m^{-3}) were observed during warm days with a pronounced light breeze ($v_s \leq 2.5$ m s^{-1}) (Sadyś, 2017). These data indicate a better release and dispersal in the air under these conditions. It was also shown that fungal fragments and conidia of *Botrytis cinerea* vary in size at changing RH and that they are released from colonies due to an airflow at low RH (Madsen, 2012).

Airborne conidia of the fungal genera including *Aspergillus*, *Penicillium*, *Talaromyces* and *Paecilomyces* are usually connected in (long) chains on top of phialides on conidiophores. When these structures are exposed to low relative humidity, the process of shape change during folding might help to separate the conidia and help dispersal into the air. Conidia are connected to each other in rows that can be very long (as for instance in the case of *Paecilomyces variotii* (van den Brule *et al.*, 2020, Supplementary Figure 2)). Cryo-planing, a technique where frozen spores are cut and subsequently observed by cryo-SEM, suggests that conidia of two *Penicillium* species are connected by a continuous layer that has to be disrupted upon separation (Dijksterhuis *et al.*, 2007, Figure 11). Conidia of *Aspergillus*

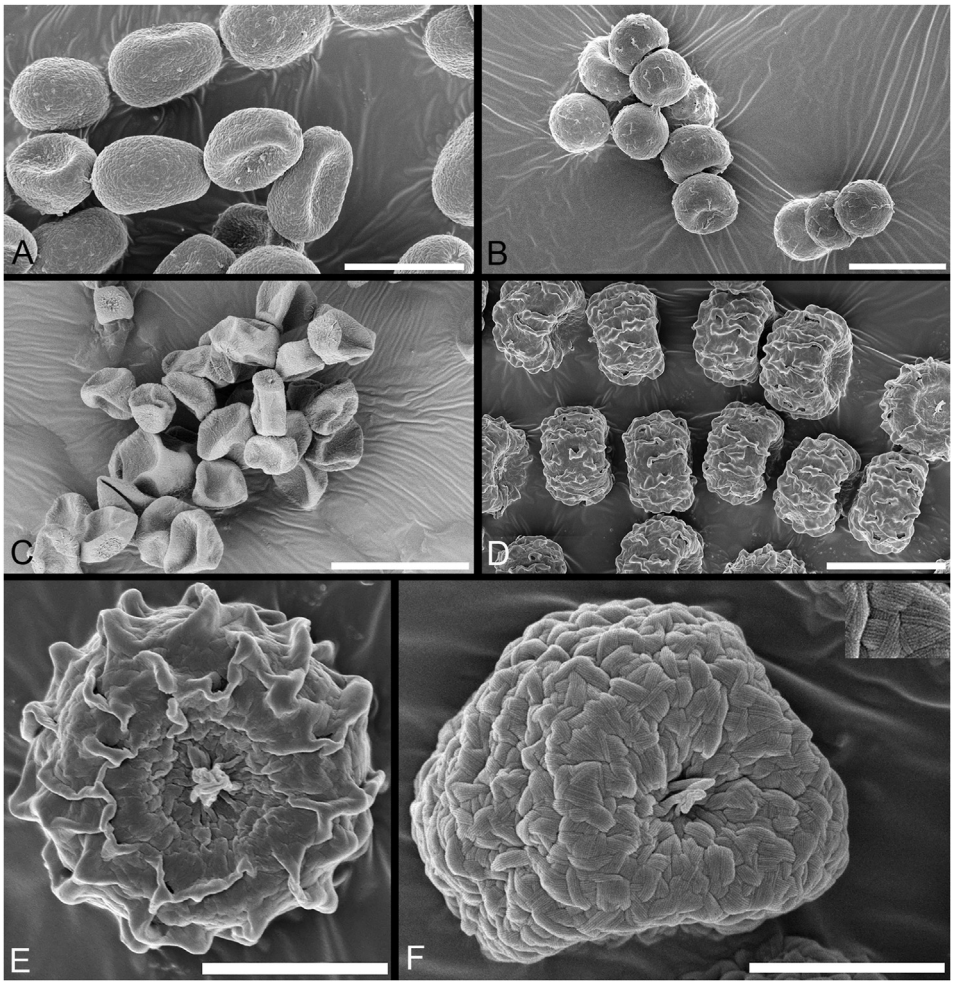

Fig. 4 – Naturally folded conidia of different *Aspergillus* species and *Neurospora crassa* collected from mature 7-day old cultures on sticky carbon tape for SEM. A. *A. fumigatus;* B. *A. niger* strain N 522 (fawn colour mutant) with simple folding. C. *N. crassa* NC111 (white mutant) with nearly square spores and D. *A. niger* diploid strain with wheel-like folded spores. E. Detail *A. niger* diploid conidium with scar-like structure in the middle (where it was connected to other conidia in the chain). F. *A. fumigatus* conidium with clear hydrophobin aggregates and scar (see also inset in the upper right corner) Bars = 10 (C); 5 (B,D); 3 (A); 2 (E); 1 (F) μm.

species also show a scar as a result of severing of this connection (Fig. 4 E, F).

b. Dispersion of naturally folded spores.

Fig. 3 clearly shows that naturally folded spores exhibit notable reduction in size and mass. Transport of spores in air is strongly influenced by both sedimentation (settling) speed and air movement. The settling speed (terminal velocity) is dependent on cell mass, shape and size and slowest for small spherical objects. A typical spore the size of an *Aspergillus* conidium would settle with typically 0.3 mm/s in still air (McCartney and West, 2007). The compaction of the folded spore and its decrease in mass due to water loss must slow down this settling speed markedly. Drag coefficients change when the shape of a spherical object changes to naturally folded or half spherical (Wong, 2013). Norros *et al.* (2014) modelled data on dispersion of *Phlebia centrifuga* (diameter 3.8 μm) and concluded on the base of their work that distribution of very small spores (in the range below 10 μm) is highly influenced by size. This was not exactly expected by Stokes law, but here a decrease in size from 4 to typically 2 μm would certainly be instrumental in dispersion for longer distances (and times). Taken together, the decreased water content, weight and size of folded conidia might increase long distance dispersal through air.

In addition, *vice versa*, an increase in RH might help these aerial particles to reach the soil or phylloplane by increasing its weight (Reponen *et al.*, 1996). An increase in RH, due to rain or dew formation, preludes favorable situations for conidia to germinate during a period of high water availability. It is suggested that fungal spores do not increase significantly in size in between 30 % RH and 90 % RH, while they do above 90 % RH, e.g. *Cladosporium cladosporioides* increases up to 27 % in diameter (Reponen *et al.*, 1996).

c. Naturally folded spores might have more resistance against UV radiation

A folded state might result in more protection against UV-radiation in air as the removal of water compresses the fungal cell wall and increases the concentration of melanin in the cell and UV resistance. The occurrence of extra folds may add to the protection as radiation passes two layers of cell wall in a fold with a minimum of cytoplasm (See Fig. 4E and F). Melanin in the cell wall may have an effect on hydrophobicity (van Veluw *et al.*, 2013), but is mainly known as a protector to UV-radiation (Esbelin *et al.*, 2013; Gessler *et al.*, 2014; Braga *et al.*, 2015). Melanin is also suggested to protect against drought and high salt concentrations (Kogej *et al.*, 2004, 2006) however several mutants of *A. niger* devoid of melanin production did not show reduced germination at lower water activity (Segers *et al.*, 2018). Melanin was however shown to be highly effective in protecting *A. niger* to space radiation, but this protection was reduced in a dry state, indicating that a dry state is not beneficial for all radiation (Cortesão *et al.*, 2020). This is an aspect that has to be studied in more detail.

d. Role in pathogenesis and allergy

Pollen grains and fungal spores are both known to cause allergy, asthma and respiratory problems in humans (Anderson *et al.*, 2001; Atkinson *et al.*, 2006; Black *et al.*, 2000; Burge, 1986, 2002; Dales *et al.*, 2003; Horner *et al.*, 1995; Pringle, 2013; Bush, 1989; Heinzerling *et al.*, 2005; Simon-Nobbe *et al.*, 2008). Pollen is usually >10 μm and do not easily reach deep into the lungs, while the smaller fungal spores can reach the alveolar surface of the lung and induce chronic inflammation or asthma (Simon-Nobbe *et al.*, 2008). A decrease in size from 5 to 3 μm will increase the fraction of particles in the alveolar region from 30 to 74% (Hinds, 1999; Kuehl *et al.*, 2012; Darquenne, 2020). Inhaling *A. fumigatus* spores is no problem for healthy people, but may result in fungal growth and various diseases in immuno-compromised individuals and are regarded as the major infecting agents in hospitals (Verweij *et al.*, 2016).

e. Future research

Collapsed fungal spores after drying are observed in many studies. Here we hypothesize that it is not an artifact, but an overseen adaptation that may be relevant in release, dispersal and survival of airborne spores in general and may also be relevant for pathogenicity of opportunistic pathogens. Research on the distribution, stress resistance and stability of spores in the folded state might add valuable information in the case of preventing infection, but also knowledge to prevent food spoilage (Dijksterhuis, 2017, 2019) where inactivation treatments are used as if conidia are in liquid suspension.

Declaration of competing interest

Authors declare no conflict of interest.

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This article is part of a Special Article Collection of Fungal Biology Reviews dedicated to the memory of Nick Read.

Author J.D.: "I worked with Nick in Edinburgh during 1998 and 1999 on rust fungi and vesicle trafficking, a time I thoroughly enjoyed. From that time on, I had regularly contact with Nick, and especially recently we worked together for a short time as co-senior Editors of Fungal Biology Reviews. I really looked forward to cooperate with Nick on this and exchange ideas about mycology, but unfortunately he was already so sick that contact became less and less. I was impressed by his positiveness and enthusiasm during these last months. He was a man interested in stories about other people; when he held a lecture for students he could, for instance, mention that a certain mycologist also was interested in antique furniture. Nick was profoundly interested in microscopy enjoying cryo-SEM and fluorescence microscopy; the movies made with Patrick Hickey and others are legendary in mycology. He also had a keen sense of beauty and design. Things were not easily perfect enough, and that could mean that the completion of a manuscript took longer time than envisioned. Nick being interested in the dynamic of the fungal cell, our contribution deals with dried conidia, the furthest opposite of this. However, Nick also was interested in fungal spores and regularly mentioned their beauty on the spore-forming structures. Here, in a way, he returned to the topic of his first publications on spores in perithecia of Sordaria. I surely will miss Nick on meetings to come and his comments on scientific and other topics."

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