

A decrease in bulk water and mannitol and accumulation of trehalose and trehalose-based oligosaccharides define a two-stage maturation process towards extreme stress resistance in ascospores of *Neosartorya fischeri* (*Aspergillus fischeri*)

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Summary

Fungal propagules survive stresses better than vegetative cells. *Neosartorya fischeri*, an *Aspergillus* teleomorph, forms ascospores that survive high temperatures or drying followed by heat. Not much is known about maturation and development of extreme stress resistance in fungal cells.

This study provides a novel two-step model for the acquisition of extreme stress resistance and entry into dormancy. Ascospores of 11- and 15-day-old cultures exhibited heat resistance, physiological activity, accumulation of compatible solutes and a steep increase in cytoplasmic viscosity. Electron spin resonance spectroscopy indicated that this stage is associated with the removal of bulk water and an increase of chemical stability.

Older ascospores from 15- to 50-day-old cultures showed no changes in compatible solute content and

cytoplasmic viscosity, but did exhibit a further increase of heat resistance and redox stability with age. This stage was also characterized by changes in the composition of the mixture of compatible solutes. Mannitol levels decreased and the relative quantities of trehalose and trehalose-based oligosaccharides increased.

Dormant ascospores of *N. fischeri* survive in low-water habitats. After activation of the germination process, the stress resistance decreases, compatible solutes are degraded and the cellular viscosity drops. After 5 h, the hydrated cells enter the vegetative stage and redox stability has decreased notably.

Introduction

Understanding the biophysics of dispersal structures in fungi is imperative, as they are masters of survival and extreme stress tolerance. Environmental challenges such as high temperatures and drought, disorder membranes and lead to aggregation of proteins and threaten cellular integrity and survival. Fungal propagules have developed adaptations to survive several stresses better than vegetative cells. Many fungi belonging to the order *Eurotiales*, that harbours the environmentally ubiquitous genera *Aspergillus* and *Penicillium*, form sexual ascospores within ascogenous cells that reside in the fruiting bodies (cleistothecia). These ascospores can survive high temperature (Dijksterhuis, 2007) and drought (Wyatt *et al.*, 2014), and can exhibit other types of extreme stress resistance, and are therefore amongst the most resistant eukaryotic cells described to date. For instance, ascospores of *Neosartorya fischeri*, a teleomorph of *Aspergillus*, survive 85°C in an aqueous environment for more than 10 min (Beuchat, 1986; Houbraken *et al.*, 2012). Moreover, in a dry state they survive a relative humidity lower than 0.5% at a temperature of 60°C for more than 7 days (Wyatt *et al.*, 2014). These properties

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enable fungi such as *N. fischeri* survive stress during adverse natural conditions including high temperatures during wet or dry conditions. This might occur in soils after (wood) fires or during illumination of dark surfaces during the day. Alternatively, these ascospores can even survive mild food-preservation treatments such as pasteurization. Knowledge about the nature of this heat resistance will reveal novel mechanisms of protection of the cell's macromolecular systems and organelles.

Factors contributing to stress resistance of ascospores include a thick cell wall, low water content, high viscosity and accumulation of protective compatible solutes (Dijksterhuis, 2007; Wyatt *et al.*, 2014). Ascospores of *N. fischeri* and *Talaromyces macrosporus* exhibit a high cytoplasmic viscosity compared with conidia (asexual spores) (Dijksterhuis *et al.*, 2007; Van Leeuwen *et al.*, 2010; Wyatt *et al.*, 2014). It has been thought that high cytoplasmic viscosity slows down the rate of metabolic reactions and therefore low amounts of reactive metabolites will be produced. Reactive metabolites such as oxygen radicals damage proteins, membranes and other cell components. The main compatible solutes that accumulate in heat-resistant ascospores of *T. macrosporus* are trehalose and mannitol (Dijksterhuis *et al.*, 2007). Trehalose and polyols have also been shown to protect desiccation-rehydration, chaotropic solutes and hydrophobic stressors in a range of microbial species (Crowe *et al.*, 1984; Mansure *et al.*, 1994; Hallsworth *et al.*, 2003; Bhaganna *et al.*, 2010). Ascospores of *Neosartorya* species such as *N. fischeri* not only contain these compatible solutes but also trehalose-based oligosaccharides (TOS) (Wyatt *et al.*, 2014). These sugars consist of a trehalose core with one (isobemisirose), two (neosartose) or three (fischerose) glucose groups α -1,6 linked to one side of the molecule. TOS with a degree of polymerization (DP) of 3–5 also occur in ascospores of other genera within the Eurotiales (Wyatt, 2014). Their presence correlated with the ability to grow at high temperature (thermophilic/thermotolerant growth style). TOS or sucrose-based oligosaccharides (SOS) (DP > 2) also accumulate in bacteria (Brique *et al.*, 2010), yeast (Iwahara *et al.*, 1993), plants (Kuo *et al.*, 1988; Blackman *et al.*, 1992) and insects (Hendrix and Wei, 1994; Wei *et al.*, 1996). It has been proposed that these molecules function as antioxidant (Chen and Yan, 2005; Van den Ende and Valluru, 2009) and/or as protectors of membranes during the processes of dehydration and rehydration (Hinch *et al.*, 2002; Cacula and Hinch, 2006). We have found indications that TOS are involved in survival of ascospores in dry heat (i.e. heat after drying of the spores). *Talaromyces macrosporus* ascospores, which do not contain TOS, were more sensitive to dry heat than *N. fischeri* ascospores (Wyatt, 2014). Conversely, *T. macrosporus* ascospores were more

more resistant to high temperatures when present in solution (i.e. wet heat).

Conner and colleagues (1987) studied ascospore maturation of *Neosartorya glabra*. It was observed that ascospores from 25-day-old cultures were more stress resistant than ascospores of 11-day-old cultures. This was accompanied by an increase of compatible solutes and changes in protein composition and cell-wall structure (Conner *et al.*, 1987). Here, we studied profiles of intracellular compatible solutes, the cytoplasmic viscosity, in relation to stress resistance of ascospores during maturation and germination of ascospores of *N. fischeri*. For the first time, these parameters were simultaneously monitored during developmental stages of ascospores, in order to elucidate the nature of the acquisition of extreme stress resistance in a eukaryotic cell. The data indicate that acquirement of stress resistance is a two-stage process. In the first stage, bulk water in the spore is reduced, while in the second stage, trehalose and TOS levels increase and mannitol levels decrease. Furthermore, in this process, the redox stability of spin probes added to ascospores (hitherto not quantified in fungal propagules) was found to be higher at later stages of maturation.

Results

Heat resistance of ascospores during maturation and germination

The heat resistance of ascospores of *N. fischeri* of 11- to 50-day-old cultures in ACES [*N*-(2-acetamido)-2-aminoethanesulfonic acid] buffer was determined. To this end, ascospores were subjected to 85°C for 0–60 min, after which they were plated out (Fig. 1). Ascospores from 11-day-old cultures did not survive 2 min at 85°C, while ascospores of 15-day-old cultures did survive this treatment (Fig. 1). More mature ascospores (> 15 days) showed a gradual increase in heat resistance. Some ascospores from 50-day-old cultures even survived 50 min at 85°C.

Ascospores of a 40-day-old culture were activated to germinate by a short (2-min) heat treatment at 85°C. Subsequently, at several stages of germination between 0 h and 4 h, ascospores were subjected to a second heat treatment (up to 4 min at 85°C) to monitor the decrease in heat resistance (Fig. 2.) Untreated dormant ascospores ($t = 0$ h) did not germinate (Fig. 2), but showed high germination after the heat treatment. After 30 min, activated ascospores survived the heat treatment, but after 1 h, the majority of the cells were killed ($81 \pm 5\%$, Fig. 2). Resistance to 85°C was completely lost 1.5 h after heat activation, even a 30-s treatment at 85°C killed all activated ascospores.

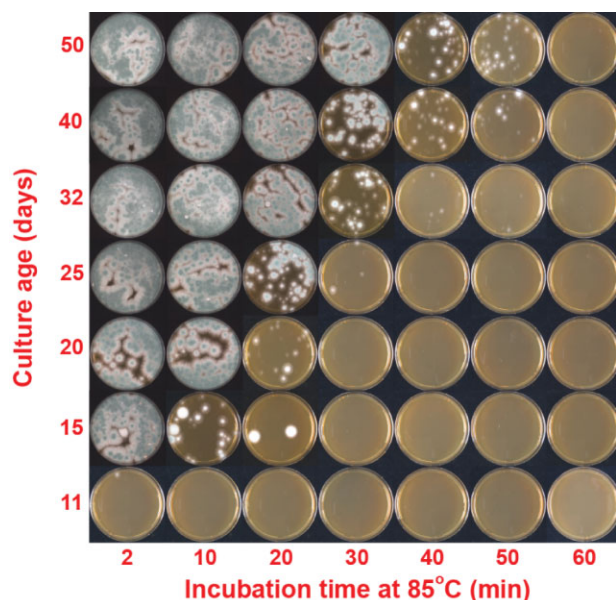


Fig. 1. Survival of ascospores of *N. fischeri* ascospores from 11- to 50-day-old cultures after a 2–60 min treatment at 85°C.

Viscosity of the cytoplasm of ascospores changes maturation and germination

The viscosity of the cytoplasm of ascospores during maturation and germination was studied with electron spin resonance (ESR) spectroscopy. Figures 3B, 3C and 4 show the narrow line spectrum from intracellular 4-oxo-2,2,6,6-tetramethylpiperidone-N-oxyl (TEMPONE). The narrow lines indicate fast rotation of the spin probe molecules. The distance of 16.1 gauss between the narrow lines of the ESR spectrum is characteristic for a polar (aqueous) environment (Golovina and Hoekstra, 2002). Therefore, this spectrum is indicative of an aqueous cytoplasm. The heights of the low-field and high-field lines and the width of the low-field line can be used to calculate a rotational correlation time and related effective cytoplasmic viscosity (Fig. 4, see Experimental procedures). The

cytoplasmic viscosity of ascospores from 15-day-old cultures was almost four times higher than that of 11-day-old cultures (5.4 and 21.2 cP respectively) (Table 1). Viscosity of spores of older cultures was similar to that of 15-day-old cultures (Table 1). The ESR spectra of the ascospores of 15-day-old cultures were subtracted from that of ascospores of 11-day-old cultures (Fig. 5A). This difference spectrum (Fig. 5B) is typical for the fast isotropic rotation of TEMPONE molecules in bulk water (Golovina and Hoekstra, 2002). The differences in ESR spectra of TEMPONE from ascospores of 15- and 20-day-old cultures (Fig. 5C and 5D) and older cultures (data not shown) are negligible. Taken together, fast isotropic motion of the spin label as in ascospores of 11-day-old cultures disappeared in spores of cultures of 15 days and older.

The cytosol of dormant ascospores of 40-day-old cultures had a viscosity of about 20 cP (Fig. 6A). This value dropped twofold within 60 min after activation. The steep decrease in viscosity slowed down between 1 and 2 h (10–9 cP) and this was followed by a second phase of reduction to 4 cP between 2 and 3 h. The viscosity decreased gradually to 1.1 cP 6 h after activation (Fig. 6A). This value is close to the viscosity of the cytosol of the vegetative mycelium.

Compatible solutes in ascospores during maturation and germination

Colonies of *N. fischeri* formed ascospores after 3–4 days of cultivation on oatmeal agar (OA). The following compatible solutes were determined in cell-free extracts of ascospores harvested from 11- to 50-day-old cultures: glycerol, mannitol, trehalose, isobemisiase, neosartose and fischerose (Table 2). The total amount of these compatible solutes was 3.9 pg per ascospore (equivalent to 454 mM) of 11-day-old cultures. This amount had increased to 12.1 pg (1027 mM) in 15-day-old cultures. During further maturation, the amount increased to a maximum of 15.5 pg per ascospore (1051 mM) of

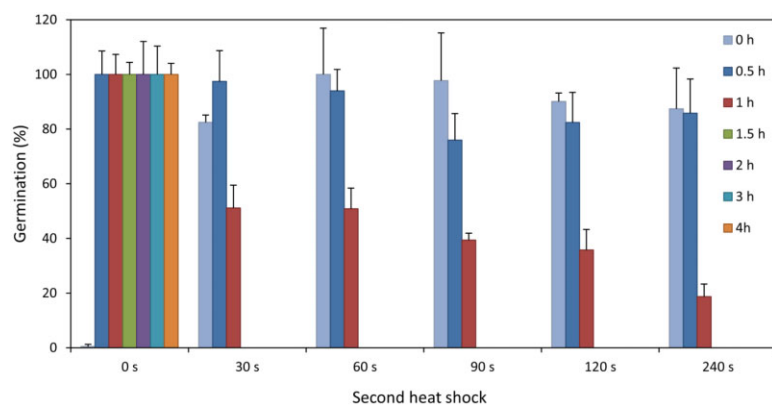


Fig. 2. Heat sensitivity of ascospores from 40-day-old cultures that were inoculated for time intervals between 0 and 4 h after heat activation (2 min at 85°C). The 0 h samples represent dormant spores. The heat sensitivity of (germinating) ascospores was measured as the percentage of germination after a second exposure to 85°C for 0–240 s.

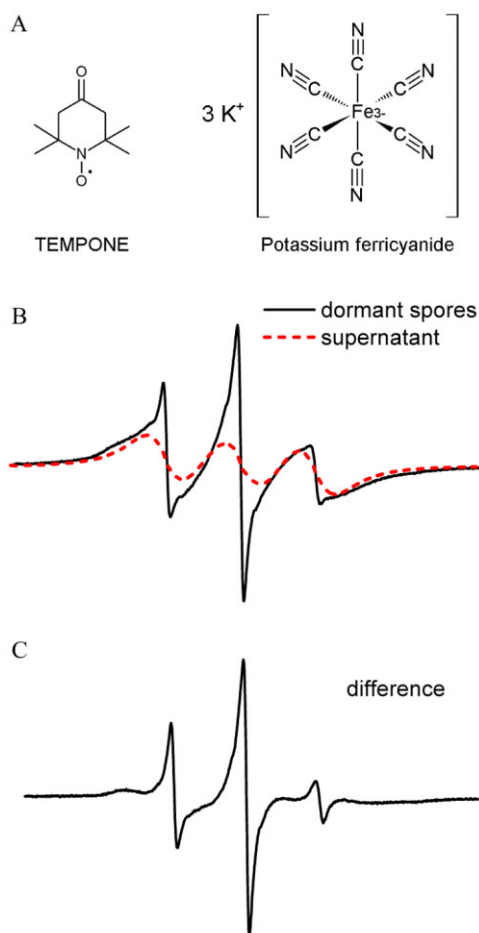


Fig. 3. ESR spectroscopy of TEMPONE of *N. fischeri* ascospores in ACES buffer. The structure of the spin probe TEMPONE and the broadening agent potassium ferricyanide (A). The decomposition of the spectrum of TEMPONE in spores and supernatant (B) and the resulting spectrum of TEMPONE in the cytoplasm at room temperature (C).

Table 1. Rotation correlation time (τ_c), effective cytoplasmic viscosity (η), and the degree of anisotropy at 295 K calculated from the spectra of TEMPONE of ascospores of *N. fischeri* of 11- to 50-day-old cultures.

Culture age (days)	Rotation correlation time τ_c (s) $\times 10^{-10}$	Viscosity η (cP)	Anisotropy h_0/h_{+1}
11	1.5	5.4	1.4
15	5.8	21.2	2.4
20	5.7	20.8	2.1
25	5.9	21.7	2.2
32	5.0	18.4	2.1
40	4.9	18.0	2.2
50	5.6	20.3	2.2

50-day-old cultures. The composition of the polyols and (oligo)saccharides changed markedly during maturation (Table 2). The ascospores of 11-day-old cultures each contained 0.7 pg glycerol. This solute was absent during later stages of culturing. Trehalose was the main solute in ascospores of 11-day-old cultures with 1.2 pg spore⁻¹. Levels of isobemisiolose, neosartolose, fischerose (all ≤ 0.7 pg spore⁻¹) and mannitol (0.5 pg spore⁻¹) were lower. All compounds showed increased abundance in ascospores of 15-day-old cultures with 3.5 pg spore⁻¹ (568 mM) for mannitol and ≥ 1.7 pg for isobemisiolose, neosartolose, fischerose and trehalose. The amount of TOS and trehalose increased further when cultures became older; i.e. 2.5–4.3 pg for TOS and 3.8 pg for trehalose per ascospore of 50-day-old cultures. In contrast, mannitol levels had decreased to 1.9 pg spore⁻¹ (Table 2).

In order to study the dynamics of compatible solute utilization during germination, ascospores of 40-day-old cultures were heat-activated and incubated in malt extract broth (MEB). Morphological changes were not apparent, upon examination using light microscopy, during the first 3 h after heat activation. Swelling,

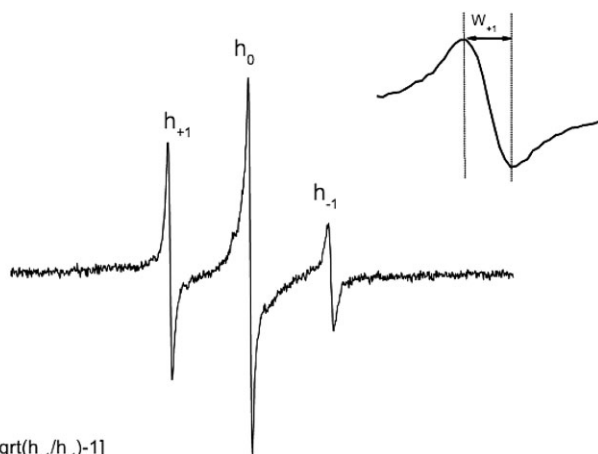


Fig. 4. ESR spectrum of TEMPONE in *N. fischeri* ascospores. Calculations of the rotational correlation time and related viscosity is based on the shape of ESR spectra of the spin label in the cytoplasm of the spores.

$$\tau_c^2 = 6.6 \times 10^{-10} W_{+1} [\sqrt{h_{+1}/h_{-1}} - 1]$$

$$\eta = 3kT \tau_c / 4\pi r^2$$

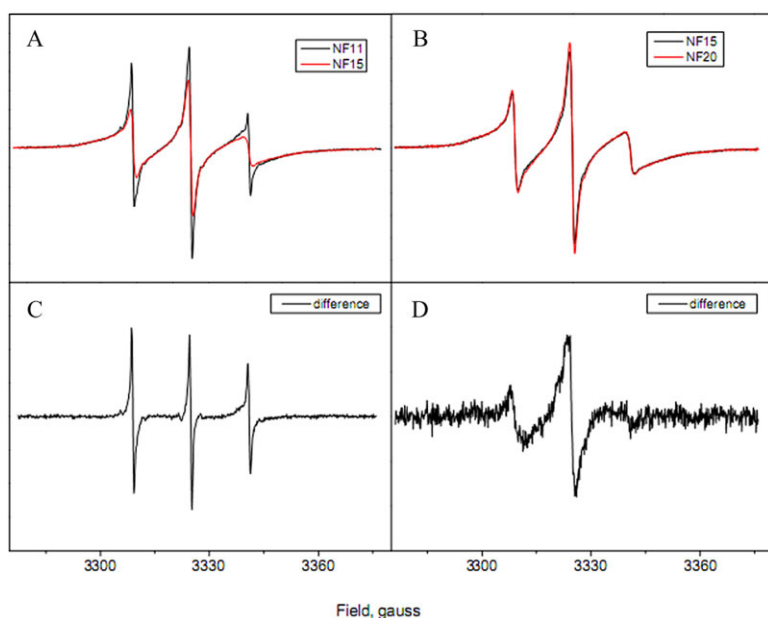


Fig. 5. TEMPONE spectra of *N. fischeri* spores from 11- and 15-day-old cultures (A), from 15- and 20-day-old cultures (C) and their difference spectra, (B) and (D) respectively. Spectrum D is enlarged.

accompanied with a slow disruption of the thick outer cell-wall along the equatorial ridge, took place between 3 and 6 h of incubation and germ tubes had formed after 6 h (data not shown). Levels of mannitol, TOS and trehalose had already decreased from 13.6 to 6.1 pg spore⁻¹ within 30 min after heat activation (Table 3; Fig. 6B). The amount of TOS dropped approximately twofold, while levels of trehalose and mannitol had even decreased fourfold. This was accompanied by the appearance of glucose (1.3 pg spore⁻¹), most probably as a result of degradation of the oligosaccharides. After 1 h levels of mannitol, TOS and trehalose had further decreased to a total amount of 1.3 pg spore⁻¹. Levels of isobemisirose, neosartose and fischerose dropped to 0.2, 0.3 and 0.1 pg spore⁻¹, respectively, while levels of mannitol and trehalose had decreased to 0.4 and 0.3 pg spore⁻¹. These solutes had disappeared by 360 min after heat activation. Glycerol started to appear after 90 min with an amount of 0.3 pg spore⁻¹ and gradually decreased to 0.1 pg spore⁻¹ between 180 and 360 min after heat activation.

Redox stability of developing spores using ESR spectroscopy

Typically, the redox stability of an environment correlates with the rate of redox conversion. The protection of the spin probe against redox conversion by the interior of the ascospores was evaluated at different stages of maturation. In our experiments, the double integral of the ESR spectrum (the integrated intensity) is used as a measure of spin-probe concentration in the cell. ESR spectra were acquired from ascospores of cultures of different age at temperatures from 250 (–23°C) to 375 K (102°C). The

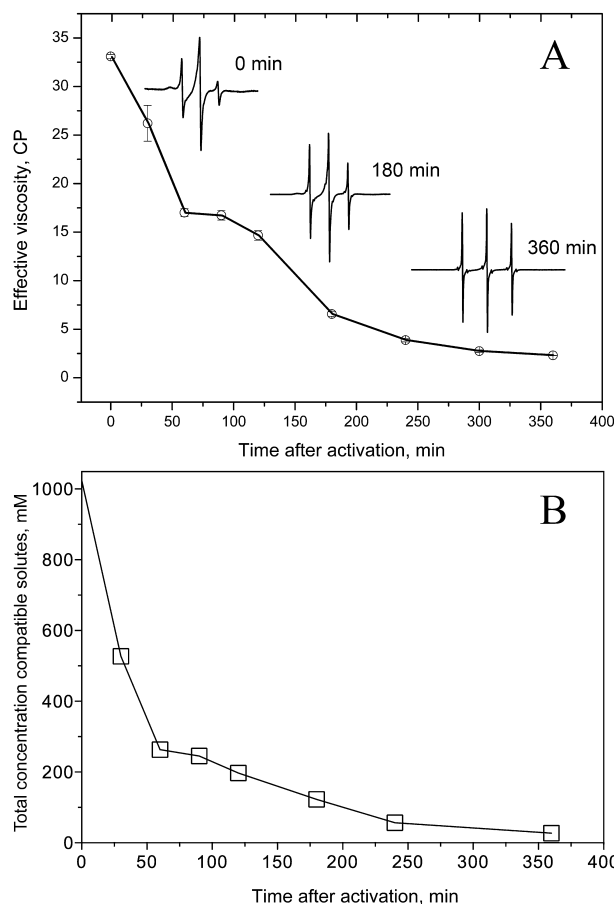


Fig. 6. Effective viscosity of the cytoplasm of ascospores of 40-day-old cultures during germination (A) calculated from the rotation correlation time of TEMPONE. The cytoplasmic viscosity of ascospores during germination decreased in two steps, which correlates with the concentration of compatible solutes in the spores (B).

Table 2. Amount (pg spore⁻¹) and intracellular concentration (mM) of solutes for ascospores from 11- to 50-day-old cultures.

	Culture age (days)													
	11		15		20		25		32		40		50	
	pg	mM	pg	mM	pg	mM	pg	mM	pg	mM	pg	mM	pg	mM
Polyols														
Glycerol	0.7	214	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0.0	0.0	0
Mannitol	0.5	77	3.5	568	3.8	614	3.4	549	2.9	468	2.7	431	1.9	310
Disaccharide														
Trehalose	1.2	92	2.0	153	2.2	172	2.2	173	2.6	199	3.0	236	3.8	293
TOS														
Isobemisiase	0.4	24	2.0	117	2.3	135	2.4	138	2.4	139	2.7	157	3.0	173
Neosartose	0.5	23	2.9	128	3.7	163	3.3	144	3.2	142	3.4	147	4.3	188
Fischerose	0.7	24	1.7	61	2.1	73	2.2	76	2.1	74	2.3	80	2.5	87
Total	4.0	454	12.1	1027	14.1	1157	13.5	1080	13.2	1022	14.1	1051	15.5	1051

TOS, Trehalose-based oligosaccharides.

integrated intensities of the spectra, normalized to the values at 295 K (22°C), were plotted against the temperature for dormant hydrated ascospores from 11- to 50-day-old cultures (Fig. 7). Three phases of temperature dependence can be discerned. At temperatures below 260 K (−13°C) (and 265 K/−8°C for spores of 11-day-old cultures) the integrated intensity of the ESR spectra against temperature decreased with increasing temperature. This pronounced change of intensity is correlated with a vitrified state of the cytoplasm as the shape of the spectrum is specific for spin probe in a solid environment. The intensities at 250 K (−23°C) are highest in the case of 25- and 50-day-old cultures. The second phase (between 260/265 and 315 K) shows nearly fixed signal strength,

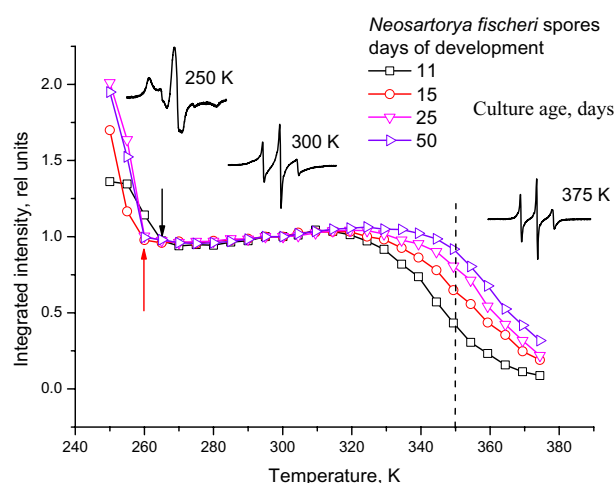


Fig. 7. The temperature dependence of the integrated intensity of ESR spectra of TEMPONE in ascospores of *N. fischeri* from 11- to 50-day-old cultures. The shape of the ESR spectra of TEMPONE in spores of 50-day-old cultures at 250 K, 300 K and 375 K are shown. The arrows at low temperature indicate the temperature of water vitrification. Integrated intensities of the ESR spectra were normalized to the integrated intensity at 295 K. The signal strength at 350 K is stronger in older spores (dashed line).

irrespective of spore age. Above this temperature (third phase), the integrated intensity decreases and the temperature at which the decrease starts depends on spore age. This phase begins at lower temperature in young ascospores, which clearly suggests that young ascospores have a higher redox conversion (activity) and hence a lower redox stability.

The degree of spin-probe redox conversion can be expressed as the ratio between integrated intensity of the spectrum at 350 K (77°C) and that at room temperature (300 K, 27°C; Fig. 8). The Boltzmann fit of these data has an adjusted R^2 of 0.932. The decrease of the spectral intensity after heating was irreversible, meaning that these silent molecules cannot be re-oxidized by ferricyanide (FC) into paramagnetic TEMPONE. Thus at high temperatures (> 60°C), paramagnetic TEMPONE is converted to paramagnetic silent molecules other than hydroxylamine. Most reduction (93.5% from the initial intensity) was observed in ascospores of 11-day-old cultures, while in ascospores of 50-day-old cultures, 73% of

Table 3. Amount (pg) of solutes per activated ascospore of 40-day-old cultures 0–6 h after heat activation.

Time after activation (h)	0	0.5	1	1.5	2	3	4	6
Polyols								
Glycerol	0.0	0.0	0.0	0.3	0.3	0.3	0.2	0.1
Mannitol	2.7	0.6	0.4	0.4	0.3	0.0	0.0	0.0
Monosaccharide								
Glucose	0.0	1.3	0.7	0.3	0.1	0.1	0.0	0.0
Disaccharide								
Trehalose	2.6	0.6	0.3	0.1	0.1	0.0	0.0	0.0
TOS								
Isobemisiase	2.5	1.0	0.2	0.1	0.0	0.0	0.0	0.0
Neosartose	4.1	1.8	0.3	0.1	0.1	0.1	0.0	0.0
Fischerose	1.7	0.8	0.1	0.0	0.0	0.0	0.0	0.0
Total	13.6	6.1	2.0	1.3	0.9	0.5	0.2	0.1

TOS, Trehalose-based oligosaccharides.

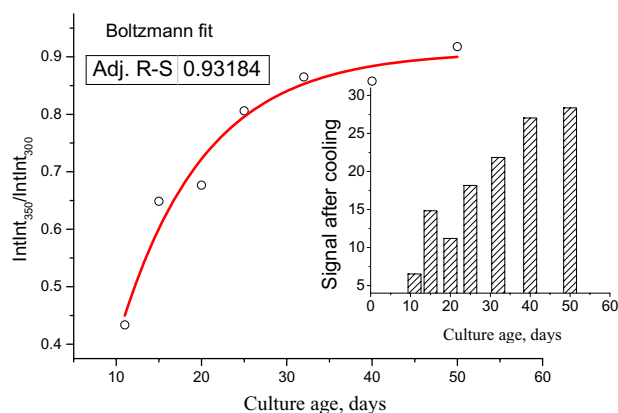


Fig. 8. Normalized integrated intensity of TEMPONE spectra at $t = 350$ K ($\text{IntInt}_{350}/\text{IntInt}_{300}$, dashed line in Fig. 7) of ascospores of *N. fischeri* from 11- to 50-day-old cultures fitted to a Boltzmann equation with an adjusted R^2 (Adj. R-S) of 0.93184. Insert: intensity of TEMPONE spectra after heating (during recording) followed by cooling of the ascospores in % from the initial intensity.

the signal was lost (Fig. 8, insert). This clearly shows that the redox stability at 350 K had increased with age.

The signal stability in ascospores was determined in the absence of FC (see Experimental procedures). Dormant ascospores of 40-day-old cultures in water did not show any reduction of spin probe signal within 25 min of incubation at room temperature, while germinating ascospores showed a distinct reduction of the signal 5 h after activation (Fig. 9). These results indicate a stable signal strength and confirm the absence of metabolic activity in these dormant ascospores.

Discussion

Ascospore maturation of *N. fischeri* is characterized by the acquisition of extreme stress resistance. Little is known about the mechanisms that play a role in this

process. In this study, maturation of ascospores was related to the accumulation of compatible solutes, the viscosity of the cytoplasm, the presence of bulk water (i.e. water in the liquid phase) and redox stability (low rate of redox reactions). Our results distinguish two phases in ascospore development. A relatively short early phase that takes place in cultures of 11–15 days followed by a much longer phase that takes place within 15–50 days. During the first phase, accumulation of compatible solutes (from a total of 0.45–1.0 M), a large increase of viscosity (5.1–21.2 cP), disappearance of bulk water, acquisition of stress (heat) resistance and an increase of redox stability occur. The second phase of maturation is characterized by a decrease of mannitol and an increase of trehalose and TOS, accompanied by a further increase of redox stability and stress (heat) resistance. The total amount of compatible solutes as well as viscosity did not change much during this stage (approximately 14 pg spore⁻¹ and 20 cP respectively). Mannitol concentration was highest in ascospores from 20-day-old cultures, while trehalose and the TOS isobemisiolose, neosartose and fischerose were most abundant in ascospores of 50-day-old cultures. Therefore, the increase of stress resistance in the second phase seems to depend on the increase of trehalose and TOS.

Several observations suggest that the fluid-phase viscosity of the cytoplasm of fungal survival structures correlates with stress resistance (Dijksterhuis *et al.*, 2007; Van Leeuwen *et al.*, 2010). Water-dispersed conidia of *Fusarium oxysporum* and *Verticillium fungicola* have a low viscosity (2 cP) and have lower stress resistance when compared with air-dispersed conidia of *Penicillium discolor* and *Aspergillus niger* (3–4 cP) (Van Leeuwen *et al.*, 2010). Constitutively dormant and extreme stress-resistant fungal and bacterial spores exhibit an even higher level of cytoplasmic viscosity (10–33 cP) (De Vries, 2006; Dijksterhuis *et al.*, 2007; Wyatt *et al.*, 2014).

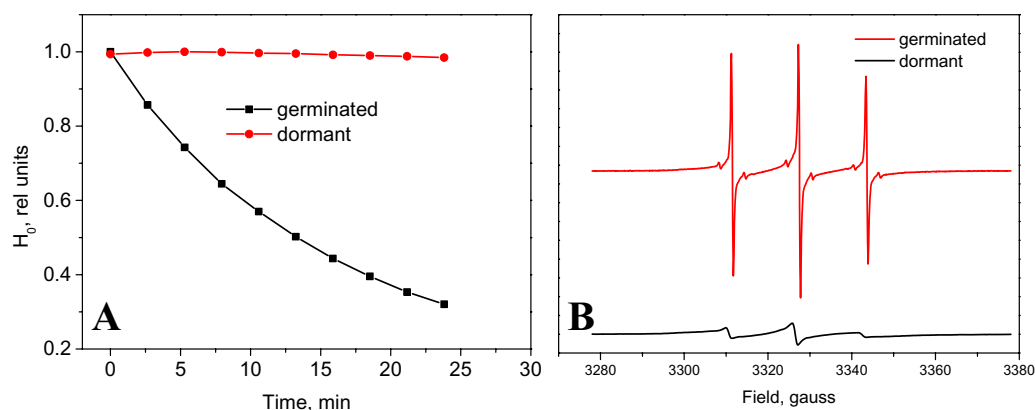


Fig. 9. Kinetics of TEMPONE reduction in dormant and 5 h germinated spores in the absence of FC in the extracellular space (A) and TEMPONE spectra in dormant and germinating spores normalized to the same number of spin probe molecules in the presence of FC (B).

The fluid-phase cytoplasmic viscosity (or microviscosity or effective viscosity) is monitored by small, non-interacting spin-probe molecules. Winther and colleagues (2012) observed that the effective viscosity derived from tumbling time (rotation correlation time) of trehalose molecules at different concentrations increases linearly up to approximately 300 mM, but at higher concentrations increases markedly faster. This was more prominently observed when the effective viscosity was plotted against the trehalose concentration (Winther *et al.*, 2012). This indicates that accumulation of solutes above a certain concentration might be an important factor in the increase of viscosity, and hence the stress resistance of, the spores. The diffusion of metabolites, and diffusion-limited enzyme kinetics, might be influenced strongly by the microviscosity. High viscosity might, as a result of these mechanisms, protect against heat stress by counteracting the effect of elevated temperature on diffusion in the cytoplasm and protein unfolding. (Blacklow *et al.*, 1988; Demchenko *et al.*, 1989).¹

Our data show a strong correlation between the intracellular viscosity and the total amount of compatible solutes during maturation and germination of ascospores. Even relatively small changes in the total amount of compatible solutes as in the case of ascospores of 32-day-old cultures (13.2 pg spore⁻¹) compared with 20-day-old cultures (14.1 pg spore⁻¹) are accompanied with similar changes in viscosity (18.4 and 20.8 cP, respectively). Viscosity and amount of compatible solutes also correlated strongly during germination of ascospores, as illustrated by a precise congruence between total solute concentration and intracellular viscosity in time (see Fig. 6). The marked increase of viscosity during early maturation coincides with the disappearance of bulk water. Trehalose that increased nearly two times in concentration during early maturation is known to be highly effective in reducing the amount of bulk water (Lerbret *et al.*, 2005). Mannitol (7.4-fold) and TOS levels (4.2-fold) increased even stronger. It is noteworthy that glycerol and fructose are the only two known compatible solutes which, at high concentrations, can have destabilizing effects on macromolecular structures via their chaotropic activity (Williams and Hallsworth, 2009; Chin *et al.*, 2010; Cray *et al.*, 2013a; Lievens *et al.*, 2014). Compatible solutes such as trehalose, as well as oligosaccharides and polysaccharides, have strong rigidifying and/or stabilizing effects on cellular macromolecules; i.e., they are highly kosmotropic (Cray *et al.*, 2013a). Kosmotropic substances are more polar than water molecules and therefore have a considerable hydration shell; the mean number of long-lived hydrogen bonds trehalose that has been found is superior than 12 other sugars (Choi *et al.*, 2006), enforcing its superior kosmotropicity (Cray *et al.*, 2013a).² The kosmotropic

compatible solutes that accumulate in ascospores of *N. fischeri* (especially trehalose and TOS; Tables 2 and 3) may also have a role in bulk water removal. Furthermore, when desiccated cells undergo rehydration (e.g. for fungal propagules, which imbibe water prior to germination) trehalose is exceptional in its ability to maintain lipid bilayer structure and thereby prevents cell lysis (Crowe *et al.*, 1984). A versatile and robust stress biology, based on capabilities such as those described above, has been shown to provide ecological success for a number of stress-tolerant fungi as well as other microbial species (Mansure *et al.*, 1994; Cray *et al.*, 2013b; Lievens *et al.*, 2014; Oren and Hallsworth, 2014; Stevenson *et al.*, 2014).

High viscosity results in a relatively low speed of adverse chemical reactions (Roos, 1995) such as the production of reactive radicals. The analysis of the redox conversion of spin probes in dormant ascospores provided an opportunity to study the protection of molecules within the matrix of the cell at different conditions, as for example temperature or after drying. The signal intensity of TEMPONE at temperatures above 320 K (47°C) remains highest in the oldest ascospores (of 50-day-old cultures). This suggests that radicals may be formed at a lower rate or that reactions of radicals with TEMPONE occur at a lower rate in older spores. Thus, the redox stability of the spore cytoplasm increased with age. Mannitol, trehalose and oligosaccharides are suggested to play a role in protection against oxidative stress (Shen *et al.*, 1997; Benaroudj *et al.*, 2001; Valluru and van den Ende, 2008; Van den Ende and Valluru, 2009). Additional or superior protective properties of trehalose and TOS in this respect could explain the accumulation of these solutes.

Dried ascospores of *N. fischeri* (containing trehalose and TOS) survive a heat treatment better than dried ascospores of *T. macrosporus* (containing only trehalose; Wyatt *et al.*, 2014). Less than 10% of *T. macrosporus* ascospores survive 1 day at 60°C, while approximately 15% of the *N. fischeri* spores survived even after 15 days at 60°C (T.T. Wyatt, unpublished results). The process of drying includes crowding of solutes in the remaining water, resulting in disturbances in the structure of proteins and membranes (Hoekstra *et al.*, 2001). Compatible solutes protect these molecules by direct interaction via their hydroxyl groups (water replacement theory) or by preferred exclusion of solutes around proteins and membranes (as a result of kosmotropic properties of the solutes, Arakawa and Timasheff, 1982; Jain and Roy, 2009). In plants, SOS are believed to act as membrane protectors under stress via direct hydrogen binding, in which the chain length is important for the insertion into the membrane (Hinch *et al.*, 2002; 2003; Milhaud, 2004; Beck *et al.*, 2007). It is also claimed that these plant

oligosaccharides protect against oxidative-stress and lipid oxidation (Cacela and Hinch, 2006; Agati *et al.*, 2007; Nishizawa *et al.*, 2008; Van den Ende and Valluru, 2009). TOS show similarities with SOS belonging to the raffinose family oligosaccharides (RFOs), which accumulate during seed maturation (Kuo *et al.*, 1988). These RFOs have like our TOS a DP of 3–5, α -1,6 linkages and their accumulation is related to increased stress resistance (Brenac *et al.*, 1997). The specific function of TOS in ascospores might be similar to the suggested function of SOS, which is providing protecting of membranes against desiccation (Hinch *et al.*, 2003).

Experimental procedures

Strain, growth conditions and culture media

Cultures of *N. fischeri* were obtained from the CBS Fungal Biodiversity Centre (CBS 317.89) and routinely grown at 30°C on OA (Samson and Houbaken, 2010). A number of 10^6 ascospores were taken up in 100 μ l 10 mM ACES buffer, pH 6.8, with 0.02% Tween-80 (Sigma-Aldrich, Zwijndrecht, The Netherlands). They were activated with a 2 min treatment at 85°C and inoculated on 20 ml OA in a Petri dish. After 11 to 50 days of growth, fungal material of three Petri dishes was collected with a glass spatula and taken up in 9 ml ice-cold ACES buffer. Approximately, 1 cm³ of sterile glass beads (diameters 0.10–0.11 mm and 1.0 mm in a 1:1 ratio) was added and the fungal material was homogenized by vortexing (2×1 min) and sonication (5 min) using a Ultrasonic cleaner 2510E-MT (Branson Ultrasonics Corporation, Danbury, USA). Debris was removed by filtration through sterile glass wool and the ascospores were spun down at 5°C at 1100 g for 5 min using a swing out rotor. The ascospores were taken up in ice-cold ACES buffer and the washing step was repeated two times. If not immediately used for experiments, the pellets of ascospores were stored in ACES buffer at –80°C.

Heat inactivation of ascospores

Ascospores (50 μ l ACES buffer containing 10^6 spores ml⁻¹) were heated for 0–70 min at 85°C in 0.5 ml Eppendorf tubes in a water bath. The ascospores were cooled on ice and germination was assessed on malt extract agar (MEA) (Samson and Houbaken, 2010) plates or on 1–2 mm thin MEA slices placed on top of object glasses using 10^2 spores and 10^4 spores respectively. Colony-forming units on the plates were counted after incubation for 2 days, while germination on the glass slides was quantified microscopically after incubation for 16 h.

Germination of ascospores

Heat-activated ascospores were inoculated in 200 ml MEB (Oxoid, Badhoevedorp, The Netherlands) using a final concentration of 2.10^7 spores ml⁻¹. The cultures were incubated at 30°C in 250 ml Erlenmeyer flasks at 150 r.p.m. Samples (10 ml) were collected after 0–6 h culturing and immediately

cooled on ice. The ascospores were pelleted at 5°C for 5 min at 1100 g using a swing out rotor. They were washed twice with ice-cold ACES buffer as described above. The samples were frozen in liquid nitrogen and stored at –80°C until further processing.

Electron spin resonance (ESR) spectroscopy

PD-TEMPONE (perdeuterated 4-oxy-2,2,6,6-tetramethylpiperidone-N-oxyl) was purchased from Sigma (St. Louis, MO, USA) and used for ESR spectroscopy. Spectra of the spin probes were recorded with an X-band 300E ESR spectrometer (Bruker Analytik, Rheinstetten, Germany) using a modulation amplitude of 1 gauss and 2–5 mW power. Under these conditions, spectra were not over-modulated and the signal was not saturated. The ESR spectra are the first derivatives of the absorbance spectra.

ESR can be used to determine the micro-viscosity, because the shape of ESR spectra is influenced by the environment of the spin label (Keith and Snipes, 1974; Knowles *et al.*, 1976; Keith *et al.*, 1977). TEMPONE was used for ESR of ascospores in an aqueous solution (Dijksterhuis *et al.*, 2007). To this end, ascospores were incubated for 1 min at room temperature in milli-Q water containing 1 mM TEMPONE and 120 mM K₃Fe(CN)₆ (FC) (Fig. 1). After a 1 min centrifugation at 2000 g the supernatant was removed and the pellet with the remaining fluid was taken up with a Hamilton syringe. The spore pellet was transferred to a glass capillary with a diameter of 2 mm and stopped with cotton wool.

The broadened TEMPONE spectrum was subtracted from the recorded spectrum (Fig. 1) to obtain the narrow lines from TEMPONE, which resides in the bulk aqueous phase of the spore cytoplasm (Fig. 1). The broad lines originate from paramagnetically broadened TEMPONE molecules. In our samples, paramagnetically broadened TEMPONE is recorded from a sample that contained ascospore cell wall labelled with TEMPONE in the presence of FC. FC was used to broaden ESR spectra of extracellular TEMPONE (Chesnut, 1977; Golovina and Tikhonov, 1994; Golovina *et al.*, 2001).

The rotational correlation time (τ_c) of intracellular spin probe in a liquid phase was calculated using the equation: $\tau_c = K \Delta W_{+1} (\sqrt{(h_{+1}/h_{-1})} - 1)$, where K is a constant with the value $6.7 \cdot 10^{-10}$ s (Kuznetsov *et al.*, 1971), ΔW_{+1} is the peak-to-peak width of the low-field (left-hand) line in gauss and h_{+1} and h_{-1} are the heights of the low-field (left-hand) and high-field (right-hand) lines respectively (Fig. 2) (Kivelson, 1960). This formula contains only low-field (h_{+1}) and high-field (h_{-1}) lines because these lines are not influenced by the presence of other free radical signals such as melanin. If present, the low- and high-field lines are also the best resolved from the anisotropic spectra of immobilized spin label. With the Stokes–Einstein relationship; $\tau_c = 4\pi(a)^3\eta/3kT$, the cytoplasmic viscosity can then be derived from the rotational correlation time (τ_c), where a is the particle radius of the spin probe molecule, η is the effective viscosity, k is the Boltzmann constant and T is the absolute temperature in Kelvin (Fig. 4). The radius of TEMPONE is approximately 3 Å (Keith and Snipes, 1974).

The change of the number of paramagnetic centres (number of paramagnetic spin-probe molecules) in a sample can be used to estimate redox conversion of the spin label.

As the shape of the ESR spectrum changes with temperature due to viscosity, the height of the spectral lines cannot be used to quantify the number of paramagnetic centres. To overcome this, the relative number of TEMPONE molecules in a sample was calculated by double integration of the ESR spectrum. Because the ESR spectrum is the first derivative of the absorption spectrum, the first integration gives the absorption spectrum, and the second derivative gives the area under the absorption spectrum, which is proportional to the number of spin probe molecules.

Metabolic processes inside the cell can reduce the spin probe TEMPONE to paramagnetically silent hydroxylamine. This redox conversion is indicative for metabolic activity. The permeability of the membrane for neutral spin probes as TEMPONE is very high and diffusion in and out the cell is much faster than the formation of paramagnetically silent spin probe molecules (Miller, 1978). Therefore, the number of paramagnetic molecules (the intensity of the ESR signal) is the result of an equilibrium between spin-probe reduction to hydroxylamine in the cell, hydroxylamine diffusion out of cells, its re-oxidation (by FC) and diffusion back into cells. To prevent re-oxidation of spin probe and estimate the metabolic activity of ascospores we measured in the absence of FC. The height of the central peak is a measurement of the signal strength.

Analysis of compatible solutes

Dormant and germinating (0–6 h) ascospores ($\pm 2.10^8$ spores) from 11- to 50-day-old cultures were broken using the Qiagen Tissuelyser (2 min at 30 strokes s^{-1}) in a stainless steel grinding jar (Qiagen, Venlo, The Netherlands) cooled with liquid nitrogen. Grinding was resumed for an additional 2 min after adding 1 ml milli-Q water. Samples were thawed, transferred to a 2 ml Eppendorf tube and centrifuged at 10 000 g for 30 min at 4°C. The supernatant was heated for 30 min at 95°C and centrifuged again at 10 000 g for 30 min. The supernatant was filtered (0.2 μm Acrodisc Cr13mm Syringe filter, Pall Life Science, Mijdrecht, The Netherlands) and stored at –80°C until further analysis.

Quantitative analysis of saccharides and polyols was carried out by high-performance liquid chromatography (HPLC) using a Sugar-Pak I cation-exchange column (Waters, Etten-Leur, The Netherlands) (Wyatt *et al.*, 2013). The column was heated (50°C) during separation (Column heater, Waters) and the oligosaccharides and polyols were detected with an infrared detector (2414 refractive index detector, Waters). The flow of the mobile phase (0.1 mM Ca EDTA) was 0.5 ml min^{-1} using a 515 HPLC pump and the pump control module II (Waters). Empower software (Waters) was used for peak integration and calculations. Trehalose, isobemisirose, neosartose, fischerose, mannitol, glucose and glycerol (0.01–0.50% w/v) were used as reference.

Notes

¹ Whereas the biophysical parameter viscosity *per se* is sometimes regarded as a stress parameter, there is a paucity of data in support of any mechanism for this (see Lievens *et al.*, 2014).

² In saline habitats kosmotropic ions, like compatible solutes, can also counter the macromolecule-disordering activities of chaotropic

substances which (like high temperature) induce otherwise-dangerous levels of cellular stress (e.g. Bhaganna *et al.*, 2010; Yakimov *et al.*, 2014).

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