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Impact of maturation and growth temperature on cell-size distribution, heat-resistance, compatible solute composition and transcription profiles of *Penicillium roqueforti* conidia

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ABSTRACT

Penicillium roqueforti is a major cause of fungal food spoilage. Its conidia are the main dispersal structures of this fungus and therefore the main cause of food contamination. These stress resistant asexual spores can be killed by preservation methods such as heat treatment. Here, the effects of cultivation time and temperature on thermal resistance of *P. roqueforti* conidia were studied. To this end, cultures were grown for 3, 5, 7 and 10 days at 25 °C or for 7 days at 15, 25 and 30 °C. Conidia of 3- and 10-day-old cultures that had been grown at 25 °C had D_{56}-values of 1.99 ± 0.15 min and 5.31 ± 1.04 min, respectively. The effect of cultivation temperature was most pronounced between *P. roqueforti* conidia cultured for 7 days at 15 °C and 30 °C, where D_{56}-values of 1.12 ± 0.05 min and 4.19 ± 0.11 min were found, respectively. Notably, D_{56}-values were not higher when increasing both cultivation time and temperature by growing for 10 days at 30 °C. A correlation was found between heat resistance of conidia and levels of trehalose and arabitol, while this was not found for glycerol, mannitol and erythritol. RNA-sequencing showed that the expression profiles of conidia of 3- to 10-day-old cultures that had been grown at 25 °C were distinct from conidia that had been formed at 15 °C and 30 °C for 7 days. Only 33 genes were upregulated at both prolonged incubation time and increased growth temperature. Their encoded proteins as well as trehalose and arabitol may form the core of heat resistance of *P. roqueforti* conidia.

1. Introduction

Food deterioration can have chemical, physical or microbiological causes. It results in a product with changed sensory properties such as visible appearance and the presence of off-odors and off-flavors. Furthermore, food spoilage can be associated with the introduction of toxic compounds. The impact of spoilage on food security is significant. Microbes alone have been estimated to spoil 25% of the global food supply (Bondi, Messi, Halami, Papadopoulou, & de Niederhausern, 2014).

Fungal conidia are ubiquitously present in the environment. These asexual spores are produced in overwhelming quantities and are effectively dispersed by wind, water, or animals like insects (Dijksterhuis, 2019). Fungal growth in food products can be precluded by preventing spores from entering the product and by food preservation processes such as heat-treatment, storage at low temperature, and addition of food preservatives (Dijksterhuis, 2017). As a single spore can cause spoilage, inactivating the most resistant spore is crucial from a food preservation perspective.

Most conidia will be killed by thermal food processing, but a resistant sub-population of cells may survive. Heterogeneity in a spore population occurs due to differences in genetic background, the developmental state of spores, and environmental growth conditions (Fujikawa & Itoh, 1996; Hallsworth & Magan, 1995; Hallsworth & Magan, 1996; Nanguy, Perrier-Cornet, Bensoussan, & Dantigny, 2010). Heterogeneity is also observed between spores from a single colony

Abbreviations: ACES, N-(2-acetamido)-2-aminoethanesulfonic acid; MEA, malt extract agar; D-value, decimal reduction time; HPLC, high-pressure liquid chromatography

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(Bleichrodt et al., 2013; Hewitt, Foster, Dyer, & Avery, 2016; Krijgsheld et al., 2013; van den Brule et al., 2019). This may not only be caused by differences in developmental state or local differences in environmental growth conditions but also by stochastic gene expression. It should be noted that survival of a small subpopulation of conidia can also be introduced by the experimental procedure allowing small numbers of spores to escape the maximal heat exposure.

Cultivation temperature is an example of an environmental condition that affects stress resistance of conidia. For example, *Aspergillus fumigatus* conidia produced at elevated temperature are more resistant to oxidative stress and heat-treatment (Hagiwara et al., 2017). This was associated with higher trehalose and mannitol levels as was observed in other Aspergilli (Fillinger et al., 2001; Ruijter et al., 2003; Wolschek & Kubicek, 1997). A similar increase in compatible solutes was found for *Penicillium expansum* and *Penicillium roqueforti* (Nguyen Van Long et al., 2017a), however, the heat resistance of the *Penicillium* sp. conidia was not assessed. Apart from compatible solutes, heat shock proteins and hydrophilins have been implicated in heat resistance (Wyatt, Wösten, & Dijksterhuis, 2013). Transcripts of their encoding genes were found to accumulate in dormant conidia of *Aspergillus niger* (van Leeuwen et al., 2013).

The genus *Penicillium* consists of hundreds of species that are very relevant in food spoilage and post-harvest damage (Samson, Houbraken, Thrane, Frisvad, & Andersen, 2019). This is in part explained by the fact that conidia of *Penicillium* are among the most abundant spores in air samples and the fact that members of this genus can grow at low temperatures. *P. roqueforti* is used in cheese making but it is a food spoiler as well (Samson et al., 2019). For instance, it spoils grain, rye bread, and dairy products including cheese (Aran & Eke, 1987; Lund, Filtenborg, & Frisvad, 1995; Lund, Westall, & Frisvad, 1996; Nielsen & Rios, 2000; Samson et al., 2019; Taniwaki, Hocking, Pitt, & Fleet, 2001). *P. roqueforti* changes the sensory properties of the product and it can also produce mycotoxins like PR toxin, roquefortine C and mycophenolic acid (Gillot et al., 2017). *P. roqueforti* is one of the fastest growing *Penicillium* species (reaching 40–77 mm in 7 days at 25 °C, Samson et al., 2019) and can grow at temperatures below 0 °C and up to 33 °C at a pH between 2.8 and 13.8 (Kalai, Anzala, Bensoussan, & Dantigny, 2017). Furthermore, it survives atmospheres containing up to 70% CO_2 (Taniwaki et al., 2001; Nguyen Van Long et al., 2017b) or having a partial O_2 pressure as low as 0.1% (Magan & Lacey, 1984; Yanai, Ishitani, & Kojo, 1980). In addition, *P. roqueforti* is one of few fungi that is classified as a preservative-resistant mould. It can spoil products containing weak organic acids such as sorbic, benzoic or propionic acid (Samson, Hoekstra, & Frisvad, 2004). In the case of sorbic acid this is due to degradation of the preservative by the fungus (Marth, Capp, Hasenzahl, Jackson, & Hussong, 1966).

Here, the impact of cultivation time and temperature on thermal resistance of *P. roqueforti* conidia was studied and related to spore size, compatible solute concentrations, and transcriptomes. To this end, cultures were grown for 3–10 days at 25 °C or at 15–30 °C for 7 days. The D_{56}-value of *P. roqueforti* conidia was up to 4-fold higher at longer cultivation time and when formed at increased temperature. Compatible solute analysis suggests that trehalose and arabitol accumulation is important for conidial heat resistance, while RNA sequencing indicates a role for hydrophilins and heat shock proteins.

2. Material and methods

2.1. Strain and cultivation conditions

Strain *P. roqueforti* LCP96 04111 (Muséum National d'Histoire Naturelle, France) was used in this study. Conidia were stored at −80 °C in 30% glycerol and spot inoculated on MEA (Oxoid, Hampshire, UK). Conidia were harvested from 7-day old cultures incubated at 25 °C with a cotton bud and suspended in 1 ml 10 mM ACES pH 6.8, 0.02% Tween 80 (called ACES buffer from now on). 100 µl of this suspension was spread on a MEA plate and incubated for 3, 5, 7 or 10 days at 15, 25 or 30 °C depending on the experiment. Conidia were harvested, taken up in 10 ml ice-cold ACES buffer, and washed twice with ACES buffer (van den Brule et al., 2019). The conidia suspension was diluted 100 times, after which conidia concentration was determined using a haemocytometer.

2.2. Heat-inactivation of conidia

Heat inactivation was performed with conidia of 3- to 10-day-old cultures grown at 25 °C and with conidia of 7-day-old cultures grown at 15 °C and 30 °C. ACES buffer (19.8 ml) was pre-heated in a 100 ml Erlenmeyer in a water bath at 56 °C and 100 rpm. 200 µl spore suspension (containing $2{\cdot}10^8$/ml conidia) was added to the Erlenmeyer and 1 ml samples were taken after 2–45 min followed by immediate cooling on ice. As a control, the untreated spore suspension was used. Decimal dilutions were made of each sample in ACES buffer and 100 µl was spread on a MEA plate and incubated at 25 °C. Colony forming units were quantified after 3–7 days depending on the lag time after the heat treatment. Experiments were done in triplicate, i.e. each experiment was performed with conidia derived from a biologically independent culture.

2.3. D-value determination

Inactivation curves were obtained by plotting Log_{10} surviving counts against the time of the heat-treatment using the modified Weibull model (Metselaar, Den Besten, Abee, Moezelaar, & Zwietering, 2013) that allows fitting of concave and convex inactivation curves:

$$\log N_t = \log N_0 - 5\left(\frac{t}{t_{5D}}\right)^{\beta} \quad (1)$$

Log N_t represents the $\log_{10}$ number of surviving spores ($\log_{10}$ CFU ml^{-1}) at time t, log N_0 the $\log_{10}$ initial number of spores ($\log_{10}$ CFU ml^{-1}), t time in minutes, $5D$ time to reach 5 $\log_{10}$ reduction in minutes, and β the shape parameters where $\beta > 1$ is concave and $\beta < 1$ is convex. Based on the experimental range a decimal reduction of $5D$ was selected. Each replicate was fitted with the R package GrowthRates (Hall, Acar, Nandipati, & Barlow, 2014) using the Levenberg-Marquardt algorithm. When β was significantly different from 1, the D_{56}-value was estimated as t_{5D} / 5. If not, the $-1/$ slope of a linear model was used to estimate the D_{56}-value. ANOVA followed by Tukey's Post Hoc test was applied to compare the D_{56}-values between the different experimental conditions.

2.4. Microscopy

Conidia chain length of *P. roqueforti* colonies that were grown for 3–7 days at 15–30 °C was monitored with a Nikon Zoom AZ-100 stereomicroscope linked to a Nikon DS-Ri2 camera (Nikon Instruments, Amsterdam, the Netherlands).

2.5. Compatible solute concentration assessment

Compatible solute extraction and quantification was performed as described (van den Brule et al., 2019) using conidia that had been harvested from 3- to 10-day-old cultures that had been cultivated at 15 °C, 25 °C or 30 °C. In short, 10^8 conidia were pelleted for 1 min at 4 °C and 21.000 g and flash-frozen in liquid nitrogen after discarding the supernatant. Conidia were crushed with stainless steel beads in pre-cooled adapters (-80 °C) using a Tissuelyzer (2 min, 25 Hz) (QIAGEN, Hilden, Germany). After adding 1 ml MiliQ and heating for 30 min at 95 °C, samples were centrifuged at 4 °C for 30 min at 20.000*g* and filtered using an Acrodisc nylon syringe filter (0.2 µm, Pall Life Science, Mijdrecht, The Netherlands). Compatible solutes were determined in a

Table 1
Effect of cultivation time and temperature on the size and number of conidia of *P. roqueforti* as measured by Coulter counter and total spore count per colony. Mean spore diameter (in μm) and spore count (log_{10} total spores / plate) are shown ± SE. Asterisk indicates statistical significant differences of the diameter of conidia compared to conidia formed at 15 °C with the same cultivation time (Tukey's HSD, $p < 0.01$). Different letters indicate statistical significant differences of diameter of conidia within each temperature group (Tukey's HSD, $p < 0.01$).

Culture age	15 °C		25 °C		30 °C	
	Diameter	Spore count	Diameter	Spore count	Diameter	Spore count
3 days	3.89 ± 0.03a	6.07 ± 0.14	3.87 ± 0.02^{ab}	9.06 ± 0.06	4.11 ± 0.02^{a*}	8.99 ± 0.07
5 days	3.75 ± 0.02b	8.43 ± 0.14	3.84 ± 0.02^{ac}	9.07 ± 0.03	4.13 ± 0.02^{a*}	8.69 ± 0.07
7 days	3.97 ± 0.03a	9.23 ± 0.05	3.94 ± 0.02^{b}	9.13 ± 0.02	4.14 ± 0.02^{a*}	8.91 ± 0.05
10 days	3.62 ± 0.01c	9.40 ± 0.02	3.77 ± 0.01^{c*}	9.11 ± 0.02	4.46 ± 0.02^{b*}	9.15 ± 0.1

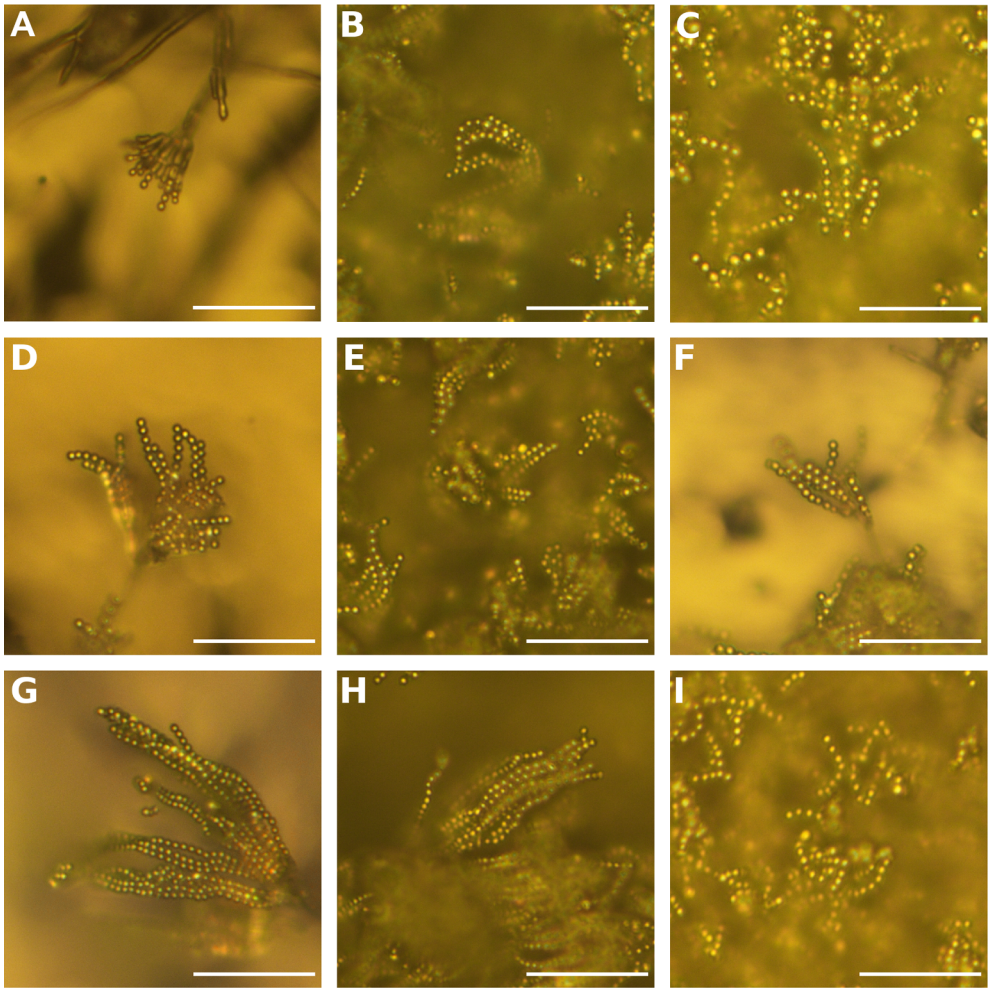

Fig. 1. Light microscopy images of chains of *P. roqueforti* conidia of 3- (A-C), 5- (D-F) and 7- (G-I) day-old cultures that had been grown at 15 °C (A,D,G), 25 °C (B,E,H) and 30 °C (C,F,I). Bar indicates 50 μm.

sample volume of 20 μl with HPLC as described (van den Brule et al., 2019). Shortly, the sample was injected in a mobile phase consisting of 0.1 mmol l^{-1} Ca EDTA in ultrapure water and were followed for 30 min. To quantify the concentration of compatible solutes in conidia, their size was measured using a Coulter counter Multisizer3 equipped with a 70 μm aperture tube with a measuring range of 1.4–42 μm (Beckman, Fichtenhain, Germany). To this end, conidial suspensions (described above) were diluted to $1{\cdot}10^5$ ml^{-1} in ISOTON II solution (Beckman Coulter) and 100 μl was used to measure spore size. At least 10^3 data points per sample were used to calculate the mean conidia diameter and volume.

2.6. DNA extraction, genome sequencing, assembly and annotation

P. roqueforti was incubated for 48 h at 25 °C and 200 rpm in an Erlenmeyer flask containing 25 ml complete medium with 1% glucose (w/v). Mycelium was harvested by filtering the culture through miracloth. DNA was extracted using the PowerPlant DNeasy kit (Qiagen) from lyophilized mycelium that was ground in a mortar and sequenced with Illumina NextSeq500 150 bp paired-end technology (Utrecht Sequencing Facility, useq.nl). The sequence reads were assembled using SPAdes v3.11.1 (Bankevich et al., 2012). The RNA-Sequencing reads (see below) were used to aid in gene prediction. These reads were pooled and aligned to the assembly using HISAT version 2.1.0, (Kim, Langmead, & Salzberg, 2015) using settings –min-intronlen 20 –max-

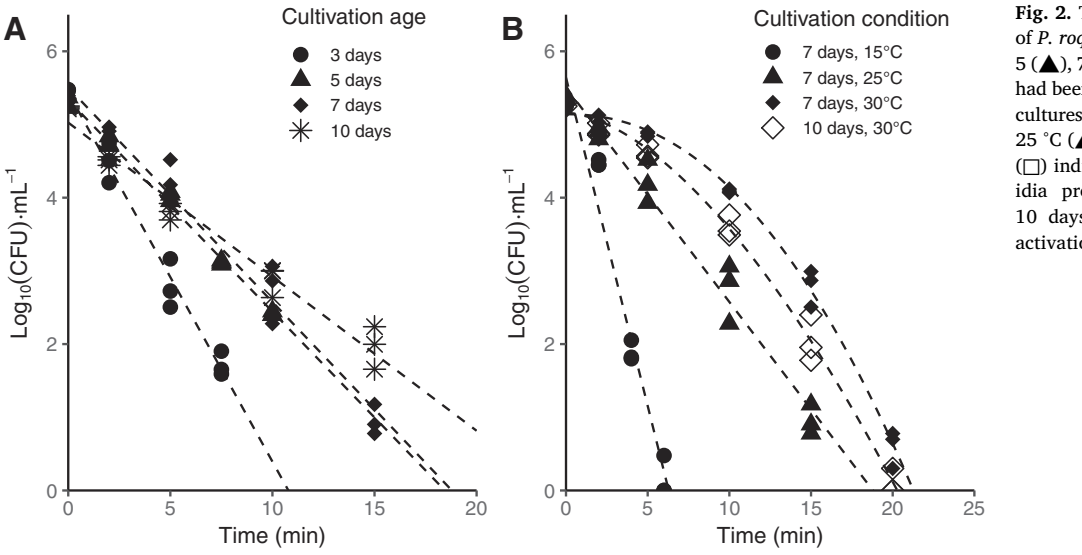

Fig. 2. Thermal inactivation curves at 56 °C of *P. roqueforti* conidia obtained from 3 (●), 5 (▲), 7 (♦) and 10-day old (*) cultures that had been incubated at 25 °C (A) or 7-day-old cultures that had been grown at 15 °C (●), 25 °C (▲) or 30 °C (♦) (B). Open diamonds (□) indicate the inactivation curve for conidia produced at 30 °C harvested after 10 days. Dashed line depicts average inactivation curve.

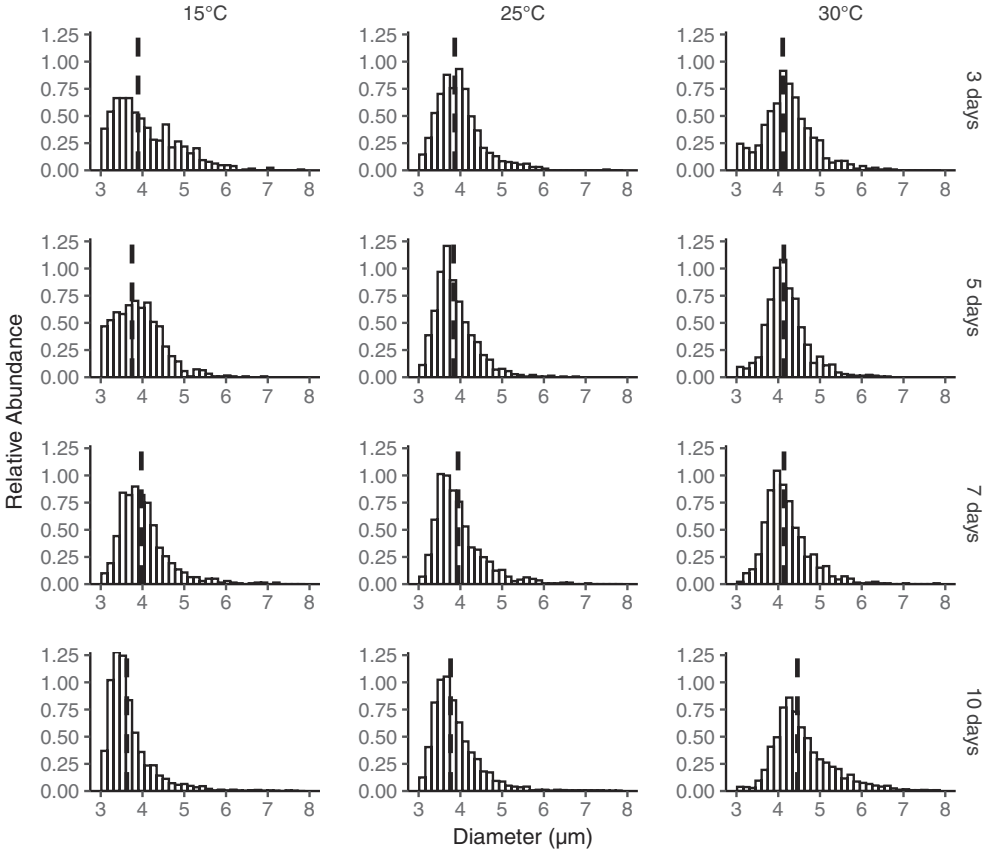

Fig. 3. Size distribution of conidia harvested from 3 to 10 day-old cultures grown at 15, 25 or 30 °C. Number of bins is equal between conditions. Dashed line highlights mean conidia diameter. Particles < 3 µm are not conidia but cellular debris.

intronlen 250 –no-unal. BRAKER version 2.1.2 (Hoff, Lange, Lomsadze A, Borodovsky, & Stanke, 2016) was used to train the *ab initio* gene predictor Augustus version 3.0.3 (Stanke, Schöffmann, Morgenstern, & Waack, 2006), which was subsequently used to generate gene predictions. Functional annotation of the predicted genes was performed essentially as described (De Bekker, Ohm, Evans, Brachmann, & Hughes, 2017), with the exception that PFAM version 32 was used (El-Gebali et al., 2019). This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession JABCSE000000000. The version described in this paper is version JABCSE010000000.

2.7. RNA extraction and sequencing

A genome-wide transcriptome analysis was performed on *P. roqueforti* conidia from biological triplicates of cultures grown for 7 days at 15, 25 and 30 °C and cultures grown for 3, 5, 7 and 10 days at 25 °C. Conidia were harvested in 10 ml ACES buffer and filtered through sterile glass wool in a 10 ml syringe. After centrifugation for 5 min at 1120*g*, the pellet was suspended in 100 µl RNA-*later* (ThermoFisher, Waltham, MA, USA), frozen in liquid nitrogen and lysed with metal beads in a Tissuelyzer (1 min, 25 Hz) (see above). Total RNA was

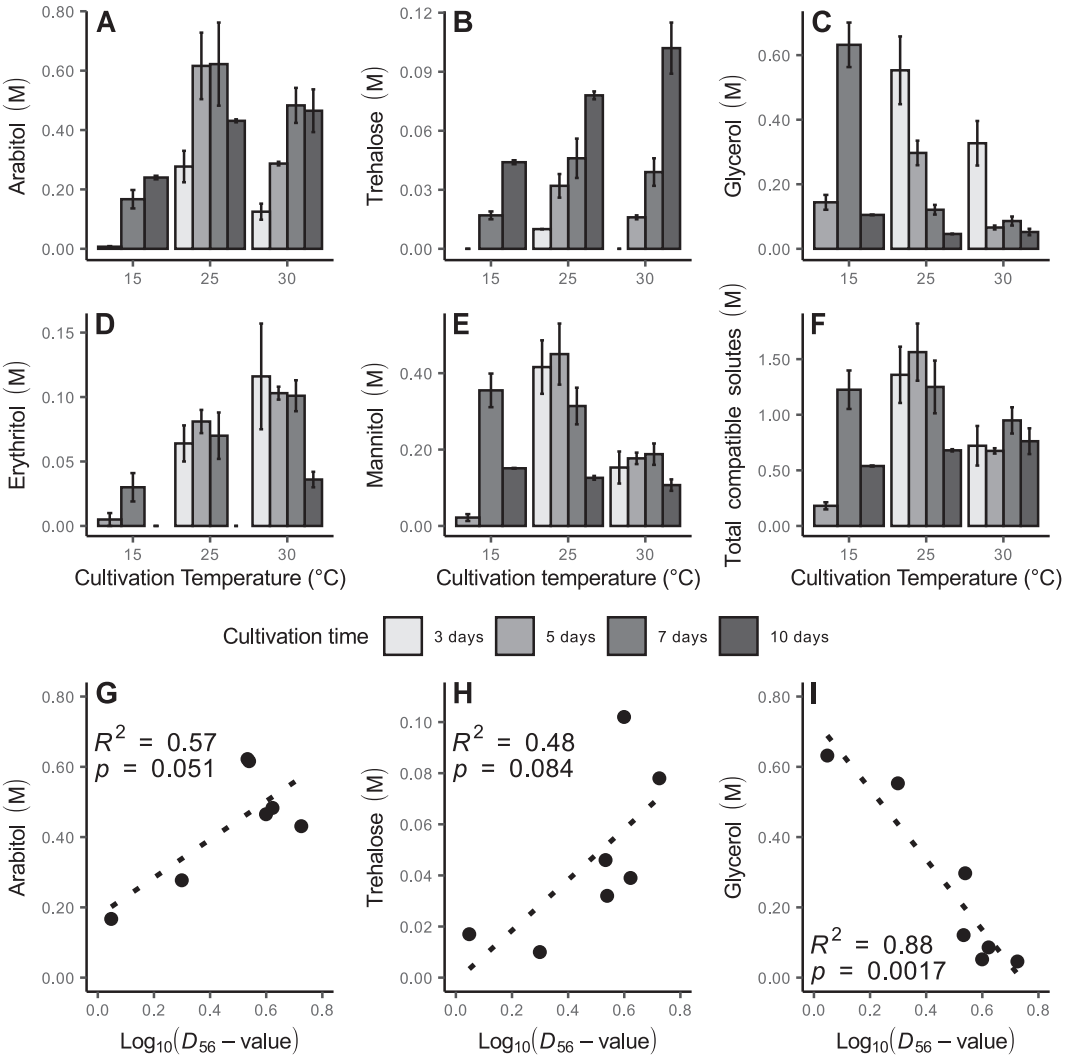

Fig. 4. Arabitol (A), trehalose (B), glycerol (C), erythritol (D), mannitol (E), and total compatible solute concentration (F) of conidia harvested from 3 to 10 day-old cultures grown at 15, 25 or 30 °C (± standard deviation). Each bar graph represents biological triplicates. Note that 3-day-old cultures grown at 15 °C did not yield sufficient number of conidia to quantify polyol content. Relationship between $\log_{10}$ (D_{56}-value) and arabitol (G), trehalose (H) or glycerol (I) concentration across all samples for which a D_{56}-value has been determined.

isolated with the RNeasy Plant Mini Kit (Qiagen) and purified by on-column DNase digestion according to the manufacturer's protocol. RNA was sequenced with Illumina NextSeq500 75 bp single-end technology (Utrecht Sequencing Facility). Transcripts were mapped to the assembled genome using HISAT v2.1.0 (Kim et al., 2015) with the following input settings: –min-intronlen 20 –max-intronlen 1000 –-no-unal –dta-cufflinks. Cuffdiff (which is part of the Cufflinks suite (version 2.2.1; Trapnell et al., 2013)) was used to determine the expression levels of each gene, normalized to the number of reads per kilobase of transcript per million reads (RPKM). Cuffdiff was also used to identify differentially expressed genes between conditions. Heatmaps were generated in R with the pheatmap and ComplexHeatmap packages (Gu, Eils, & Schlesner, 2016). The RNA-Sequencing expression data are available from the NCBI GEO database under accession GSE149235.

3. Results

3.1. Spore formation and thermal inactivation curves

Cultures of *P. roqueforti* were grown for 3, 5, 7 and 10 days at 15 °C, 25 °C and 30 °C. These temperatures were selected based on the cardinal temperatures of strain FM163 (Kalai et al., 2017). The selected temperature of 25 °C is close to the optimal growth temperature of this fungus, while growth rate at the suboptimal temperature 15 °C is similar to that at 30 °C (Kalai et al., 2017). At 15 °C, 1000 times less spores were harvested from 3-day-old cultures when compared to 7-day-old cultures; 90% of the spores were formed between 5 and 10 days (Table 1). The conidia chain length was observed to increase over time at 15 °C (Fig. 1) thus explaining, at least in part, the increase in spore formation over time. In contrast, spore numbers had reached their maximum values already after 3 days in the case of cultures grown at 25 °C and 30 °C.

The effect of cultivation time and cultivation temperature on heat resistance of conidia was determined by treatment of spores at 56 °C (Fig. 2). The inactivation curves were fitted with the modified Weibull model and a linear model. In the case β (the shape parameter) was significantly different from 1 the modified Weibull model was used to estimate the D_{56}-value, the linear model was used if β was not significantly different from 1 (Table S1). The time needed to inactivate conidia increased with increasing cultivation time at 25 °C (Fig. 2A). Conidia of 3- and 10-day-old cultures grown at 25 °C had estimated D_{56}-values of 1.99 ± 0.15 min and 5.31 ± 1.04 min, respectively. No significant difference in D_{56}-values was found between 5-day old (3.46 ± 0.08 min) and 7-day old (3.41 ± 0.09 min) cultures.

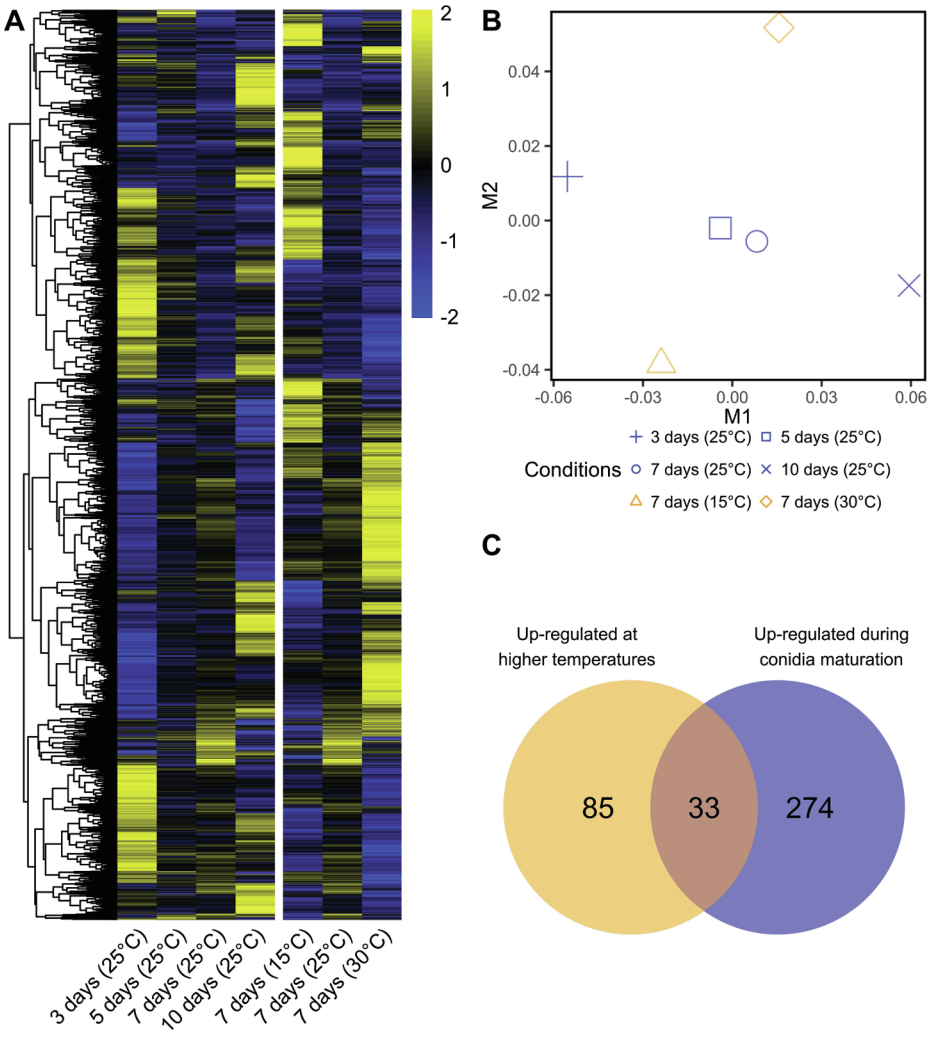

Fig. 5. (A) Heat map depicting hierarchically clustered expression data of 9723 genes expressed in conidia formed by 3, 5, 7 and 10-day-old 25 °C grown cultures and formed by 7-day-old cultures that had been grown at 15, 25 or 30 °C. Values represent mean RPKM values transformed into Z-scores. Yellow and blue shading represents higher and lower expression, respectively. (B) Multi-dimensional scaling plot of RNA-seq samples used in this study. (C) Venn-diagram with number of genes ≥4-fold differentially expressed in conidia from 10-day-old cultures compared to 3-day-old cultures (blue circle) or ≥4-fold differentially expressed genes in conidia from 7-day-old cultures grown at 30 °C compared to 15 °C (orange circle).

Remarkably, conidia formed at a temperature of 30 °C, showed a clear shoulder in the (concave) inactivation curve. This indicates that a longer heat- treatment results in an accelerated killing of conidia. An almost 4-fold increase in D_{56}-value was found when conidia of 7-day-old cultures had been formed at 30 °C (D_{56}-value 4.19 ± 0.11 min) when compared to 15 °C (D_{56}-value 1.12 ± 0.05 min) (Fig. 2B). Notably, no further increase in thermal resistance was found when conidia were tested of cultures grown at 30 °C for 10 days (D_{56}-value 3.97 ± 0.11 min) (Fig. 2B). This demonstrates that thermal resistance of the conidia was maximal after 7-days of cultivation at 30 °C. Together, these results show that D_{56}-values of *P. roqueforti* conidia differ 4-fold within the tested cultivation time and temperature range.

3.2. Conidia size and compatible solute content

The average diameter of the conidia and its size distribution were determined with a Coulter counter using a population of ≥ 1000 conidia (Table 1). The average diameter of the conidia that had been produced at 15, 25 and 30 °C by 7-day-old cultures was 3.97 ± 0.03 μm, 3.94 ± 0.02 μm and 4.14 ± 0.02 μm, respectively (Fig. 3). These data show a significant difference in cell size with conidia formed at 30 °C being on average 12–14% bigger compared to those formed at lower temperatures.

The compatible solute content of conidia was determined with HPLC. Mean conidial volume was determined with a Coulter counter (Table 1) and used to estimate the intracellular concentration of trehalose, glycerol, mannitol, arabitol and erythritol (Fig. 4A–E). Combined accumulation of the measured compatible solutes was fastest in the case of conidia that had been formed at 25 °C, reaching 1.36 M after 3 days. In contrast, conidia of cultures grown at 30 °C or 15 °C reached > 1.2 M total polyol content after 5 and 7 days, respectively (Fig. 4F). The highest total compatible solute levels were observed in conidia of 5-day-old cultures grown at 25 °C. This was mainly due to the glycerol and arabitol levels.

Trehalose concentration increased significantly with increasing culture age at all incubation temperatures. The highest trehalose concentration (0.102 M) was found in conidia of 10-day-old cultures that had been grown at 30 °C. Similar maximal levels of erythritol were found at 30 °C but in this case they were reached at day 3 and had declined at day 10. Glycerol, arabitol and mannitol were the most abundant compatible solutes. Glycerol reached the highest levels in conidia of 3-day-old cultures when grown at 25 and 30 °C and in conidia of 7-day-old cultures that had been grown at 15 °C. In fact, the highest concentration of glycerol (0.63 M) was observed in the latter condition. Arabitol levels were generally highest at day 7 and 10 and reached maximum levels of 0.62 M in conidia of 10-day-old cultures that had been grown at 25 °C. Heat resistance correlated with arabitol (R^2 = 0.57; p-value = 0.051) and trehalose (R^2 = 0.48; p-value = 0.084) levels, while a negative correlation (R^2 = 0.88; p-value = 0.0017) was observed in the case of glycerol (Fig. 4G-I). No strong correlation (p-value > 0.1) was found between heat-resistance and erythritol, mannitol or total compatible solute content (Figure S1).

Table 2
Genes that were at least 4-fold up-regulated both in conidia formed at higher cultivation temperature (30 °C versus 15 °C) and cultivation time (3- versus 10-days of culturing). Each gene had RPKM levels > 50 in one or more conditions.

Gene ID	Temperature: 25 °C				Culture age: 7 days		Ratio		Functional annotation	Gene name
	3 days	5 days	7 days	10 days	15 °C	30 °C	10d : 3d	30 °C : 15 °C		
2891	0.9	49.4	200.9	715.5	0.8	362.7	775.0	443.4	Conidiation-specific protein	con-10
3236	19.7	146.4	415.9	1270.0	17.8	149.2	64.4	8.4	Aegerolysin	
8873	4.0	29.0	66.5	173.7	17.2	82.6	43.3	4.8	Conidiation-specific protein	con-6
1335	37.8	241.0	510.3	1333.8	30.1	357.1	35.3	11.9	hypothetical protein	
8393	5.9	49.2	72.0	189.1	13.7	82.5	32.1	6.0	hypothetical protein	
2892	2.4	11.6	30.5	59.5	4.5	27.2	25.1	6.1	Hemerythrin HHE cation binding domain	
4038	4.3	18.4	45.9	104.4	8.8	43.5	24.1	5.0	hypothetical protein	
3817	101.5	763.9	888.5	2296.0	61.8	1089.1	22.6	17.6	hypothetical protein	
8946	17.1	104.0	158.2	361.0	25.8	117.1	21.1	4.5	Arylsulfotransferase (ASST)	
263	303.0	1482.7	2633.4	6381.2	666.9	2781.3	21.1	4.2	hypothetical protein	
8700	7.1	38.3	57.8	144.5	7.5	65.0	20.4	8.6	hypothetical protein	
3247	20.0	52.6	112.5	393.5	17.9	79.8	19.7	4.5	Zinc-regulated transporter	
8125	4.6	23.3	47.3	90.3	0.8	51.9	19.5	63.2	hypothetical protein	
3238	54.2	227.2	298.4	961.9	58.6	241.1	17.8	4.1	hypothetical protein	
9274	8.0	33.2	61.9	139.2	9.5	69.8	17.5	7.3	hypothetical protein	
3532	3.5	17.2	21.1	54.2	9.7	73.1	15.7	7.5	Ankyrin repeat-containing domain	
1797	29.9	104.7	180.4	393.1	30.4	151.5	13.2	5.0	hypothetical protein	
71	44.4	161.5	226.0	579.3	39.4	352.4	13.1	8.9	hypothetical protein	
3818	9.1	37.1	46.4	117.8	6.5	113.5	12.9	17.6	Fungal Zn(2)-Cys(6) binuclear cluster domain	
3096	6.3	21.8	39.4	76.5	7.1	46.0	12.2	6.5	Aflatoxin biosynthesis regulatory protein	aflR
5339	10.9	38.7	52.7	100.2	6.7	59.9	9.2	8.9	hypothetical protein	
4209	17.8	38.7	70.5	145.2	14.8	59.9	8.1	4.1	hypothetical protein	
2064	1575.6	4650.9	5570.8	11916.9	890.5	7382.7	7.6	8.3	hypothetical protein	
6013	11.5	36.1	38.3	81.9	13.9	62.5	7.1	4.5	Pyridoxal phosphate-dependent decarboxylase	
4965	36.4	105.3	139.5	232.0	67.5	276.7	6.4	4.1	Beta-1,3-glucanase	
5161	15.3	42.2	54.5	97.5	19.3	89.5	6.4	4.6	Platelet-activating factor acetylhydrolase	
3533	22.8	75.2	69.3	142.4	48.4	206.7	6.3	4.3	hypothetical protein	
6552	2.7	3.4	6.4	15.0	0.2	58.2	5.6	258.5	hypothetical protein	
4451	33.7	64.9	84.4	181.5	17.6	93.0	5.4	5.3	Extradiol ring-cleavage dioxygenase	
6680	26.9	49.6	61.2	140.1	8.3	76.6	5.2	9.2	Glutathione S-transferase, N-terminal domain	
3246	39.6	63.7	82.1	194.7	21.1	87.8	4.9	4.2	Conidial pigment biosynthesis oxidase	Laccase-1
7290	27.1	47.7	56.1	109.6	13.1	95.5	4.0	7.3	hypothetical protein	
1023	13.6	27.1	32.1	55.0	4.9	72.0	4.0	14.6	Acetolactate synthase	

3.3. Transcriptome analysis

The genome of *P. roqueforti* strain LCP96 04,111 was sequenced as a reference for a genome-wide transcriptome analysis. Sequencing was performed with approximately 126x coverage. Genome assembly and gene prediction resulted in a 26.97 Mbp assembly and 9762 predicted genes (Table S2).

RNA was isolated from dormant *P. roqueforti* conidia harvested from cultures that had been grown for 7 days at 15, 25 and 30 °C and grown for 3, 5, 7 and 10 days at 25 °C. RNA-sequencing produced at least 18.5 million 75 bp reads per biological replicate of which > 98% mapped to the reference genome. Transcripts of 9723 genes were detected in the conidia that had been formed at these various conditions (Table S3). The expression profiles of all 9723 genes are visualized in a heat map (Fig. 5A), which shows that gene expression changed over time as well as at increasing temperatures. Overall, cultivation time and temperature have distinct effects on gene expression. This is further illustrated by a multi-dimensional scaling (MDS) plot of the expression data (Fig. 5B), which shows that samples from increasing cultivation times aligned along the M1 axis in order of increasing temperature. A similar trend was observed along the M2 axis for the samples differing in cultivation temperature.

Gene expression data was filtered to identify differentially expressed genes with similar expression profiles in time as well as increased cultivation temperature. First, genes were selected that were significantly differentially expressed ($\alpha < 0.05$) in any of the conditions (6047 genes). This selection was used to compare expression levels between conidia that had been formed by cultures that had been grown at different temperatures or cultivation times. Next, genes were selected with an expression value of > 50 RPKM in at least one condition (3247 genes, Table S3). Among these 3247 genes, 85 (Table S4) and 274 genes (Table S5) were at least 4-fold up-regulated in conidia from 7-day old cultures produced at 30 °C compared to 15 °C and from 3-day-old cultures compared to 10-day-old cultures produced at 25 °C, respectively. Only 33 genes were shared in these two gene sets (Fig. 5C). These genes included 17 unknown genes, the hydrophilin genes *con-6* and *con-10*, the conidial pigmentation biosynthesis oxidase gene *laccase-1* and the predicted aflatoxin biosynthesis regulatory gene *aflR* (Table 2) (Note that it is unlikely that aflatoxin is produced in *P. roqueforti;* Fontaine et al., 2015).

In an all-versus-all comparison, the largest number of differentially expressed genes was found when comparing conidia from 3-day-old and 10-day-old cultures grown at 25 °C (2062 up / 2051 down) (Table S6). The smallest number of differentially expressed genes (87 up / 55 down) was found when conidia from 5-day-old and 7-day-old 25 °C-cultures were compared (Table S6). The top 40 genes showing the highest RPKM ratio between conidia of 10-day-old and 3-day-old cultures grown at 25 °C were analyzed (Table 3). This set contained 20 genes that are also part of the 33 differentially expressed genes shared between the sets of ≥ 4-fold upregulated genes found at increased cultivation time and cultivation temperature. Notably, the hydrophilin genes were among the 4 genes with highest 10-day:3-day ratio. Next, the top 40 genes were analyzed that were at least 4-fold up-regulated between 15 °C and 30 °C of 7 day old-cultures (Table 4). In this selection, *con-10* was the gene with the highest 30 °C:15 °C RPKM ratio. In addition, this gene set contained two genes encoding heat-shock proteins, a gene encoding a GPI-anchored cell wall protein and a gene encoding a protein with a NLPC/P60 domain. 16 genes of this gene set were also part of the 33 differentially expressed genes that were shared between the sets of ≥4-fold upregulated genes found at increased

Table 3
Top 40 of genes with the highest RPKM ratios between 10- and 3-day-old cultures grown at 25 °C. Each gene had RPKM levels > 50 in one or more conditions. Grey shading indicates genes that are part of the 33 genes that are both 4-fold upregulated at increased cultivation time and increased cultivation temperature (see Table S5).

Cultivation temperature								
	25 °C				15 °C	30 °C	Ratio	
Gene ID	3 days	5 days	7 days	10 days	7 days	7 days	10d:3d	Functional annotation
2891	0.9	49.4	200.9	715.5	0.8	362.7	775.0	Conidiation-specific protein
3236	19.7	146.4	415.9	1270.0	17.8	149.2	64.4	Aegerolysin
3235	11.9	58.9	147.3	575.6	22.8	79.6	48.5	Multicopper oxidase
8873	4.0	29.0	66.5	173.7	17.2	82.6	43.3	Conidiation protein 6
1335	37.8	241.0	510.3	1333.8	30.1	357.1	35.3	hypothetical protein
8393	5.9	49.2	72.0	189.1	13.7	82.5	32.1	hypothetical protein
2892	2.4	11.6	30.5	59.5	4.5	27.2	25.1	Hemerythrin HHE cation binding domain
4038	4.3	18.4	45.9	104.4	8.8	43.5	24.1	hypothetical protein
3817	101.5	763.9	888.5	2296.0	61.8	1089.1	22.6	hypothetical protein
3259	3.2	19.1	26.4	69.8	7.6	21.9	22.2	Major Facilitator Superfamily
4035	802.8	3559.3	7076.0	17536.5	10914.6	4379.8	21.8	hypothetical protein
8946	17.1	104.0	158.2	361.0	25.8	117.1	21.1	Arylsulfotransferase (ASST)
263	303.0	1482.7	2633.4	6381.2	666.9	2781.3	21.1	hypothetical protein
3613	4.0	20.2	37.4	82.7	14.1	17.1	20.8	hypothetical protein
8700	7.1	38.3	57.8	144.5	7.5	65.0	20.4	hypothetical protein
3247	20.0	52.6	112.5	393.5	17.9	79.8	19.7	ZIP Zinc transporter
8125	4.6	23.3	47.3	90.3	0.8	51.9	19.5	hypothetical protein
4008	103.9	510.3	976.6	1895.6	361.5	549.5	18.2	CVNH domain
3238	54.2	227.2	298.4	961.9	58.6	241.1	17.8	hypothetical protein
9274	8.0	33.2	61.9	139.2	9.5	69.8	17.5	hypothetical protein
4036	143.5	818.2	1101.3	2501.4	499.1	1177.2	17.4	hypothetical protein
9209	78.1	436.1	634.4	1334.5	272.7	384.1	17.1	hypothetical protein
3532	3.5	17.2	21.1	54.2	9.7	73.1	15.7	Ankyrin repeat-containing domain
8439	6.1	26.5	60.3	90.1	11.3	28.0	14.7	Membrane dipeptidase (Peptidase family M19)
7080	77.8	354.7	461.7	1091.5	207.0	440.2	14.0	Phenazine biosynthesis-like protein
4034	21.2	66.8	125.4	296.8	507.6	121.4	14.0	hypothetical protein
4123	24.8	131.4	164.4	329.9	163.0	153.1	13.3	hypothetical protein
1797	29.9	104.7	180.4	393.1	30.4	151.5	13.2	hypothetical protein
71	44.4	161.5	226.0	579.3	39.4	352.4	13.1	hypothetical protein
85	42.7	171.1	272.1	553.9	100.2	183.6	13.0	non-haem dioxygenase in morphine synthesis N-terminal
3818	9.1	37.1	46.4	117.8	6.5	113.5	12.9	Fungal Zn(2)-Cys(6) binuclear cluster domain
4021	406.2	1841.9	3089.7	5166.2	600.9	2401.5	12.7	hypothetical protein
704	21.4	77.5	127.7	264.3	79.6	83.2	12.3	hypothetical protein
8036	24.1	105.3	149.7	294.8	60.5	125.8	12.2	hypothetical protein
3096	6.3	21.8	39.4	76.5	7.1	46.0	12.2	Fungal Zn(2)-Cys(6) binuclear cluster domain
3993	579.1	2228.8	3120.2	6939.2	861.9	3026.8	12.0	Membrane transport protein
7452	248.8	967.6	1341.9	2957.6	299.5	977.4	11.9	hypothetical protein
454	2019.0	7702.0	10383.1	23272.9	5435.4	9066.7	11.5	Steryl acetyl hydrolase
959	8.0	24.9	43.8	91.5	32.4	43.5	11.5	MULE transposase domain
6230	89.9	368.5	474.2	1023.4	200.9	341.6	11.4	hypothetical protein

cultivation time and cultivation temperature.

Expression profiles of genes involved in polyol biosynthesis and genes encoding heat-shock proteins and hydrophilins were assessed. To this end, orthologs were identified in *P. roqueforti* LCP96 04111 of genes characterized in *Aspergilli* (polyol biosynthesis and heat-shock proteins) and *Neurospora crassa* (hydrophilins) (Table S7). A heat map was generated from the Z-scores of the expression patterns of stress- and polyol-related genes (Fig. 6). Only genes that were significantly differentially expressed and showed > 50 RPKM in at least one condition were included. Trehalose-6-phosphate synthase (*tpsB* & *tpsC*) and α,α-trehalose glucohydrolase (*treA*) showed increased expression levels in older conidia compared to conidia derived from 3- or 5-day-old cultures. This coincided with an increased trehalose content in these conidia (Fig. 4A). Conversely, expression of *tpsC* and *tppB* decreased in conidia formed at 30 °C compared to conidia of the same age produced at 15 or 25 °C. Yet, trehalose content was not lower under these conditions. No clear correlation between the expression profiles of erythrose reductases (*err1*) and erythritol content was observed. Expression levels of the hydrophilins *lea*, *con-6* and *con-10* were higher in older cultures at 25 °C. At 25 °C and 30 °C, expression levels of *con-6* and *con-10* also were higher when compared to 15 °C, but this was not the case for *lea*.

In total 17 predicted heat-shock proteins (HSP's) were identified in the transcriptome, of which only two showed a > 4 fold change in expression levels between 10- and 3-day-old cultures grown at 25 °C or 7-day-old cultures grown at 30 °C or 15 °C (Table 4). Among this set of 17 HSPs, *hsp12* has been previously described to play a role in the protection against heat stress in *Aspergillus fischeri* (van Leeuwen et al., 2016) but the *P. roqueforti* homolog of *hsp12* did not show a significant change in expression.

In addition to changes in expression patterns, the 20 most highly expressed genes in each condition were analyzed, yielding 37 different genes of which the 4 most highly expressed genes were shared among all conditions (Table S8). Notably these included 2 glucose-repressible proteins (*grg1* & *grg2*), hydrophobins 1 (*hpb1*) and 2 (*hpb2*), an extracellular membrane protein (CFEM) and a 30 kDa heat shock protein (*hsp30*).

4. Discussion

P. roqueforti conidia that are present in air at a certain location and time will not all have matured for the same time on the conidiophores before they had been released and will not have been formed under the same environmental conditions. Here we showed that both incubation

Table 4
Top 40 of genes with the highest RPKM ratios between 7-day old cultures produced at 30 ˚C compared to 7-day cultures produced at 15 ˚C. Each gene had RPKM levels > 50 in one or more conditions. Grey shading indicates genes that are part of the 33 genes that are both 4-fold upregulated at increased cultivation time and increased cultivation temperature (see Table S4).

Cultivation temperature	25 ˚C				15 ˚C	30 ˚C	Ratio	
Gene ID	3 days	5 days	7 days	10 days	7 days	7 days	30C:15C	Functional annotation
2891	0.9	49.4	200.9	715.5	0.8	362.7	443.4	Conidiation-specific protein
6552	2.7	3.4	6.4	15.0	0.2	58.2	258.5	hypothetical protein
5882	245.8	88.4	19.8	20.8	6.7	1026.5	152.1	NlpC/P60 family
8119	96.7	45.4	11.2	5.4	1.2	102.8	82.3	Major Facilitator Superfamily
8125	4.6	23.3	47.3	90.3	0.8	51.9	63.2	hypothetical protein
3417	21.5	15.8	50.9	23.9	6.6	171.4	26.0	hypothetical protein
8792	2.8	1.9	2.3	3.0	4.8	111.8	23.2	Sugar (and other) transporter
8545	29.4	11.4	7.4	16.1	10.5	241.4	22.9	hypothetical protein
152	37.6	22.4	25.0	33.1	45.4	899.7	19.8	hypothetical protein
3817	101.5	763.9	888.5	2296.0	61.8	1089.1	17.6	hypothetical protein
5090	16.4	13.9	5.3	15.1	4.7	83.0	17.6	Putative peptidase family
3818	9.1	37.1	46.4	117.8	6.5	113.5	17.6	Fungal Zn(2)-Cys(6) binuclear cluster domain
8798	46.1	60.0	61.8	70.6	7.9	123.7	15.8	Hsp70 protein
2807	61.2	26.5	41.5	31.8	4.3	67.8	15.6	hypothetical protein
9099	46.4	43.8	42.0	37.1	12.8	196.8	15.4	HSP20-like domain found in ArsA
7966	48.1	67.1	51.9	72.8	112.9	1683.1	14.9	hypothetical protein
1023	13.6	27.1	32.1	55.0	4.9	72.0	14.6	Thiamine pyrophosphate enzyme
4065	31.3	50.1	73.4	95.1	8.0	104.3	13.0	hypothetical protein
8031	19.5	38.1	45.1	55.4	2.4	28.9	11.9	hypothetical protein
1335	37.8	241.0	510.3	1333.8	30.1	357.1	11.9	hypothetical protein
8064	306.6	258.8	243.2	426.2	80.0	939.8	11.7	hypothetical protein
770	8.3	19.9	20.5	31.0	10.7	117.6	10.9	Sugar (and other) transporter
1081	63.5	88.3	93.6	213.0	23.3	235.2	10.1	glycosyl-phosphatidyl-inositol-anchored membrane family
1351	14.1	8.0	7.5	8.0	6.3	61.9	9.8	hypothetical protein
6680	26.9	49.6	61.2	140.1	8.3	76.6	9.2	Glutathione S-transferase, N-terminal domain
6473	26.4	26.0	26.5	20.3	23.3	212.5	9.1	Tryptophan dimethylallyltransferase
71	44.4	161.5	226.0	579.3	39.4	352.4	8.9	hypothetical protein
5339	10.9	38.7	52.7	100.2	6.7	59.9	8.9	hypothetical protein
3287	34.5	37.9	50.6	32.7	28.2	246.1	8.7	hypothetical protein
8700	7.1	38.3	57.8	144.5	7.5	65.0	8.6	hypothetical protein
3236	19.7	146.4	415.9	1270.0	17.8	149.2	8.4	Aegerolysin
2064	1575.6	4650.9	5570.8	11916.9	890.5	7382.7	8.3	hypothetical protein
8469	33.3	31.1	33.9	69.9	12.4	102.4	8.3	RNA recognition motif
8882	15.9	18.2	27.2	10.9	9.8	80.1	8.1	O-methyltransferase domain
3532	3.5	17.2	21.1	54.2	9.7	73.1	7.5	Ankyrin repeats (3 copies)
7509	11.1	11.8	9.9	11.7	16.8	123.7	7.4	hypothetical protein
9274	8.0	33.2	61.9	139.2	9.5	69.8	7.3	hypothetical protein
7290	27.1	47.7	56.1	109.6	13.1	95.5	7.3	hypothetical protein
4272	11.6	14.4	13.3	20.4	7.6	53.7	7.1	hypothetical protein
4248	37.4	24.3	26.3	34.9	9.7	65.7	6.8	hypothetical protein

temperature and culture age affect heat resistance (D_{56}-values) of *P. roqueforti* conidia and that this correlates with compatible solute composition and RNA profiles. Our data imply that airborne *P. roqueforti* conidia are heterogeneous with respect to their maturation, and as a consequence, to their heat resistance and their composition. This is expected to be a more general phenomenon. For instance, survival of *A. fumigatus* conidia to a heat-shock increases when they are formed at a higher temperature (Hagiwara et al., 2017). In contrast to our findings with *P. roqueforti*, no increase in heat-resistance was found between conidia obtained from *A. fumigatus* cultures with different age (3–14 days). It should be noted that heat resistance is not only the result of the composition of conidia but also of the environmental conditions during stress exposure. For example, *P. roqueforti* conidia are less sensitive to heat treatment when they are exposed to a matrix with low water activity such as bread (Bröker, Spicher & Ahrens, 1987; Garcia, da Pia, Freire, Copetti, & Sant'Ana, 2019; Raynaud & Lelieveld, 1997).

P. roqueforti conidia cultivated at 30 ˚C showed a 12–14% increased volume compared to conidia cultivated at 15 ˚C and 25 ˚C. Conversely, Nguyen Van Long et al. (2017a) demonstrated that cultivation at 5 ˚C increases spore diameter by about 25% compared to *P. roqueforti* conidia produced at 20 ˚C and 27 ˚C. Combined with our results this suggest that conidia size increases under suboptimal conditions as both 30 ˚C and 5 ˚C are close to the growth boundaries of *P. roqueforti* (Kalai et al., 2017). Significant differences in conidia size distributions have been observed within a single *P. variotii* colony and it has been postulated that the larger conidia are more heat resistant (van den Brule et al., 2019). Maturation time also impacted the average size of the conidia. Size reduced when conidia matured at 15 ˚C and 25 ˚C, while it increased at a growth temperature of 30 ˚C. The former can be explained by evaporation of water from the spores, the explanation for increased spore size is not yet clear.

Trehalose has been associated with heat-resistance of fungal conidia (van den Brule et al., 2019; Hagiwara et al., 2017; Nguyen Van Long et al., 2017a; Ruijter et al., 2003; Sakamoto et al., 2009; Wyatt et al., 2015). Indeed, trehalose concentration increased in older conidia of *P. roqueforti* and in conidia that had been grown at higher temperature. Similar results have been found for *A. fischeri* ascospores and conidia of *A. niger, Beauveria bassiana, Cordyceps farinose* (formerly described as *Paecilomyces farinosus*) and *Metarhizium anisopliae* (Hallsworth & Magan, 1996; Teertstra et al., 2017; Wyatt et al., 2015). It should be noted that the conditions that yielded conidia with the highest trehalose concentration (10 days, 30 ˚C) did not result in the most heat-resistant conidia. This suggests that trehalose has other functions and, indeed, trehalose serves as a carbon storage molecule in fungi (Perfect, Tenor, Miao, & Brennan, 2017). Notably, *P. roqueforti* conidia produced at 27 ˚C contain more trehalose and have an extended germination time

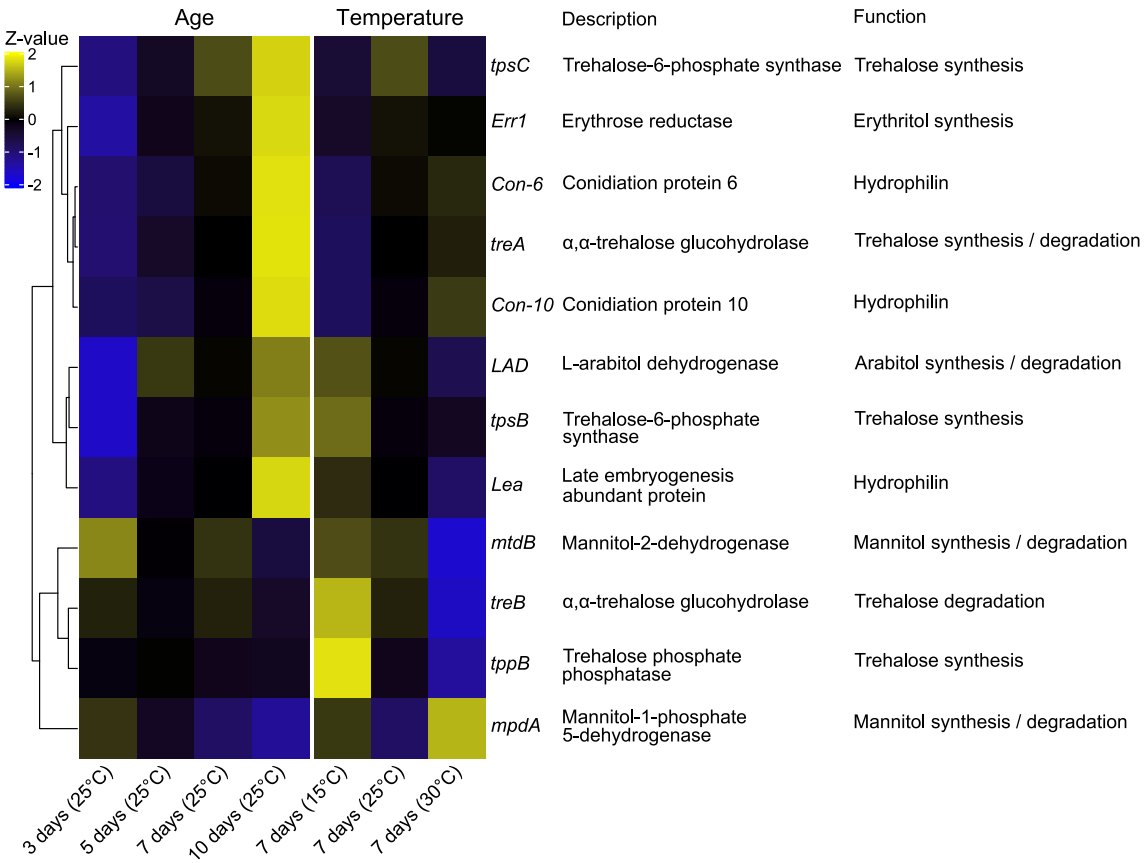

Fig. 6. Heat map depicting hierarchically clustered expression data of 19 *P.* roqueforti that are orthologous to genes related with compatible solute biosynthesis and heat-shock proteins in other fungi. Values represent mean RPKM values transformed into Z-scores. Profiles for conidia obtained from 3, 5, 7 and 10 day old cultures incubated at 25 °C and for 7 day old cultures produced at 15 °C, 25 °C or 30 °C are shown. Yellow and blue shading represent higher and lower expression, respectively.

compared to conidia produced at 5 °C (Nguyen Van Long et al., 2017a). Possibly, the high levels of trehalose under these conditions delay the germination time, as intracellular trehalose is shown to reduce germination speed in *Beauveria bassiana*, *Metarhizium anisopliae* and *Paecilomyces farinosus* (Hallsworth & Magan, 1995). Delaying the germination time may be a strategy to prevent premature germination; i.e. at the site where the spores are produced. The fact that conidia with the highest trehalose concentration are not the most heat resistant also implies that other factors, in particular arabitol, contribute to heat resistance of *P. roqueforti* conidia. No clear relation was found between heat resistance and mannitol or erythritol concentration, while a negative correlation was observed for heat-resistance and glycerol levels. The neutral effect of mannitol contrasts other studies that link concentration of this compatible solute with heat-resistance of fungal conidia (Dijksterhuis & de Vries, 2006; Wyatt et al., 2013). The effect of glycerol was in line with other studies. This compatible solute as well as erythritol has been related with desiccation resistance (Beever & Laracy, 1986; Hallsworth & Magan, 1995).

Gene expression profiles of spores were analyzed to identify genes that are potentially involved in heat resistance. Overall, cultivation time and temperature had distinct effects on gene expression. Notably, only 33 genes were ≥4-fold upregulated both in the most heat resistant spores resulting from different incubation temperature and from different incubation time. This set included 17 predicted proteins with unknown function as well as the hydrophilins con-6 and con-10. The hydrophilins have been implicated in heat resistance (see below) and are therefore expected to fulfil such a role in *P. roqueforti* conidia as well. The set of 274 genes that are > 4-fold higher expressed in conidia of 10-day-old cultures when compared to 3-day-old cultures included *tppA*, *tpsC* (involved in trehalose synthesis) and *err1* (erythritol synthase), whereas the 85 genes that are 4-fold higher expressed in conidia produced at 30 °C when compared to conidia of cultures grown at 15 °C included *mtdB* (mannitol-2-dehydrogenase). Thus, some of the genes involved in production of trehalose, erythritol, and mannitol are upregulated in conidia that are more heat resistant.

Hydrophilins have been linked to increased resistance of conidia against heat, desiccation and osmotic stress in *A. fischeri* (van Leeuwen et al., 2016). Absence of *con-6* and *con-10* promotes accumulation of the desiccation-related compatible solutes glycerol and erythritol in *Aspergillus nidulans* conidia (Suzuki, Sarikaya Bayram, Bayram, & Braus, 2013). Interestingly, we observe a similar effect; decreased glycerol and erythritol concentrations correlate with increased *con-6* and *con-10* expression levels.

Together, the unknown upregulated genes, the hydrophilins, trehalose, arabitol and increased spore size may contribute to heat resistance of *P. roqueforti* conidia. The roles of these molecules still need to be assessed in more detail. Apart from the fundamental point of view, our results also have applied implications. Industry should screen various conidiation conditions for their thermal inactivation protocols since optimal growth conditions might not result in the most stress-resistant spores.

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Author contributions

HABW and MP devised the study; experiments were performed by MP and TB; RNA-seq and genome sequencing was performed by MP and supervised by RAO; JD assisted with microscopy; HB verified analytical methods and WRT helped supervise the study. The manuscript was drafted by MP and revised by HABW and RAO. All authors read and approved the final version of the manuscript.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2020.109287.

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