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Fungal spores: Highly variable and stress-resistant vehicles for distribution and spoilage

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ABSTRACT

This review highlights the variability of fungal spores with respect to cell type, mode of formation and stress resistance. The function of spores is to disperse fungi to new areas and to get them through difficult periods. This also makes them important vehicles for food contamination. Formation of spores is a complex process that is regulated by the cooperation of different transcription factors. The discussion of the biology of spore formation, with the genus *Aspergillus* as an example, points to possible novel ways to eradicate fungal spore production in food. Fungi can produce different types of spores, sexual and asexually, within the same colony. The absence or presence of sexual spore formation has led to a dual nomenclature for fungi. Molecular techniques have led to a revision of this nomenclature. A number of fungal species form sexual spores, which are exceptionally stress-resistant and survive pasteurization and other treatments. A meta-analysis is provided of numerous D-values of heat-resistant ascospores generated during the years. The relevance of fungal spores for food microbiology has been discussed.

1. The fungal kingdom

Representatives of the fungal kingdom, although less overtly visible in nature than plants and animals, are nevertheless present in all habitats. The variability within the kingdom Fungi equals that of the kingdoms Planta and Animalia, but like the kingdoms Archaea, Bacteria and Protozoa (Ruggiero et al., 2015) in the majority of the cases, fungi perform their biological function in a less visible way.

A hallmark of the fungi is their network of branched tubular cells, called mycelium, but there are many variations on this theme. Mycelium can be very condensed, with a tissue-like appearance (for example in the case of *Cladosporium halotolerans*, Segers et al., 2016) or can be very loosely organized. The tubular cells, called hyphae, grow from the tips, a feature shared with root hairs and pollen tubes in the Plant Kingdom, although marked differences occur within the flow of vesicles at the tips of pollen tubes (Kroeger and Geitman, 2012). This apical growth mode is also observed with organisms in the Kingdom Bacteria such as the filamentous bacterium *Streptomyces* (Flärdh et al., 2012) and members of the phylum Pseudofungi (Oomycota) within the kingdom Chromista (Brent Heath et al., 2000; Guerriero et al., 2010).

The fungal hyphae are able to excrete compounds, including a multitude of different enzymes that can degrade numerous complex biopolymers, which highlights the important role of fungi during recycling of the elements of life. In addition, they produce many smaller molecules, often called “secondary” metabolites, but with many primary functions including communication or antagonism. However, fungi can also be superb collaborators as is illustrated by their ability to form close associations with members of other kingdoms. These include the lichens (fungi and algae) and the associations between fungi and plants within mycorrhizae. In the latter cooperation, limited nutrients as phosphate are recruited from soil and “traded” with plants for sugars and other nutrients.

Fungi thrive in soil and degrade leaves and wood, but also colonize many surfaces (e.g. plant, rock, indoor surfaces in dwellings, surfaces of food) and water environments.

Remarkably, mycology, the science of studying fungi, initially was seen as part of botany, as fungi were regarded as plants. Nowadays, fungi are often discussed in the context of microbiology, for example, food mycology is seen as a branch of food microbiology.

While fungi belong to the largest organisms on earth (e.g. *Armillaria bulbosa,* see among others Smith et al., 1990), they also have a microscopic dimension. A fungal colony is macroscopic, but identification of (a)sexual fruiting structures requires a microscope. This is illustrated most clearly by the production and dispersion of fungal spores.

2. Fungal spores; stabilized cells

Fungal spores usually are of microscopic size (most species are

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between 3 and 30 μm), often markedly smaller than pollen. They show an enormous variety in shape, pigmentation, ornamentation and stress resistance. Seifert et al. (2011) illustrate this, depicting a large variation of spore shapes including the occurrence of curls, elaborated appendages or multi-cellularity.

Spores enable the distribution of fungi in space and time. Distribution in space means that fungal cells travel by air, water or other vehicles (e.g insects) towards new possible areas for colonization. For example, many fungal species produce spores that can travel through air and do so for long distances (Schmale and Ross, 2017; Damialis et al., 2017). Distribution in time means that stress-resistant cells remain at a location for extended times awaiting conditions that are more conducive for growth. The latter might include the absence of competitors as such may occur after a wood fire or when water is present after a long period of drought.

Fungal spores are stabilized cells (see Wyatt et al., 2013), often metabolism is low (Novodvorska et al., 2016) and precious cell constituents are protected by a thick, often pigmented, cell wall and accumulated compatible solutes. The latter molecules are polyols (glycerol, mannitol, arabitol and erithritol) and the (di)saccharide trehalose. The molecules are rich in hydroxyl groups and accrue to high concentrations within the cells. The extent of accumulation correlates with the microviscosity of the cytoplasm (Dijksterhuis et al., 2007; Van Leeuwen et al., 2010; Wyatt et al., 2015ab) and the (heat)-resistance of the spores. The micro-viscosity is measured by electron spin resonance spectroscopy (ESR) using a spin probe (TEMPON) that can easily enter the spores and subsequently be recorded (Dijksterhuis et al., 2007). The mobility of the spin probe molecule (correlation rotation time) is calculated from the ESR spectrum and interpreted as a measure of viscosity.

There are indications that other protective mechanisms such as the protein chaperones LEA-like proteins, dehydrins and hsp 9 that also play a role in stabilization (van Leeuwen et al., 2013ab; Van Leeuwen et al., 2016). Spores (conidia) of *Penicillium expansum*, after a damaging heat treatment of 30 min at 54 °C showed 20-fold higher germination after being kept in aerated water at room temperature for 3 days, compared to the cells that were plated out on growth medium directly (Baldy et al., 1970). The authors observed that during recovery, cells became more insensitive for radiation damage and concluded that repair of DNA after heat damage might be responsible the observed recovery. Interestingly, in a recent study (Shin et al., 2016) a strain of *A. fumigatus* deleted for DefA, a factor that plays a role in the degradation of stalled RNA polymerase II after DNA damage, produce spores that were hypersensitive for mutagenic agents. These data show that repair mechanisms after damage also play a role in the survival of spores that subsequently can germinate.

Spores are relatively stress-resistant structures, but show a large variation in their ability to survive adverse conditions. As an example, microconidia of *Fusarium oxysporum* are similar to vegetative cells (e.g. growing hyphae), with respect to relative low cytoplasmic viscosity, plasma membrane composition (as judged by staining with filipin that stains ergosterol) and sensitivity for the fungistatic antifungal natamycin (Van Leeuwen et al., 2008, 2010). In contrast, ascospores of the fungus *Talaromyces macrosporus* belong to the most resilient eukaryotic cells described to date, surviving high temperatures and pressure (Dijksterhuis and Teunissen, 2004; Houbraken et al., 2012b).

Before and during dispersion spores remain dormant and do not germinate. This might be the logical consequence of the mechanisms of stabilization described above. For example, the high concentrations of compatible solutes do not interfere with biochemical functions, but slow down biochemical reactions (Wyatt et al., 2013). This state is called dormancy and different types are described by Sussman and Halvorsson (1966). Additionally, certain molecules might play a role to prevent pre-mature germination. The molecule 1-octen-3-ol has been stated as a volatile self-inhibitor preventing germination of conidia at high density (Chitarra et al., 2004, 2005; Nemčovič et al., 2008; Herrero-Garcia et al., 2011; Gillot et al., 2016), but the precise way of action is not clear and has been discussed (Miyamoto et al., 2014).

After dispersion of the spores to suitable locations, humidity may be enough to result in spore germination as is suggested by Segers et al. (2017), but alternatively, various inducing nutrients are described for conidia of *Aspergillus niger* (Hayer et al., 2013, 2014) to be necessary for effective germination. Germination is characterized by isotropic growth (swelling) and polarized growth (germ tube formation). Conidia of *A. fumigatus* show an increase of sensitivity for antifungal compounds during early germination (Russell et al., 1975). This suggests that the protective mechanisms discussed above are dismantled. For example, a steep drop in the content of compatible solutes (Thevelein et al., 1981, 1982) characterizes the first stage of spore germination. Heat-resistant ascospores of *A. fischeri* (previously designated as *Neosartorya fischeri*) show a degradation of compatible solutes, accompanied with a decrease in cytoplasmic viscosity and heat resistance (Wyatt et al., 2015c). In addition, transcripts that encode for proteins that provide protection against heat and oxidative damage strongly drop within 2 h after the onset of germination in conidia of *Aspergillus niger* (Van Leeuwen et al., 2013a). This implies that resistance of spores against stressors might drop quickly during early germination and that (novel) treatments that exploit this phenomenon might be effective.

3. The formation of spores is a highly complex phenomenon and is a source of heterogeneity

Fungi form spores in a bewildering variation and the morphology of the spore-forming apparatus is an important means for identification of species. In time, an extended vocabulary has evolved for different types of spores, including names such as aplanospores, aleuriospores, chlamydospores, phragmospores etc. (see Hawksworth et al., 1995). Often, conidia are mentioned in literature dealing with food-related fungi. These are mitotic (asexual), non-motile spores that are freely dispersed via water, air or other means.

A complex terminology is dedicated to the way spores are formed which include many different schemes of cell wall deposition, delineation of spores and order of formation. Spore formation can be acropetal, with the youngest spore at the apex, or basipetal with the youngest spore at the base of the chain. Thallic, blastic, phialidic, annellidic spore formation are among other terms used (see Cole and Samson, 1979). Several main groups of fungi such as zygomycetes (sporangiospores), and zoosporic fungi have their spores formed by cytoplasmic cleavage and subsequent cell wall deposition.

Airborne conidia, asexual spores, need to survive drying and are produced by fungal genera such as *Aspergillus*, *Penicillium*, *Paecilomyces* and *Cladosporium*. They are produced in overwhelming numbers and are present in every cubic meter of air. As an example of their ability to form so many conidia: *Penicillium roqueforti* and *Paecilomyces variotii* produce up to 10^8- 10^9 spores in a single Petri dish containing malt extract agar medium within 1–2 weeks of cultivation. This might be the reason that a species such as *Penicillium chrysogenum* (including the closely related species *P. rubens*, Houbraken et al., 2012a) has a cosmopolitan distribution (Henk et al., 2011). The near omnipresence of air- and waterborne fungal spores makes then suitable as vehicles of contamination of food products during the food production chain as well as in human dwellings. For example, Fig. 1 shows fungi that were able to grow on the blades of a ventilator in a refrigerator. When sampled, 5 fungal species including *Cladosporium psychrotolerans*, *C. ramotenellum* and *C. halotolerans* as well as *Penicillium brevicompactum* and *P. radicicola* were identified (in collaboration with M. Meijer, Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands).

Such abundant spore formation is characterized by the multiplication of a single growth end point into multiple spore-forming cells. *Penicillium* and *Aspergillus* produce several to numerous phialidic cells on one basal stem. Each phialide can form a chain of spores, the latest one formed closest to the phialide neck. Thus, more spores can be

Fig. 1. Fungi and fungal spores on the blades of a ventilator in a refrigerator.

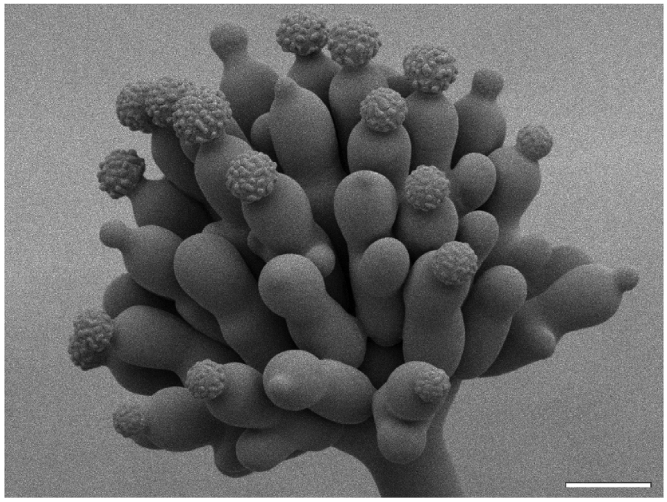

Fig. 2. Spore formation on *Aspergillus niger* mutant showing both metulae and phialidic cells on a round vesicle resting on a broad stipe (stalk). Bar = 5 μm.

formed at once; enabling the colony to produce spores in high numbers rapidly. The complexity of this process is discussed below with members of the genus *Aspergillus* as the example (Fig. 2. *Aspergillus niger*). With these fungi, the whole spore-forming structure (conidiophore) rests on a broad specialized aerial hypha called the stipe or stalk. In this way several ten-fold or in the case of larger conidiophores, hundreds of spore-forming cells (phialides) form thousands of conidia. In *A. niger* numerous phialides are formed on a swollen vesicle (see Fig. 3 and also Wang et al., 2015). A number of *Aspergillus* species form a layer of metulae sprouting from the vesicle, with each metula forming several phialides. This ensures a further layer of multiplication of spore-producing ends. This feature defines the difference between uniseriate (only phialides) and biseriate Aspergilli. The very early stages of metulae and/or phialide formation in *Aspergillus* include very shallow dome shaped expansions of the vesicle cell wall at a very regular distance as shown in Fig. 3. This suggests a very precise positioning of the nuclei that have to enter the phialides, inside the vesicle. Perhaps the nuclei are tethered to the membrane and the cell wall of the vesicle and initiate a localized outgrowth (bulging) on the vesicle. Fig. 3 indicates that the most apical initials are further developed in comparison to those close to the stipe. This observation suggests the existence of a gradient of a so far unidentified factor involved in stipe development.

Nuclei have to be transported from the vesicle into spore-forming cells; otherwise no chain of viable conidia can be formed. Ishi et al. (2005) provide evidence for transport of nuclei into phialides from the

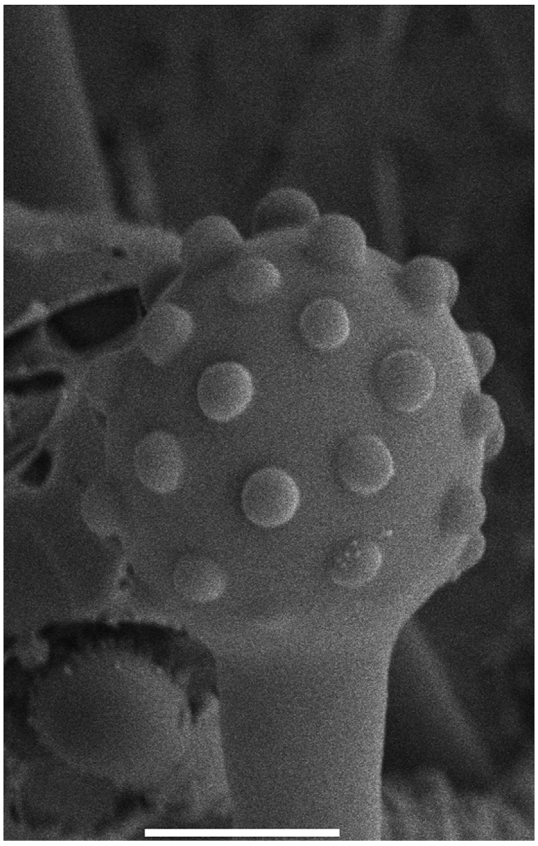

Fig. 3. First formation of metulae/phialides on a vesicle of *A. niger*. Note the regular distance of these initials and the very shallow bulge of an initial next to the stipe. Bar = 5 μm.

vesicle, although one would not expect that 20–40 nuclei have to pass through a phialide from a subapical source into a conidial chain. Therefore, inside the phialides, continuously dividing nuclei might provide daughter nuclei that transfer into the youngest conidium. In general, the transport of cell organelles, including nuclei, nutrients and metabolites, during all stages of formation of the spore-bearing structure is vital for successful spore formation and may be a possible target for novel antifungal compounds to prevent fungal spoilage.

In *Aspergillus*, vegetative hyphal cells differentiate into a “foot cell”, a thick-walled cell that will form the base of the stipe. The foot cell and the subsequent stipe formation are distinct first stages dedicated towards spore formation. The broad stipe can be regarded as an active transport highway for large amounts of biological building blocks to ensure the proper formation of up to 10.000 conidia. Fig. 4 illustrates the large number of nuclei present in the stipe and vesicle of *Aspergillus niger*. For comparison, a “normal” hyphae, which is visible in the lower right of the micrograph, contains single or duplicated nuclei in a row. The formation of these very early stages towards spore formation must be carefully orchestrated by transcription factors that enable the simultaneous onset of expression of many different genes. However, a transcription factor for this onset of asexual development is not decisively identified. The fluG factors and the products of the fluffy genes (fluA-E and G, Lee and Adams, 1996) are candidates for this function. Overexpression of fluG leads to sporulation in submerged cultures (Lee and Adams, 1994). There is also evidence that not activators, but repressors such as nsdD play a role that can block the stages of the initial structures during asexual spore formation (Lee et al., 2014).

Two transcription factors involved in further stages of spore formation have already been known for about 30 years as described in the fungus *Aspergillus nidulans* (Adams et al., 1998). These are brlA (bristle, Adams et al., 1988) and abaA (abacus, Sewall et al., 1990;

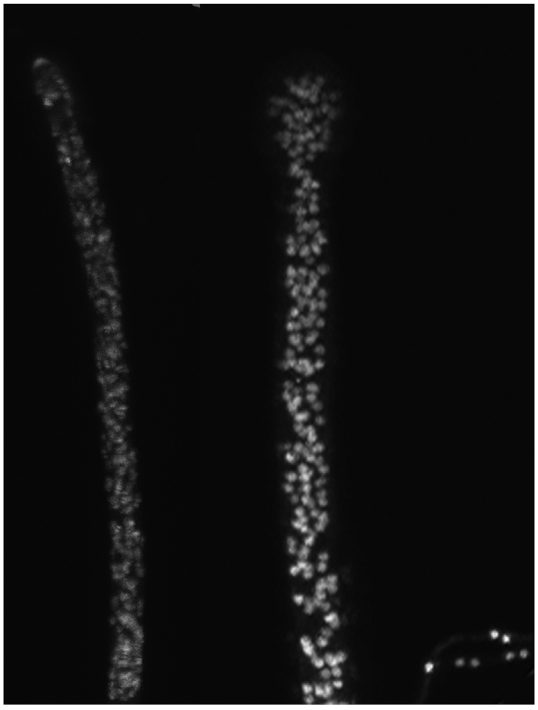

Fig. 4. Accumulation of numerous nuclei in a stipe of a strain of *A. niger* that transport fluorescent histones into the nuclei, before (left) and after metulae (right) formation. In the lower right a normal hyphae is visible containing markedly lower densities of nuclei (in collaboration with R. Bleichrodt, Utrecht University, Netherlands.

Andrianopoulos and Timberlake, 1994) and involved in vesicle and metulae/phialide formation, respectively. Further stages of spore development and dormancy include the activity of other factors that influence the expression of numerous genes. These include factors as wetA, aftA, mycA, veA (Lara-Rojas et al., 2011; Hagiwara et al., 2016; Valsecchi et al., 2017; Wang et al., 2015; Wu et al., 2017) reported in different *Aspergillus* species.

In general, it is of interest to realize that transcription factors that have a role in asexual spore formation, namely wetA (in *Aspergillus flavus*, Wu et al., 2017) and mybA (in *Aspergillus fumigatus*, Valsecchi et al., 2017) are reported to affect a thousand to 5000 genes. In addition, for asexual spore formation, several transcription factors enhance or decrease each other's expression (Valsecchi et al., 2017). Further, there is clear overlap between many target genes of these transcription factors. It is a challenge to understand how the interplay of these factors results in a phenotype.

As many of the transcription factors mentioned above have orthologues in other fungal species, similar networks of influence/orchestration/regulation must be present, but without doubt will show different patterns and specificities. Fig. 5 shows spore formation of a fungus belonging to the *Cladosporium cladosporioides* complex. Fungi of this genus are very common in air (among others, Damialis et al., 2017) and are formed in a very different way from *Aspergillus*. Every cell in a conidiophore is, in fact, conidiogenous; spores develop at the end of the chain, a variation on spore formation called acropetal spore formation. Three different morphological types of spores are formed (see Bensch et al., 2010) including ramoconidia (elongated and septated), intercalary conidia (rounded, with typical scars at both sides) and terminal conidia (smaller and round). Conidiophores are formed quickly during the life cycle (see Segers et al., 2017). Thus, large variations in spore morphology occur between the fungal species, but also within the same species different types of spores are formed.

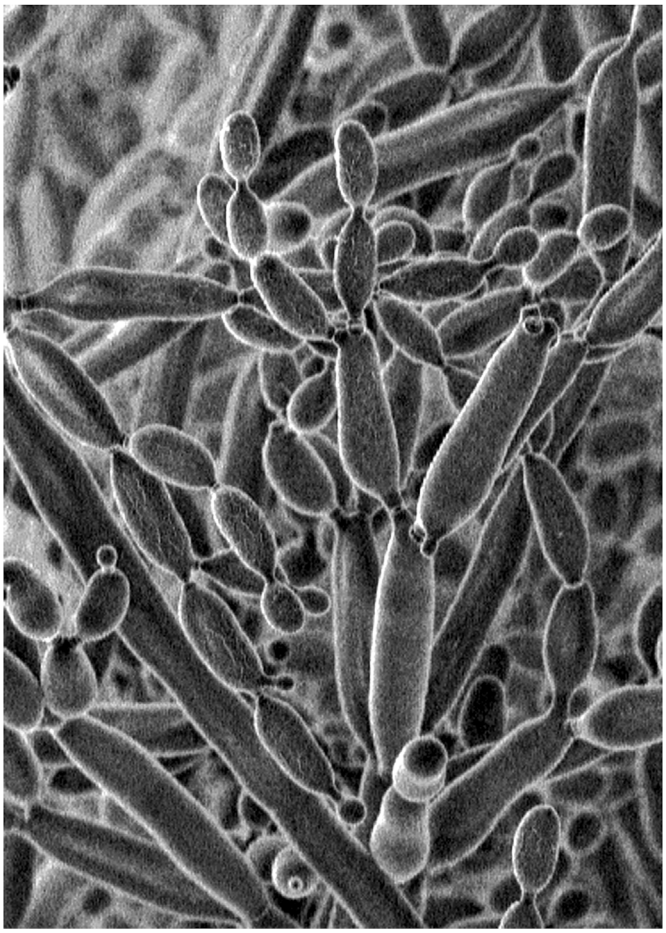

Fig. 5. Formation of spores on a conidiophore of a fungus belonging to the *Cladosporium cladosporioides* species complex. A tree-like structure consists out of longer branch-like cells (primary ramoconidia) formed on a basal aerial hyphae; branches hereof (secondary ramoconidia), and rounded intercalary and terminal conidia.

4. Homo- and heterothallic spores are formed by the same fungi

It is important to note that many fungal species are able to form different types of spores within the same colony. The production of different types of asexual spores by a colony adds to the complexity, as both aerial conidia and chlamydospores are formed. The fungus *Botrytis cinerea,* that spoils many crops, for example strawberries, forms both chlamydospores inside hyphae, as spores on conidiophores (spore bearing structures). *Fusarium oxysporum* that spoils cereals, forms both micro- and macroconidia for dispersion as well as chlamydospores for survival in time. Macroconidia have more than 2 compartments and are roughly banana shaped, and microconidia are one to two celled.

Many fungi form asexual and sexual spores within the same colony. *Aspergillus nidulans* forms conidia, but dependent on the growth conditions (light, nutrients) also forms sexual ascospores in a structure called cleistothecium. The regulation of this process must be at least as complex as highlighted above for asexual spores (Braus et al., 2002). The sexual cycle starts with loose intertwined hyphae around a cleistothecium initial containing nuclei (see Sohn and Yoon, 2002). The surrounding hyphae will form the protective peridium and the initial develops into many ascogenous cells forming numerous asci, each containing 8 ascospores. Asci are a hallmark of the Ascomycetes, the most species-rich phylum of fungi. The formation of sexual spores includes meiosis and recombination of DNA ensuring new combinations of properties during evolution. Fungi are enigmatic in this respect that many species may form both the ascospore forming (sexual) and the

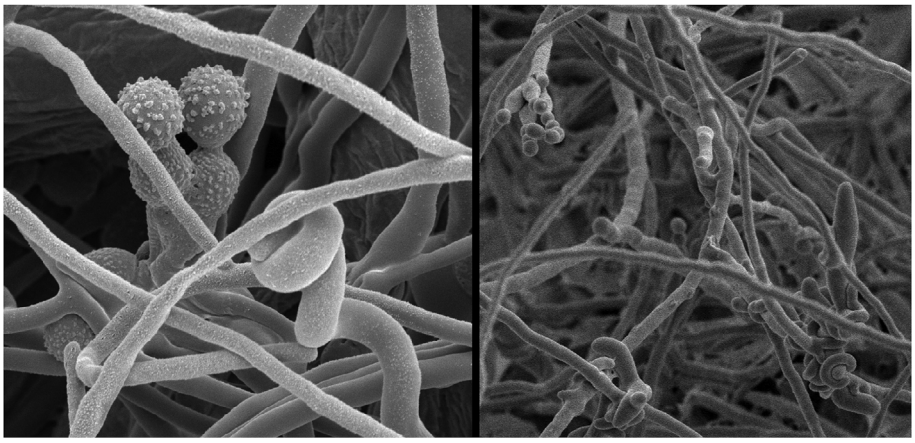

Fig. 6. Formation of both asexual spores and initials of sexual structures that will lead to ascospores in *Aspergillus* (left, with an *Eurotium*-morph) and *Talaromyces* (right, *T. calidicanium*). Asexual spores are formed on phialidic cells as round cells. The initials of ascomata are characterized by hyphae that spiralize. In the case of *Talaromyces* a swollen initial is visible (lower right in the picture), with spiralizing hyphae below.

conidia forming (asexual) structures next to each other within the same colony (isolate, clone) as is illustrated in Fig. 6. These fungi are called homothallic (self-fertile), in contrast to the heterothallic fungi that only form sexual structures as a result of fusion between two different, competent strains. *A. fumigatus* and *Paecilomyces variotii* are heterothallic fungi, forming ascomata solely at the border between two parent colonies (O'Gorman et al., 2009; Houbraken et al., 2008). These species have mat1-1 and mat 1–2 loci dispersed among different isolates, while both loci are present in one genome of the homothallic fungi. *A. nidulans* and many other fungi of the order *Eurotiales*, the group to which *Aspergillus* and *Penicillium* belong, can form ascospores within the same colony.

The question rises if the occurrence of meiosis and the formation of fruiting bodies and ascospores could have any benefit when both "parents" are identical. Bruggeman et al. (2003) state that the sexual processes in *A. nidulans* result in a lower accumulation of mutations in the mycelium compared to strains that only can form asexual spores. The recombination process may result in a higher removal of deleterious mutations compared to the "simple" mitosis needed for phialidic spore formation. Sexual reproduction, counterintuitively, would serve the *diminishing* of (deleterious) variability in the genome. Of course, different strains of homothallic fungi will out-cross effectively in nature (see Houbraken and Dyer, 2015) and use sexual crossing in the way heterothallic fungi do. Interestingly, in *A. nidulans*, Hoffmann et al. (2001) have observed that nuclei with some differences preferably fuse within the same fungal species compared to (near) identical nuclei.

The occurrence of pleomorphic life-cycles in fungi, such as the sexual state (called the teleomorph) and the asexual state (the anamorph) have resulted in a dual nomenclature for fungal species (Taylor, 2011). For instance, the fungus *Aspergillus glaucus* is the anamorph of *Eurotium herbariorum*. The difficulty of relating both states to each other resulted in uncertainties in fungal nomenclature. In the case of many fungi, a teleomorph was never observed in culture and the name of the anamorph was the only one available (these fungi were called the Deuteromycota). It has been the rise of DNA sequencing technology using PCR, which has resulted in a reorganization of many fungal genera (Taylor, 2011). DNA sequence comparison showed that many clades contain both fungi that express only anamorphic states and others that also show teleomorphic states. During recent years, a shift from dual to single nomenclature designated as, "One fungus, one name" is already implemented for many different fungal genera Of interest, *Aspergillus fumigatus* was identified as *Neosartorya fumigata* (O'Gorman et al., 2009), but now remains to have the name *Aspergillus*. As a rule of thumb, the historically oldest name used for a genus will be selected as the single name. In the case of food-spoilage related fungi this is relevant for heat-resistant fungi. For example, the name *Paecilomyces variotii* replaces *Byssochlamys spectabilis*. The name *Byssochlamys*, however, is well-known in food industry as a producer of highly heat-resistant ascospores found in pectin, and several other food products and we expect that the implementation of this transition in nomenclature will need time. The topic of single nomenclature is discussed in more detail in Houbraken and Samson (2017) and more references on this are mentioned in this review.

5. Sexual spores can be extremely stress-resistant

In many cases, ascospores have higher stress resistance than conidia and some species produce ascospores with prolonged resistance at temperatures above 70 °C. *Aspergillus spinosus* (with a neosartorya morph), *Paecilomyces variotii* (with a byssochlamys morph) and *Talaromyces macrosporus* exhibit stress resistance similar to some bacterial spore-formers such as *Bacillus subtilis*. In Fig. 7A the extent of heat-resistance of ascospore heat resistance, based on 455 D-values out of 32 studies performed during 39 years, is presented for 22 fungal species. In this graph, the logarithm of the D-values (in minutes) is plotted against the temperature of treatment at the Y-axis. The D-values of well-known heat-resistant fungi as *Paecilomyces niveus, Pae. fulvus*, *Aspergillus fischeri*, *Talaromyces flavus/macrosporus* as well as other species are given. Table 1 is summarizing, the old and the new nomenclature (as highlighted in the previous section) for these fungi. Fig. 7B is showing the same graph, but now with three main groups of heat resistance highlighted. Group I includes ascospores of the yeast *Saccharomyces cerevisi* (Put and De Jong, 1982; Milani et al., 2015) and *Aspergillus glaucus* (Splittstoesser et al., 1989; Yildiz and Çoksöyler, 2002) that are in a similar range and show relatively low resistance. The second group (II) contains ascospores of *Pae. niveus* (Michener and King, 1974; Splittstoesser and Splittstoesser, 1977; Aragão, 1989; Engel and Teuber, 1991; Kotzekidou, 1997; Sant'Ana et al., 2009; Evelyn and Silva, 2015), *Monascus ruber* (Panagou et al., 2002), part of the *Talaromyces flavus/macrosporus* samples (Beuchat, 1986; Scott and Bernard, 1987; King and Halbrook, 1987; Casella et al., 1990; King and Whitehand, 1990; King, 1997; Dijksterhuis and Teunissen, 2004) and form a group of heat-resistant fungi with D-values of several minutes at 80–85 °C. Interestingly, the D-value of *X. bisporus* (Pitt and Hocking, 1892) is close to that of *M. ruber*, to which it is related (Barbosa et al., 2017). Also one measurement done at our laboratorium on *Talaromyces helicus* (with J. Eleveld, Westerdijk Institute, Utrecht, the Netherlands)) falls into this series of moderate heat-resistant species. *Talaromyces trachyspermus* (Tranquillini et al., 2017) mentioned with respect to food- and dairy spoilage (Enigl et al., 1993), *Talaromyces stipitatus* (with J. Eleveld) and *Penicillium javanicum* (Casella et al., 1990) also fit into group II.

The highly heat-resistant fungal species (group III, see also Supplementary Fig. 1) include several *Aspergillus* species with a neosartorya-morph including *A. fischeri*, *A. hiratsukae*, *A. thermomutatus*, *A. neoglaber*, *A. aureolus* and an *Aspergillus* (neosartorya-morph) strain WR-

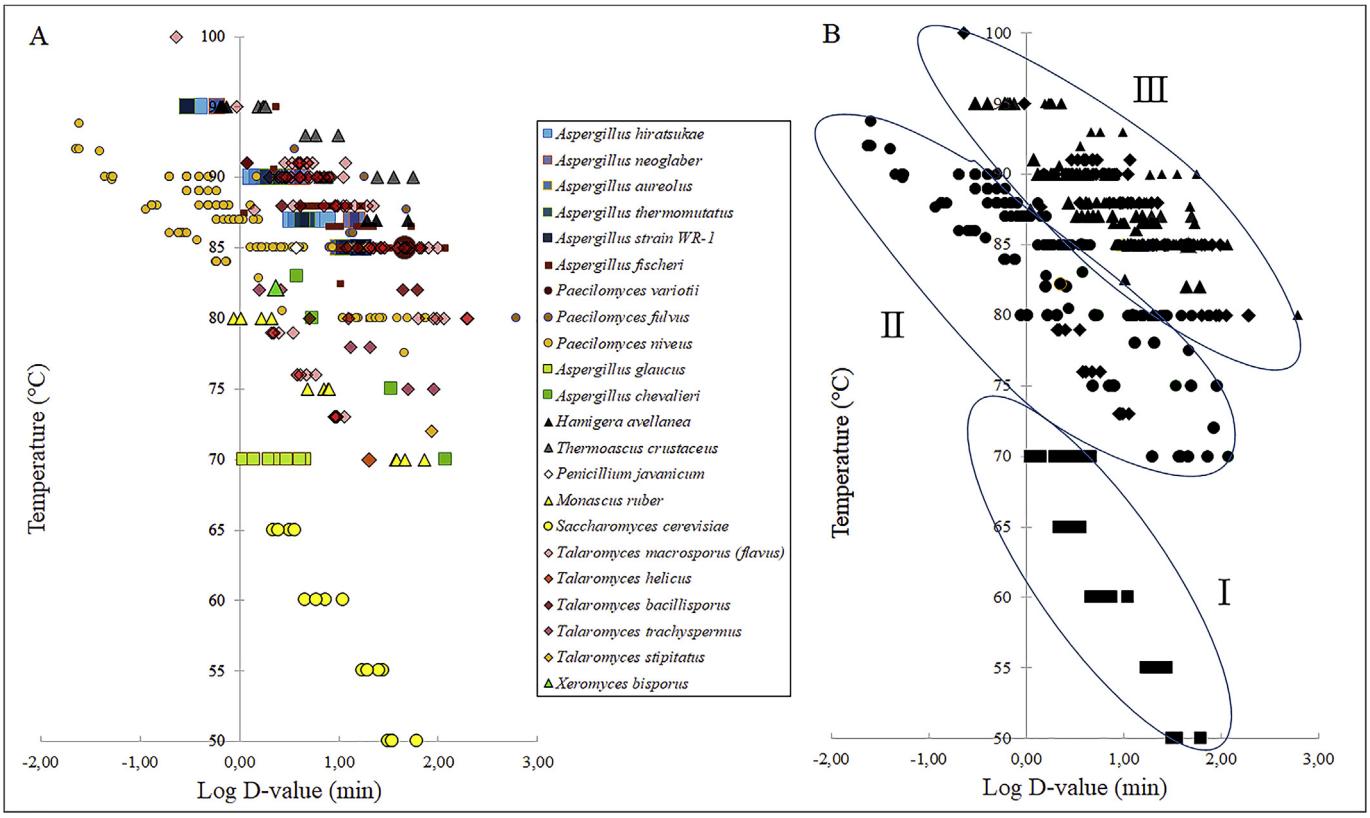

Fig. 7. A) Graphical representation of 455 D-values of 22 fungal species from 32 studies over a period of 39 years. The log of the D-value in minutes is plotted against the temperature. The novel nomenclature of ascosporic heat-resistant fungi is used and the change in names given in Table 1. B.) Recognition of three major groups of heat resistance among the data of Fig. 7. Group I consists out of ascospores with relatively low resistance. A second group (II) consist out of ascospores with heat-resistance of several minutes at 80 °C to 85 °C, Group three exists out of very resistant ascospores with resistance of ten or more minutes at 90 °C.

Table 1
New nomenclature for heat-resistant fungi.

Old nomenclature	New nomenclature
Byssochlamys fulva	*Paecilomyces fulvus*
Byssochlamys nivea	*Paecilomyces niveus*
Byssochlamys spectabilis	*Paecilomyces variotii*
Neosartorya aureola	*Aspergillus aureolus*
Neosartorya hiratsukae	*Aspergillus hiratsukae*
Neosartorya glabra	*Aspergillus neoglaber*
Neosartorya pseudofischeri	*Aspergillus thermomutatus*
Eurotium herbariorum	*Aspergillus glaucus*
Eupenicillium javanicum	*Penicillium javanicum*

1 (Splittstoesser and Churey, 1989). The 111 data points related to *A. fischeri* (Beuchat, 1986; King and Halbrook, 1987; Scott and Bernard, 1987; Conner and Beuchat, 1987b; Splittstoesser and Churey, 1989; Casella et al., 1990; King and Whitehand, 1990; Gómez et al., 1994; Tournas and Traxler, 1994; Gumerato, 1995; Kotzekidou, 1997; Rajashekhara et al., 1998, 2000; Slongo and Aragão, 2006; Dijksterhuis, unpublished results; Evelyn et al., 2016) show a variation of > 1 log-scale in D-value at 85 and 88 °C.

Within a fungal species, variation in heat resistance results from many factors as ascospores are formed by different strains during different growth conditions. For a given environment, strains belonging to the same species can exhibit heat resistance variability associated with intraspecific biodiversity. The extent of variation between strains (often a large number of strains, typically > 20) is mostly studied in the case of food-spoiling bacteria (Den Besten et al., 2016). In addition, for a given fungal strain, treatments in different environments during heat stress measurements also cause variation in D-values. In the presented figures (Fig. 7 and Supplementary Figs. 1–4), all data were taken into account. The other *Aspergillus* species with a neosartorya-morph fit very well into this set of data (Splittstoesser and Splittstoesser, 1977; Splittstoesser et al., 1993; Berni et al., 2017). In our laboratory we observed an increase of heat resistance of ascospores in the case of *A. fisheri*, *A. hiratsukae* and *A spinosus* strains respectively, and this may be related to specific spoilage problems, but the attribution of intraspecific and interspecific variation to the total distribution of heat resistances has to be researched in further detail.

The ascospores of *Pae. fulvus* (see Supplementary Fig. 2, Michener and King, 1974; Splittstoesser and Splittstoesser, 1977; Kotzekidou, 1997; Sant'Ana et al., 2009), *T. macrosporus* (see references above and Supplementary Fig. 3) and *T. bacillisporus* (Tranquillini et al., 2017) are also well dispersed within this group of extreme heat-resistant fungal species. Since 1990 (Frisvad et al., 1990), *T. flavus* and *T. macrosporus* are recognized as different species. Ascospores of the latter species are markedly larger and have a higher heat resistance. It has to be expected that 2 of the 3 strains tested in Beuchat (1986) are in fact *T. macrosporus*. The third isolate is *T. flavus*, falling into the group of fungi that form ascospores with lower heat resistance.

More recently *Hamigera avellanea* and *Thermoascus crustaceus* (Supplementary Fig. 4, Scaramuzza and Berni, 2014) are studied and also belong to this group of fungi. *T. crustaceus* shows the highest heat-resistance of all. It has to be expected that the fungi *A. fumigatus* (O'Gorman et al., 2009) and *Pae. variotii* (Houbraken et al., 2008) belong to the extreme stress-resistant fungi. One measurement done with the latter fungus already fits in the middle of this group (Houbraken et al., 2006).

Several fungal species show a robust dormancy and remain dormant even within rich growth media (see Dijksterhuis et al., 2002; Dijksterhuis and Samson, 2006; Dijksterhuis, 2007). This so-called constitutive dormancy (Sussman and Halvorsson, 1966) can be broken

by extreme environmental triggers. For instance, ascospores can be activated and subsequently germinate after treatments, such as pasteurization or high-pressure processing, that are developed to prevent food spoilage (Reyns et al., 2003; Kikoku, 2003; Dijksterhuis and Teunissen, 2004; Slongo and Falcão de Aragão, 2006). With these ascospores, spoilage occurs in food products after these treatments. Further, these ascospores survive for many years and contain high levels of protective compatible solutes with an intracellular concentration above 1M (as is calculated for *Talaromyces macrosporus* and *Aspergillus fischeri*, Dijksterhuis et al., 2002; Wyatt et al., 2015a). The compatible solutes include mannitol, trehalose and a for fungi, a newly reported class of oligosaccharides (TOS, trehalose-based oligosaccharides) characterized by trehalose with glucose moieties bound via an α-1,6 bond, (Wyatt et al., 2015c). Different fungal species have different mixtures of compatible solutes that may provide protection against different environmental stresses. For instance, ascospores of *T. macrosporus*, that contain mainly trehalose, survive heat better in liquid, whereas *A. fischeri*, containing trehalose, mannitol and TOS, better survive dry heat. At low water levels or temperatures, the already viscous cytoplasm of the ascospores with high concentrations of solutes, may transfer into a glassy state. This physical state is non-crystalline (amorphous) and is characterized by extreme low molecular movement, hence very protective against damaging chemical reactions between (bio)molecules. Thus, the glassy state might provide these ascospores even more resistance during dry or cold conditions. Wyatt et al. (2013) provide an extensive review on protection within spores and describe different protective mechanisms. The concentration of compatible solutes and the (micro)viscosity of the cytoplasm are very clearly correlated. This is clear during maturation of ascospores (Wyatt et al., 2015b), a process that takes several weeks, characterized by a strong increase of compatible solutes, viscosity and stress resistance. Conversely, these three parameters decrease strongly during germination (Dijksterhuis et al., 2007; Wyatt et al., 2015b) within approx. 1 h.

Germination of ascospores of *T. macrosporus* is shown in Fig. 8 and characterized by a sudden jump from the inner cell through the outer thick cell wall. There is clear evidence that ascospores of heat-resistant fungi are enclosed and separated from the environment by these protective cell walls and shedding is necessary for effective respiration and development (see Dijksterhuis et al., 2002, 2007; Wyatt et al., 2013).

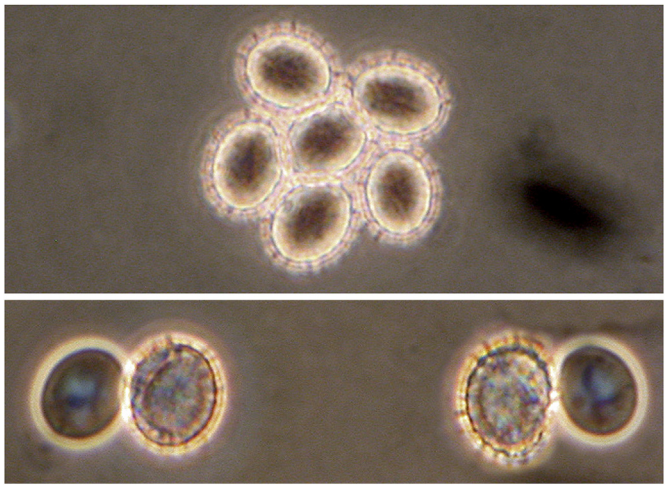

Fig. 8. Dormant ascospores of *T. macrosporus* possess a thick ornamented cell wall and appear as bright cells with contrast microscopy, due to the relatively high cell density. Upon synchronization of germination by a heat flash, the inner cell encompassed within a thin cell wall, jump through a fissure in the outer cell wall. The jumped cells have much lower concentrations of compatible solutes and appear dark.

6. Spores and food microbiology

In food microbiology, fungal spores are important vehicles for colonization and subsequent spoilage of food products. As they are ubiquitously present in outdoor and indoor air (Fradkin et al., 1987; Flannigan, 2011; Flannigan and Miller, 2011) in numbers of typically one hundred to thousands per cubic meter of air, any contact of a food product with air leads to the deposition of spores on the product. These spores may or may not germinate to a visible colony on the product and in the latter case shorten the shelf life. There are several preventive methods; including air filtration that reduces the numbers of fungal spores in air (see a discussion on these measures in dairy products, Garnier et al., 2017).

However, the air within a production facility also can also contain spores originating from the spoilage fungi that develop in the environment of food production. The presence of novel spores because of spoilage can result in a positive feedback loop, unless spore levels are continuously kept at a low level due to cleaning and air filtering.

As spores (conidia) are present in low numbers on or within the heterogenous matrix of food, early detection will be extremely difficult to realize. Further, prediction of fungal outgrowth and therefore the shelf life is difficult as the lag time of germination of a single spore is variable compared to contamination with larger numbers (a population) of spores on one spot (Dagnas et al., 2017). Gougouli and Koutsomanis (2013) conclude that the variation in germination of the spores determines the variation in the time needed for a colony to become visible and as such the shelf life. This variation increases if the limits of growth are approached, making food spoilage from single spore even more difficult to predict under these conditions (see Dagnas et al., 2017). It has to be expected that germination conditions are suboptimal as food products often have properties that discourage growth of spoilage fungi, such as low water activity, high sugar and a modified atmosphere.

Not only airborne spores are present in low numbers. Ascospores of heat-resistant fungi are not dispersed via air and also occur in low numbers on a large proportion of tested fruit samples (Tranquilini et al., 2017; Berni et al., 2017; Dos Santos et al., 2018).

The heterogeneity of stress resistance and germination capacity within a population of spores produced by one species or even one colony (reviewed by Dijksterhuis, 2017) is still scarcely studied. Subpopulations of different spores may exist that show resistant to one stressor, but maybe be more sensitive for another stress. Hagiwara et al. (2017) show that conidia of the fungus *A. fumigatus* formed at lower temperatures are more resistant to UV- radiation, but have a lower temperature resistance. The opposite, higher temperature- and lower UV resistance, was observed in the case of conidia were formed at higher temperature. Novel insights in the limits of germination and the existence of subpopulations may lead to novel ways to prevent spoilage.

During history mankind has evolved many techniques to prevent spoilage of food by fungi. These include among others, lowering of water activity, storage at low temperature, pasteurization and the application of high salt or sugar. Some fungi have an ecological niche (e.g. low water activity, low temperature or osmophily) that makes them suitable to survive in the food matrix. It is very important to realize that specific fungal species are associated with certain food products or crops, a topic that is recently covered in several reviews and books (Frisvad et al., 2007; Pitt and Hocking, 2009; Samson et al., 2010; Dijksterhuis et al., 2013). For example, *Penicillium expansum* is known from apple rot, but *P. digitatum* is related to the green rot on citrus fruit. This specificity is also reflected in the behavior of spores. Conidia of the latter fungus are adapted to infection, as they germinate markedly faster in the presence of wounds in the peel of the fruit (Eckert and Ratnayake, 1994).

Fungi also have co-evolved with our food products for prolonged times. Strains of the fungus *Rhizopus oligosporus* that are used for tempe production show deviations in the process of sporangiospore formation,

compared to “wild” strains of the fungus (Jennessen et al., 2008). This suggests that spore dispersion is less important for these strains as generations of people have transferred the fungus by hand, and defects occurred in the delicate process of cytoplasmic cleavage and cell wall deposition during sporangiospore formation.

New studies on the behavior of asexual and sexual fungal spores during formation and germination may provide novel tools to prevent fungal spoilage from a very early stage on, but also methods that “only” delay germination lead to a longer shelf life.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2018.11.006.

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