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Maturation of conidia on conidiophores of *Aspergillus niger*

Wieke R. Teertstra [a,*], Martin Tegelaar [a], Jan Dijksterhuis [b], Elena A. Golovina [c], Robin A. Ohm [a], Han A.B. Wösten [a]

[a] Microbiology, Department of Biology, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands
[b] CBS-KNAW Fungal Biodiversity Centre, Uppsalalaan 8, Utrecht 3584CT, The Netherlands
[c] Laboratory of Biophysics, Wageningen University Research Center, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands

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ABSTRACT

Conidia of *Aspergillus niger* are produced on conidiophores. Here, maturation of conidia on these asexual reproductive structures was studied. Pigmented conidia that had developed on conidiophores for 2, 5, and 8 days were similarly resistant to heat and were metabolically active as shown by CO_2 release and conversion of the metabolic probe Tempone. A total number of 645–2421 genes showed a ⩾2-fold change in expression when 2-day-old conidia were compared to 5- and 8-day-old spores. Melanin was extracted more easily from the cell wall of 2-day-old conidia when compared to the older spores. In addition, mannitol content and germination rate of the 2-day-old conidia were higher. Dispersal efficiency by water was lower in the case of the 8-day-old conidia but no differences were observed in dispersal by wind and a hydrophobic moving object. These data and the fact that only a minor fraction of the conidia on a conidiophore were dispersed in the assays imply that a single colony of *A. niger* releases a heterogeneous population of conidia. This heterogeneity would provide a selective advantage in environments with rapidly changing conditions such as availability of water.
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1. Introduction

The genus *Aspergillus* comprises a diverse group of fungi (Galagan et al., 2005; Hawksworth, 2011) that are among the most abundant fungi on the globe. The success of aspergilli is explained by the fact that they are not very selective with respect to abiotic growth conditions (Krijgsheld et al., 2013). For instance, they grow over a wide range of temperatures (6–55 °C) and at relatively low humidity. Moreover, aspergilli can feed on a large variety of organic material (Bennett, 2010) and can also adopt a pathogenic life style (Krijgsheld et al., 2013). The enormous number of conidia that are released by *Aspergillus* colonies also contributes to the success of this genus. These asexual spores are among the most dominant fungal structures in the air (Bennett, 2010). More than 10 *A. fumigatus* spores m^{-3} were found in outdoor air (Mullins et al., 1984). This implies that humans inhale several hundred conidia of this *Aspergillus* species at a single day, and a multitude of spores when all *Aspergillus* conidia are taken into account.

Conidia are formed after a period of vegetative growth. To this end, specialized aerial hyphae differentiate into conidiophores (Adams et al., 1998). These stalks extend about 100–3000 μm into the air, after which a so-called vesicle is formed by swelling of the hyphal tip. Biseriate species like *A. nidulans* and *A. niger* form a layer of primary sterigmata termed metulae at the surface of the vesicle by budding. The metulae in turn bud twice resulting in a second layer of sterigmata. These phialides give rise to chains of uninucleate conidia. As a result, more than 10,000 conidia can be produced per conidiophore. In the case of uniseriate aspergilli (e.g. *A. fumigatus*) spore producing phialides are positioned directly at the surface of the conidiophore vesicle.

Conidiophores of *A. nidulans* and *A. niger* are formed 20 h post-inoculation when grown on complete medium and at 37 °C and 30 °C, respectively (Krijgsheld et al., 2013). Here it is shown that pigmented conidia (i.e. 2 days after the start of conidiation) mature on the conidiophores after completion of the spore chains. Maturation is accompanied by differences in RNA and compatible solute

Abbreviations: ESR, electron spin resonance; FEP, fluorinated ethylene propylene; FPKM, fragments per kilobase of exon model per million fragments; GO, gene ontology; HPLC, high-performance liquid chromatography; MM, minimal medium; PDT, perdeuterated 2,2,6,6-tetramethyl-4-piperidon N-oxide; SE, standard error; ST, saline tween; TM, transformation medium.

* Corresponding author.
E-mail addresses: w.r.teertstra@uu.nl (W.R. Teertstra), m.tegelaar@uu.nl (M. Tegelaar), j.dijksterhuis@cbs.knaw.nl (J. Dijksterhuis), elena.golovina@wur.nl (E.A. Golovina), r.a.ohm@uu.nl (R.A. Ohm), h.a.b.wosten@uu.nl (H.A.B. Wösten).

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composition, melanin extractability, water dispersal efficiency, and germination rate.

2. Material and methods

2.1. Strains and culture conditions

A. niger strain N402 (Bos et al., 1988) forms conidiophores that are about 10-fold shorter than those of a wild-type strain. The spore chain length of N402 and wild-type strains is similar with 10–20 conidia per chain (de Hoog et al., 2000; Wang et al., 2015). N402 was grown at 30 °C on 20 ml minimal medium (MM; de Vries et al., 2004) with 2% glucose and 1.5% agar. Conidia used for inoculation of cultures were harvested from 4-day-old colonies with 0.8% NaCl and 0.005% Tween-80 (ST). They were taken up at 2×10^8 ml^{-1} in 30% glycerol and 0.67% peptone and stored at −80 °C. Plates were inoculated by spreading 10^7 conidia over the agar surface. The 2-, 5-, and 8-day-old conidia of the resulting cultures were studied.

2.2. RNA isolation

RNA was isolated from conidia as described (van Leeuwen et al., 2013) using biological duplicates. Conidia were harvested in ST and centrifuged at 4 °C for 5 min at 1700*g*. Pellets were frozen in liquid N_2 in 500 µl ST and homogenized with a TissueLyser II in a stainless steel grinding jar that was cooled with liquid N_2 (Qiagen, www.qiagen.com). After 2 min at 30 strokes s^{-1}, 1 ml RTL buffer (Qiagen RNeasy® Kit) was added and homogenization was continued for another 2 min with liquid N_2 cooling. The material was transferred to 14 ml RTL buffer and 150 µl β-mercaptoethanol. RNA was isolated using a combination of the maxi RNeasy kit (Qiagen RNeasy; www.qiagen.com), the Master Pure Yeast RNA purification kit (Epicentre, www.epibio.com) and the mini RNeasy kit (Qiagen RNeasy; www.qiagen.com). Concentration and quality of RNA was assessed using a Nanodrop spectrophotometer (NanoDrop Tech; www.nanodrop.com) and 1% TBE gel electrophoresis, respectively.

2.3. RNA sequencing

RNA sequencing was performed at BaseClear (www.baseclear.com) using an Illumina HiSeq2500 system. Quality of total RNA was checked on a Bioanalyzer using a RIN value ⩾8.0. Strand-specific mRNA libraries were prepared (Parkhomchuk et al., 2009; Levin et al., 2010) by purifying mRNA from total RNA with oligo-dT beads and conversion to cDNA following fragmentation. DNA adapters including sample-specific barcodes were ligated and a PCR amplification was performed. The library was size-selected using magnetic beads, resulting in libraries with an insert size of 100–400 bp. Quality of the libraries was checked on a Bioanalyzer. Single-end sequence reads were generated using the Illumina Sequence system. The RNA-Seq pipeline used the TRIMMOMATIC read trimmer version 0.32 (Bolger et al., 2014) to remove low quality regions and the Illumina adapters from the reads. RNA sequencing data have been deposited at NCBI under accession GSE83819.

2.4. RNA sequence analysis

STAR (Dobin et al., 2013) was used to align sequence reads to the *A. niger* ATCC 1015 genome (version Aspni7; Andersen et al., 2011), which was obtained from Mycocosm (Grigoriev et al., 2014). The program Cuffdiff (version 2.2.1), which is part of Cufflinks (Trapnell et al., 2010), was used to identify reads mapping to predicted genes and to identify differentially expressed genes. The expression level of each predicted gene was normalized to fragments per kilobase of exon model per million fragments (FPKM). For differential expression a ⩾2-fold change was used and a minimal expression level of 4 FPKM in at least one of the samples. The bias correction method was used while running Cuffdiff (Roberts et al., 2011). The quality of the latter results were analyzed using CummeRbund (Goff et al., 2013) and Integrative Genome Viewer (Robinson et al., 2011). The correlation coefficients of the replicates were ⩾0.98.

Conserved protein domains were predicted using PFAM version 27 (Finn et al., 2014) and mapped to the corresponding gene ontology (GO) terms (Ashburner et al., 2000; Hunter et al., 2009). Metabolic genes were predicted using KEGG on the KAAS server (Moriya et al., 2007), while proteases were predicted using the MEROPS database (Rawlings et al., 2014) with a blastp E-value cutoff of 10^{-5}. Secretion signals and transmembrane domains were predicted using SignalP 4.1 (Petersen et al., 2011) and TMHMM 2.0c (Krogh et al., 2001), respectively. Proteins with a secretion signal, no transmembrane domain from amino acid 41 onwards and a total length <300 amino acids were considered small secreted proteins. Genes and gene clusters involved in secondary metabolism were predicted using a pipeline based on SMURF (Khaldi et al., 2010). SMURF parameters *d* (maximum intergenic distance in base pairs) and *y* (the maximum number of non-secondary metabolism genes upstream or downstream of the backbone gene) were set at 3000 bp and 6, respectively.

Custom scripts were developed in Python and R to analyze over- and under-representation of functional annotation terms in sets of differentially regulated genes using the Fisher Exact test. The Benjamini-Hochberg correction was used to correct for multiple testing using a p-value < 0.05.

2.5. Quantification of compatible solutes

Conidia were harvested in ST, passed over Miracloth (Millipore, www.merckmillipore.com), washed twice with ST, centrifuged at 4 °C for 5 min at 1700*g*, and frozen in liquid N_2. They were homogenized with a TissueLyser II in a stainless steel grinding jar that was cooled with liquid N_2. After 2 min at 30 strokes s^{-1}, 1 ml water was added and homogenization was continued for another 2 min at 30 strokes s^{-1} with intermittent cooling using liquid N_2. Remnants of conidia were removed by centrifugation at 4 °C for 30 min at 10,000*g*. The supernatant was heated for 30 min at 95 °C, centrifuged at 4 °C for 30 min at 10,000*g*, and passed over a 0.2 µm PTFE CR13 mm Acrodisk filter (Pall Life Science, www.pall.com). Compatible solutes (mono- and oligosaccharides and polyols) were quantified using a Waters high-performance liquid chromatography (HPLC) system (www.waters.com) equipped with a Waters Sugar-Pak I cation-exchange column (Wyatt et al., 2014). Trehalose, glucose, erytritol, glycerol, mannitol, and arabitol (0.01–0.50% w/v) were used as reference (Wyatt et al., 2015). A two-way ANOVA with post hoc tests with a Bonferroni correction was used for statistical analysis of data obtained with biological duplicates ($p < 0.05$).

2.6. Electron spin resonance for determination of conidia metabolic activity

Metabolic activity of conidia was determined by spin probe electron spin resonance (ESR) spectroscopy. Cells can reversibly reduce most spin probes to non-paramagnetic ESR silent hydroxylamines, resulting in a decrease of ESR signal intensity. Bioreduction of nitroxides depends to a large extent on the activity of the electron transport chain of mitochondria (Kocherginsky and Swartz, 1995). Spin probes can be also reduced by ascorbic

acid, sulfhydryl groups of proteins and other reducing metabolites. Perdeuterated Tempone (2,2,6,6-tetramethyl-4-piperidon N-oxide) or PDT was used as the spin probe to characterize metabolic activity of conidia. Conidia (1.5×10^8) were washed twice with H_2O, each step followed by centrifugation at 4 °C for 5 min at 1700*g*, taken up in 25 µl ice cold H_2O, and mixed with 25 µl 2 mM PDT (Sigma-Aldrich, www.sigmaaldrich.com). The conidia were tightly packed in a glass capillary (surface area 1 mm^2) by centrifugation at 2000*g*. The supernatant was removed and measurements were started 45 min after addition of PDT. At this time the equilibrium distribution of PDT across the sample was reached. Twenty ESR spectra, with a 1 min interval were recorded at 30 °C with a ELEXSYS E500 CW-EPR X-band spectrometer (Bruker, Germany, www.bruker.com) using a modulation amplitude of 0.5 Gauss and 1 mW power. Under these conditions, spectra were not overmodulated and the signal was not saturated. Spectra were recorded within the range of 100 gauss (1024 points) with the central field around 3324 gauss. Conversion time and time constant were 40.96 ms and 10.24 ms respectively.

2.7. CO_2 release assay for metabolic activity

Conidia (10^{10}) were taken up in a final volume of 600 µl H_2O and transferred to a well of a 24 well suspension culture plate (Cellstar, Greiner Bio-One, www.greinerbioone.com). The well was closed with a CO_2 MG811 sensor (DFRobot; www.dfrobot.-com) that was connected to an Arduino UNO (ArduinoBoardUNO; www.arduino.cc). The well and the sensor were sealed with PTFE tape and Poly Max glue (Bison. www.bison.net), respectively, to prevent CO_2 exchange.

2.8. Melanin isolation

Melanin was isolated from conidia as described (Wargenau et al., 2011). Conidia were harvested in ST (10^8 conidia ml^{-1}), centrifuged at 4 °C for 5 min at 1700*g*, and washed with 500 µl H_2O. The conidia were incubated in 1 ml 1 M NaOH at 98 °C and pelleted for 10 min at 17,000*g*. Melanin in the supernatants resulting from spore harvesting and washing and from NaOH extraction was precipitated for 30 min at pH 1 by addition of 12 N HCl, after which it was pelleted at 17,000*g* for 10 min. It was washed once with 500 µl H_2O and dissolved in 500 µl 0.05 M NaOH. The pH of the melanin solution was set at 7.5 using 1 M HCl, after which the absorption spectrum of melanin was analyzed at 360–625 nm using a Unicam UV1 spectrophotometer (www.spectronic-camspec.co.uk). Absorption at OD_{450} was used to quantify melanin in biological duplicates.

2.9. Spore dispersal assays

Pieces of 2.5 × 7.5 cm were cut from agar cultures and placed on an object glass. To assay air flow dispersal, the object glass was transferred to a 50 ml Greiner tube, resting on the conical part of the tube. The lower 1 cm of the Greiner tube had been removed by cutting. The tube was closed with a lid into which a 3 cm by 1 mm slit had been made. In a second parallel slit (0.25 mm × 2.5 cm) a piece of Teflon (FEP; 0.25 mm thick, Permanento, www.permanento.com) was inserted and sealed with gum. The Teflon served to direct the air flow over the conidia containing slab. The tube was placed on a slit air sampler, slit size 3 cm × 1 mm, connected to a VacTorr 25 vacuum pump with a capacity of 25 l air min^{-1} (www.torrtech.com). Slits of the sampler and tube lid were positioned parallel to create a jet stream of air. The sampler was run for 20 min and conidia were collected in a Petri dish containing 14 ml ST. Conidia were pelleted by centrifugation for 30 min at 10,000*g*. The pellet was taken up in 100 µl ST and conidia were counted using a haemocytometer.

To assess dispersal by water droplets, object glasses with 2.5 × 7.5 cm agar slabs were transferred to 50 ml Greiner tubes. These tubes were placed at a 20° angle and the top of the slide aligned with the top of the tube. Ten droplets of 15 µl H_2O were allowed to run over the length of the slide and were collected in the conical part of the tube. The track covered by 1 droplet was calculated to be 230 mm^2. After removing the object glass, the droplets coated with conidia were taken up in 850 µl ST and counted with a haemocytometer.

For surface attachment, siliconized cover slips of 22 × 22 mm were used (Hampton Research, www.hamptonresearch.com) that had a water contact angle of 89.7° as determined with a Krüss Dropshape Analysis system DSA10Mk2 (www.kruss.de). The coverslips were placed on top of sporulating *A. niger* cultures, pressed gently to remove air bubbles, and transferred to a well (diameter 35 mm) filled with 2 ml ST. The side of the slip with the attached conidia was facing the liquid. Slides were shaken at 30 rpm for 30 min to remove weakly adsorbed conidia. Strongly adhering conidia were taken up in ST by rubbing with an inoculation spreader and counted using a haemocytometer.

A two-way ANOVA with post hoc tests with a Bonferroni correction was used for statistical analysis of biological sextuplicates of the dispersal assays ($p < 0.05$).

2.10. Germination analysis

Conidia were harvested in ST, passed over Miracloth, and washed twice with H_2O with intermittent centrifugation at 4 °C for 5 min at 1700*g*. Aliquots of 10^7 conidia ml^{-1} H_2O were exposed to 55 °C for 5 min. Conidia were diluted to 5×10^4 conidia ml^{-1} in 750 µl transformation medium (TM; Kusters-van Someren et al., 1991) with 25 mM maltose and seeded in a well of a 24 wells suspension culture plate (Greiner bio-one, Cellstar 662102, www.gbo.com). Germination of conidia was monitored on line at 30 °C using the oCelloScope (Philips Biocell, www.philips.com/biocell) (Fredborg et al., 2013) with UniExplorer software version 6.0.0.5419, and the segmentation plugin 6.0.0.811. Measurements were started after 2 h incubation, enabling settling of the conidia at the bottom of the well. Objects were scanned every hour. The scan area length was set at 2205 µm, the scan area (min-max) at 70–700 pixels, and the maximum number of objects at 1000. Individual objects were tracked over time by comparing successive measurements using oCelloScope XY coordinates and a custom Python script. Scanning was stopped at the moment microcolonies started to overgrow dormant or germinating conidia or other micro-colonies. Conidial aggregates and non-conidial objects were distinguished based on size and circularity and removed from the data set of the first scan. Remaining objects were screened visually to represent single conidia. Object area and circularity of the selected conidia was followed in time. The objects were classified into dormant conidia, swollen conidia, germinating conidia, and hyphae based on surface area and circularity. Heat-killed (15 min, 65 °C) spores in TM were used to measure the dimensions of dormant spores. These dimensions (21–42 μm^2) agreed with non-killed spores that had settled for 2 h. Swelling is a gradual process and is characterized by increase in circularity and diameter (from 4.1 µm in the case of dormant conidia to 8.1 µm in the case of swollen conidia; resulting in a surface area of 42–76 μm^2). Circularity drops below 1.0 at the moment of germination and this starts at a surface area $\geqslant$76 μm^2. Hyphal growth objects were defined as objects with a surface area $\geqslant$106 μm^2. These objects have a hyphal tube length $\geqslant$1.5 times longer than the width of the hypha. χ^2-tests with Bonferroni corrected post hoc tests were carried out to assess whether differences in progression of

germination were statistically significant with $p < 0.05$. To determine differences in swelling speed between different spore ages and heat-stressed spores, ANCOVA and repeated measures ANOVA were carried out.

3. Results

MM plates were inoculated with a confluent layer of *A. niger* conidia resulting in a synchronized mycelium covering the agar surface. Conidiophores were formed between day 1 and 2 and pigmentation of conidia had completed after 3 days. Approximately 1.5×10^9 conidia were isolated from 4-day- (2-day-old conidia), 7-day- (5-day-old conidia), and 10-day-old cultures (8-day-old conidia) per plate of 5500 mm^2 (Table 1, labelled as control). Since there is no significant change in the number of conidia between the different days of harvesting we conclude that indeed no additional conidia are formed after 4 days of cultivation.

3.1. Changes in RNA profiles of conidia

RNA was sequenced from 2-, 5- and 8-day-old conidia. Expression data were confirmed with genes known to be constitutively expressed, genes known to be sporulation specific, or genes specifically expressed in the vegetative mycelium (Supplemental Table 1). To this end, ratios of expression in the conidia and liquid shaken cultures were calculated. Sporulation specific genes showed ratios >3.3 < 189, constitutive genes >0.2 < 2.3, and vegetative mycelium specific genes <0.13.

RNA levels of 572 and 785 genes were ⩾2-fold increased or decreased, respectively, when profiles of 5-day-old and 2-day-old conidia were compared (Table 2; Supplemental Table 2). These numbers were 1175 and 1246 when 8-day-old conidia were compared with 2-day-old spores, while 399 and 246 genes showed ⩾2-fold increased and decreased levels when 8-day and 5-day-old conidia were compared. Between 33 and 39% of the up-regulated genes had no predicted function according to PFAM or KEGG annotation, while this was only 11–18% of the down regulated genes (Table 2). The genes with no predicted function, which represent 26% of the total number of *A. niger* genes, were also abundant in the top 25 genes with the highest fold changes, with 44–60% and 8–36% of the up- and down-regulated genes, respectively (Supplemental Table 2).

A number of 26 and 27 GO terms were over-represented in the up-regulated genes when 5- and 8-day-old conidia were compared with 2-day-old conidia, respectively (Supplemental Table 3). Notably, 16 and 20 of these functional gene classes, respectively, were related to regulation. No GO terms were over-represented in the up-regulated genes of 8-day-old conidia when compared to the 5-day-old spores. In the case of the down-regulated genes 64, 201, and 95 GO terms were enriched when 5- and 8-day-old conidia were compared with 2-day-old spores and when 5-day-old conidia were compared with 8-day-old conidia, respectively. Many of the GO terms were related to metabolism and biosynthetic processes (Supplemental Table 3).

Genes encoding protective proteins and hydrophobins and genes encoding proteins involved in compatible solute metabolism, DHN melanin synthesis, and pyomelanin synthesis and tyrosine degradation were analyzed in more detail (Table 3). Transcript levels of compatible solute metabolism had generally decreased when 8-day-old conidia were compared to the 2-day-old conidia. Transcript abundance of the trehalose synthesis gene *tpsC* and the mannitol-1-phosphate 5-dehydrogenase gene *mpdA* had dropped ⩾2-fold in 8-day-old conidia. Eight out of 15 genes encoding protective proteins were differentially expressed, of which 1 was ⩾2-fold upregulated (*hsp30*) and 3 were ⩾2-fold down-regulated (*lea-like*, *dehydrin-like*, and *hsp9*). The latter 3 genes belonged to the top 50 of most highly expressed genes at all time points. Transcripts of genes involved in DHN melanin formation had disappeared in the pigmented 2-, 5-, and 8-day-old conidia with the exception of *pptA*. In contrast genes involved in pyomelanin formation and tyrosin formation were expressed. Five out of six genes were differentially expressed at day 8 compared to day 2 with a 3.2-fold increase in the case of the *hmgA* gene that is involved in tyrosin degradation. Transcripts of 7 out of 8 hydrophobin genes were found in the pigmented conidia, 4 of them showed a huge drop in mRNA levels at day 8, while the *hypH* hydrophobin gene showed a 4-fold increase at this time point.

3.2. Changes in compatible solutes in conidia

The amount of glycerol, trehalose, and mannitol was determined in 2-, 5- and 8-day-old conidia (Fig. 1). Glycerol was only detected in young conidia at an amount of 0.04 pg $spore^{-1}$. Mannitol was the most abundant compatible solute. The decrease in the amount of mannitol from 7.7 to 5.6 pg $spore^{-1}$ in 2- and 5-day-old spores was significant. No significant differences in trehalose levels could be detected between the different spore ages although there was a trend that conidia that had matured longer on the conidiophore had increased levels of this compatible solute.

Table 1
Release of conidia from the conidiophore. Number of conidia (±2 × SE) that were dislodged from confluent inoculated MM glucose plates by 20 min of wind (~6 Beaufort), 15 µl water droplets, or by attachment to siliconized hydrophobic glass. Rubbing spores from the surface with ST (normal harvesting procedure) served as a control. Numbers represent an average of 6 biological replica's.

	Dislodged conidia mm^{-2}			
	Wind	Rain	Hydrophobic surface	Control
2-day-old conidia	$5.5 \times 10^1 \pm 4.5 \times 10^1$	$5.0 \times 10^2 \pm 4.1 \times 10^2$	$3.3 \times 10^4 \pm 3.2 \times 10^3$	$2.7 \times 10^5 \pm 7.7 \times 10^4$
5-day-old conidia	$3.6 \times 10^1 \pm 3.0 \times 10^1$	$4.8 \times 10^2 \pm 7.3 \times 10^2$	$3.5 \times 10^4 \pm 4.5 \times 10^3$	$3.0 \times 10^5 \pm 3.2 \times 10^4$
8-day-old conidia	$5.4 \times 10^1 \pm 4.4 \times 10^1$	$2.6 \times 10^2 \pm 4.4 \times 10^2$	$3.4 \times 10^4 \pm 1.0 \times 10^4$	$2.4 \times 10^5 \pm 8.4 \times 10^4$

Table 2
Total number of genes and number of genes with no predicted function (i.e. without PFAM or KEGG annotation) that were ⩾2-fold up- or down-regulated when comparing conidia that had matured on the conidiophore for 2, 5, and 8 days. Data are based on biological duplicates.

	Upregulated		Downregulated	
	Total	Unknown	Total	Unknown
5-day-old conidia vs 2-day-old conidia	572	190	785	144
8-day-old conidia vs 2-day-old conidia	1175	412	1246	188
8-day-old conidia vs 5-day-old conidia	399	154	246	29

Table 3
Expression of genes belonging to selected functional groups. ProteinID were retrieved from Aspni7 (JGI) based on the ATCC1015 strain of *A. niger* and identified by bidirectional hits. Shaded fold-change cells represent genes that are differentially expressed ($p \leqslant 0.05$), while bold font indicates genes that have a 2-fold difference in expression with a FPKM $\geqslant 4$ in at least one of the time points. Shaded FPKM cells represent RNA levels of the top 50 genes that are most highly expressed. Names of genes encoding protective proteins are according to van Leeuwen et al. (2013), names of genes involved in pyomelanin synthesis and tyrosine degradation are according to Heinekamp et al. (2013), names of hydrophobins are according to Pel et al. (2007), while all other names are according to the *A. niger* AspGD database. Data are based on biological duplicates.

ProteinID	Name or Description (AspGD)	FPKM 2 day	FPKM 5 day	FPKM 8 day	Fold change 8 day / 2 day
	Compatible solute metabolism				
1180293	tpsA	266.4	142.7	176.9	0.66
1105321	tpsB	31.6	13.6	21.6	0.68
1080687	tpsC	155.3	61.5	66.1	**0.43**
1139489	tppA	169.8	110.7	89.7	0.53
1156210	tppB	401.6	204.4	276.7	0.69
1120960	tppC	31.8	17.3	41.0	1.29
1149855	treB	513.3	510.9	450.9	0.88
1144666	mpdA	142.3	45.1	59.3	**0.42**
1146052	mannitol 2-dehydrogenase	1054.9	993.1	686.5	0.65
1093505	glycerol kinase	512.0	425.5	441.9	086
1098376	glycerol-3-phosphate dehydrogenase	487.1	287.6	308.8	0.63
	Protective proteins				
1174991	LeA-like protein	10883.4	4370.3	5010.9	**0.46**
1161909	dehydrin	2452.8	1506.0	1310.2	0.53
1181320	dehydrin	7721.4	3269.7	3063.6	**0.40**
1189150	Hsp9	10757.3	3520.0	4553.8	**0.42**
1142755	Hsp104	700.1	437.0	367.6	0.53
1173423	Hsp30	28.8	18.3	71.8	**2.49**
1147735	Hsp30	89.5	52.4	102.3	1.14
1121080	Hsp70	1320.9	886.5	1164.1	0.88
1186352	Hsp42	74.8	74.4	45.4	0.61
1147735	Hsp30	89.5	52.4	102.3	1.14
1095276	Hsp10	689.9	363.5	412.0	0.60
1148082	Hsp70	304.7	342.6	315.6	1.04
1157207	GroEl	218.7	156.1	201.5	0.92
1145960	Hsp70	792.0	867.7	821.9	1.04
1113616	Hsp78	102.1	102.1	87.9	0.86
	Pyomelanin formation and tyrosine degradation				
1146950	hppD	95.2	91.9	67.5	0.71
1167569	hmgX	68.1	103.0	97.0	1.43
1096049	hmgA	26.1	86.7	82.9	**3.17**
1127224	fahA	7.2	4.7	4.0	0.56
1187486	maiA	6.8	4.7	5.8	0.86
1187485	hmgR	9.8	12.1	16.3	1.67
	DHN melanin				
1143106	pptA	20.2	29.8	21.2	1.05
1099425	fwnA	1.2	0.9	0.8	0.67
1156112	brnA	1.0	0.3	0.4	0.37
1202726	olvA	2.7	1.3	0.9	0.33
	Hydrophobins				
1182392	hypA	72.4	38.4	43.3	0.60
1146047	hypB	69.1	27.6	2.2	**0.03**
1186439	hypC	62.6	22.9	1.2	**0.02**
194815	hypD	123.8	43.2	46.5	**0.38**
1184024	hypE	178.8	18.2	18.6	**0.10**
1134034	hypF	2.3	2.2	1.9	0.80
1180858	hypG	17.4	10.0	5.7	0.33
1189071	hypH	45.1	197.7	181.5	**4.02**

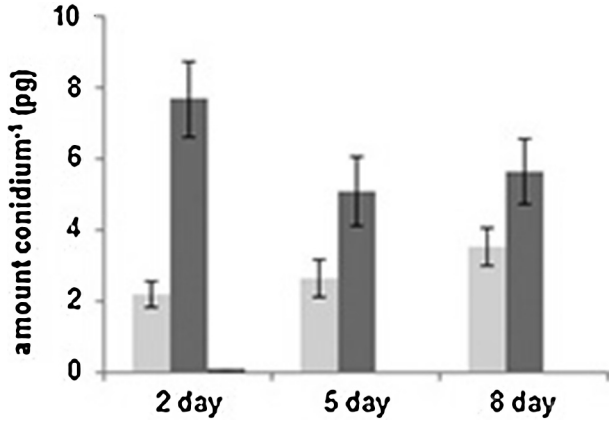

Fig. 1. Amount (pg conidium^{-1}) of trehalose (light grey shading), mannitol (dark grey shading), and glycerol (black shading) in 2-, 5-, and 8-day-old conidia of *A. niger*. Error bars represent standard deviation of technical and biological duplicates.

3.3. Changes in extractability of melanin

Harvesting of conidia resulted in release of part of the melanin in the harvesting liquid. The amount of released melanin was 1.8-fold higher in 2-day-old conidia when compared to 5- and 8-day-old conidia (Fig. 2A). A subsequent extraction with NaOH also released more melanin from the 2-day-old spores. At the same time, the amount of non-extractable melanin was higher in the case of 5- and 8-day-old conidia as shown by the intensity of the black color of the spore pellets (Fig. 2B).

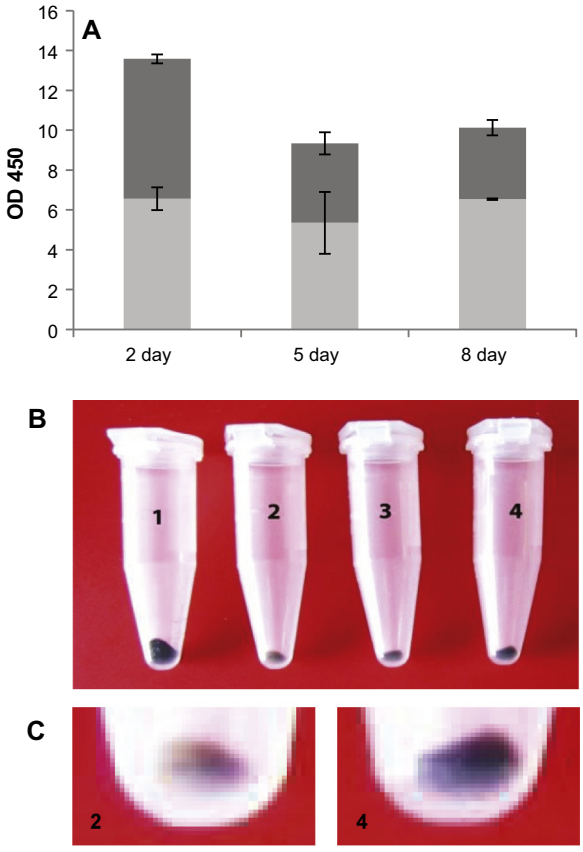

Fig. 2. Amount of melanin of 2-, 5-, and 8-day-old conidia of *A. niger* extracted with saline Tween (dark grey shading) and NaOH (light grey shading) (A) and pigmentation of 2- (tube 2), 5- (tube 3), and 8-day-old (tube 4) conidia after extraction with water and NaOH, non-extracted 2-day-old conidia serving as a control (tube 1) (B). (C) shows magnification of the extracted spore pellets of tube 2 and 4. Contrast and brightness was increased identically. Error bars represent standard deviation of biological duplicates.

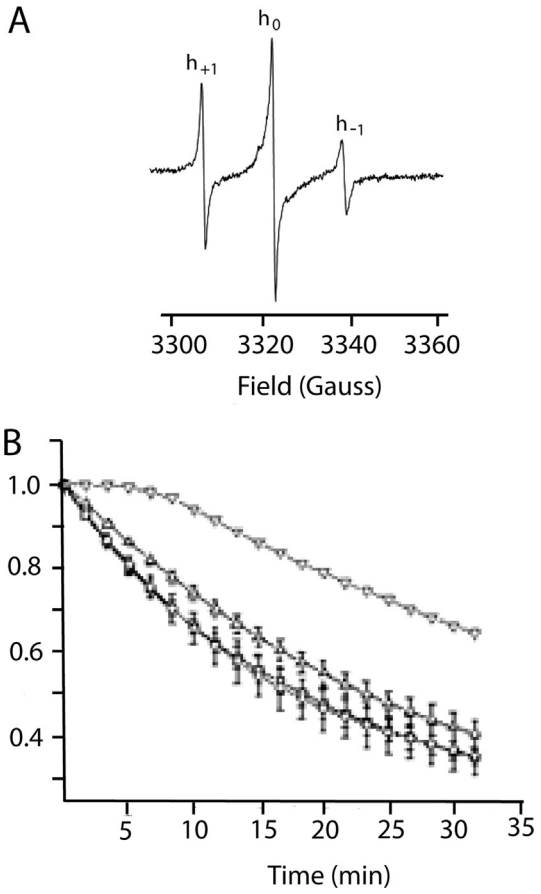

Fig. 3. Reduction of PDT indicative of metabolic activity in 2-, 5-, and 8-day-old conidia of *A. niger*. (A) ESR spectra of 1 mM PDT in the presence of conidia at t = 0 (upper profile) and t = 30 min (lower profile). The height of the high-field (right-hand) line (H_{-1}) was used to quantify PDT. (B) The decrease of the H_{-1} height in time of 2- (squares), 5- (circles), and 8- (triangles) day-old conidia. Heat-killed-2-day-old conidia were used as a control (triangles ▽). Error bars represent standard deviation of biological duplicates, r.u. are relative units.

3.4. Changes in metabolic activity of conidia

Incubation of 2-, 5-, and 8-day-old conidia with 1 mM PDT caused gradual decrease of the ESR signal intensity, indicative of metabolic activity (see Material and Methods). The ESR spectrum of PDT has three equidistant narrow lines (Fig. 3A), of which the height of the high-field (right-hand) line is used to quantify ESR signal intensity. The spin probe reduction after 30 min of incubation was 35% in heat-killed conidia, proposed to be caused by reducing agents that were still present in the cells. Reduction of ESR signal intensity was 64, 64, and 59% in the case of 2-, 5-, and 8-day-old conidia, respectively (Fig. 3B). These data indicate that all 3 spore types were metabolically active. This was confirmed by monitoring CO_2 release by conidia in an aqueous environment (Fig. 4). In all cases, a decrease of 5–10% mV (i.e. an increase in CO_2) was observed in a 120 min time frame.

3.5. Germination before or after heat stress

Germination of conidia of different age was followed in time with the oCelloScope enabling us to monitor germination of a

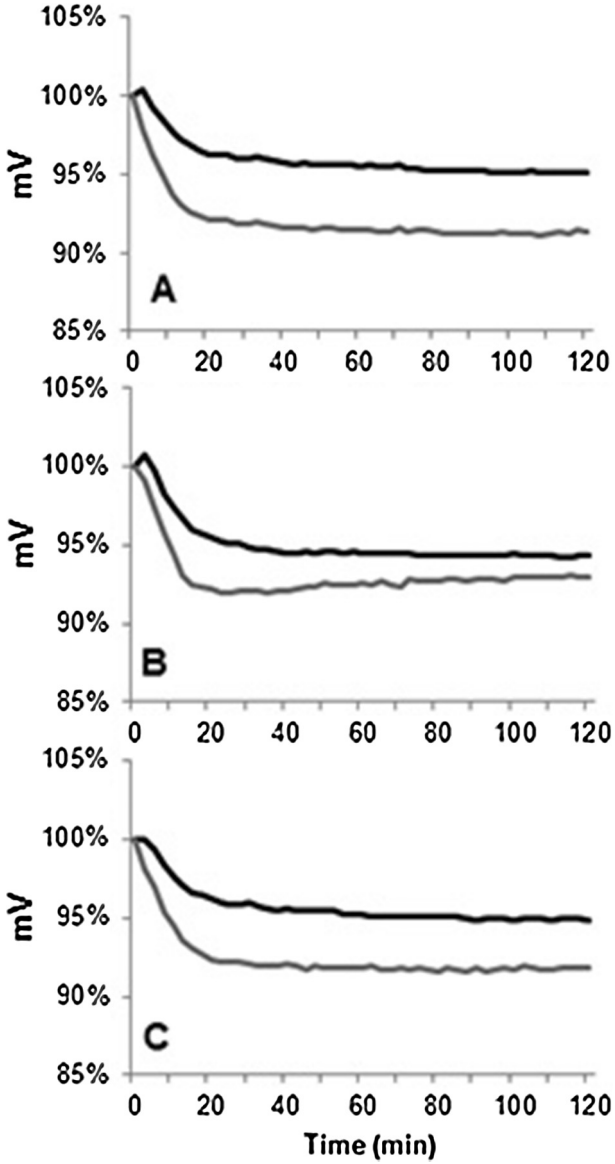

Fig. 4. Increase in CO_2 levels, indicative of metabolic activity, in the case of 2- (A), 5- (B), and 8- (C) day-old conidia of biological duplicates as monitored by the reduction in voltage (mV) of CO_2 sensors.

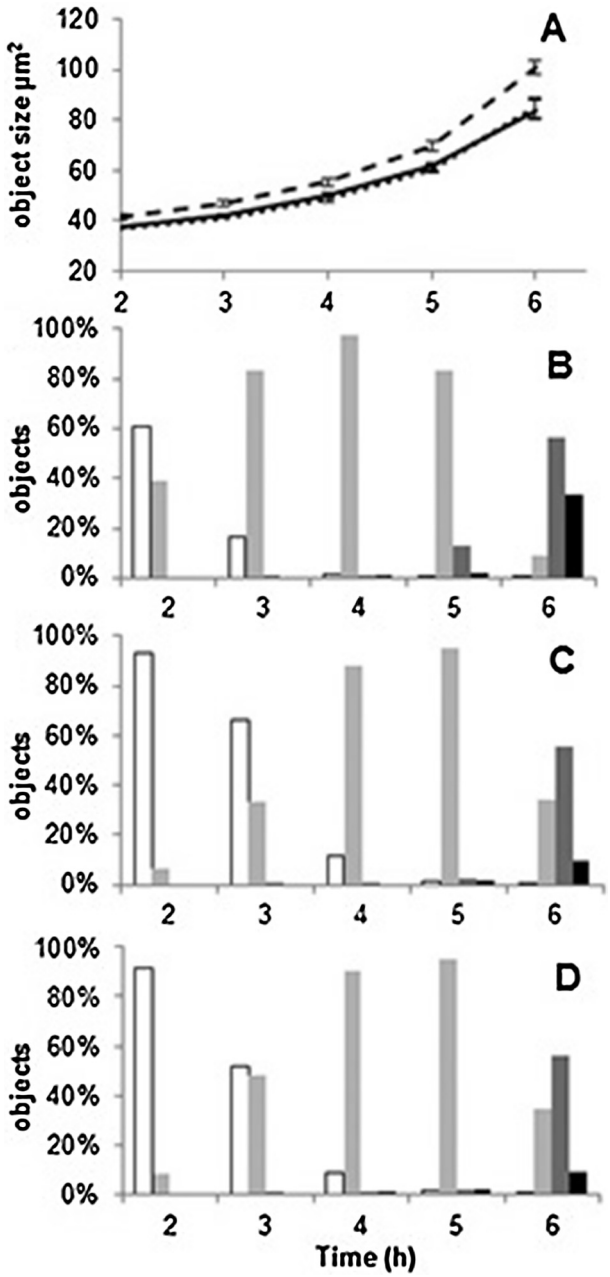

Fig. 5. Size distribution of 2- (A), 5- (B), and 8- (C) day-old spores after incubation for 2–6 h in medium. Bars represent resting conidia (21–42 µm², non-shaded bars), swollen conidia (42–76 µm², light grey bars), germinating conidia (76–106 µm², dark grey bars), and hyphal growth (106–212 µm², black bars). Average size of all objects (D) resulting from 2- (striped line), 5- (dotted line) and 8- (black line) day-old conidia. Error bars represent standard deviation of biological duplicates.

few hundred spores within a single experiment. Two-day-old conidia germinated faster as illustrated by the increase in size of the spore objects in time (Fig. 5D). After 2 h 40% of the conidia had started to swell while this was <10% in the case of the 5- and 8-day-old spores (Fig. 5A–C). Faster swelling was accompanied by fast outgrowth. After 6 h 40% of the 2-day-old conidia had formed hyphae, while this was <10% in the case of the 5- and 8-day-old spores.

Treatment of 2-, 5-, and 8-day-old conidia at 55 °C for 5 min slowed down germination by about 4 h (compare Figs. 5 and 6). The slope of the increase in surface area of the objects was similar between the different spore ages. Thus, no differences were observed in heat resistance between the 2-, 5-, and 8-day-old conidia (Fig. 5D).

3.6. Spore dispersal

Wind, water, and insect (i.e. contact with a hydrophobic solid surface) dispersal assays were set up to explore differences in dispersal efficiency among the differently-aged spores. The wind assay, simulating 6 Beaufort, was 10- and 1000-fold less effective in dispersal of spores when compared to water and insect dispersal assays, respectively (Table 1). No differences were observed between the different spore ages in the case of wind and insect dispersal. Yet, 8-day-old conidia were less effectively dispersed by water.

4. Discussion

The mycelium of filamentous fungi can be highly heterogeneous (Wösten et al., 2013). For instance, zones of *A. niger* colonies grown on minimal medium with for instance xylose or maltose as carbon

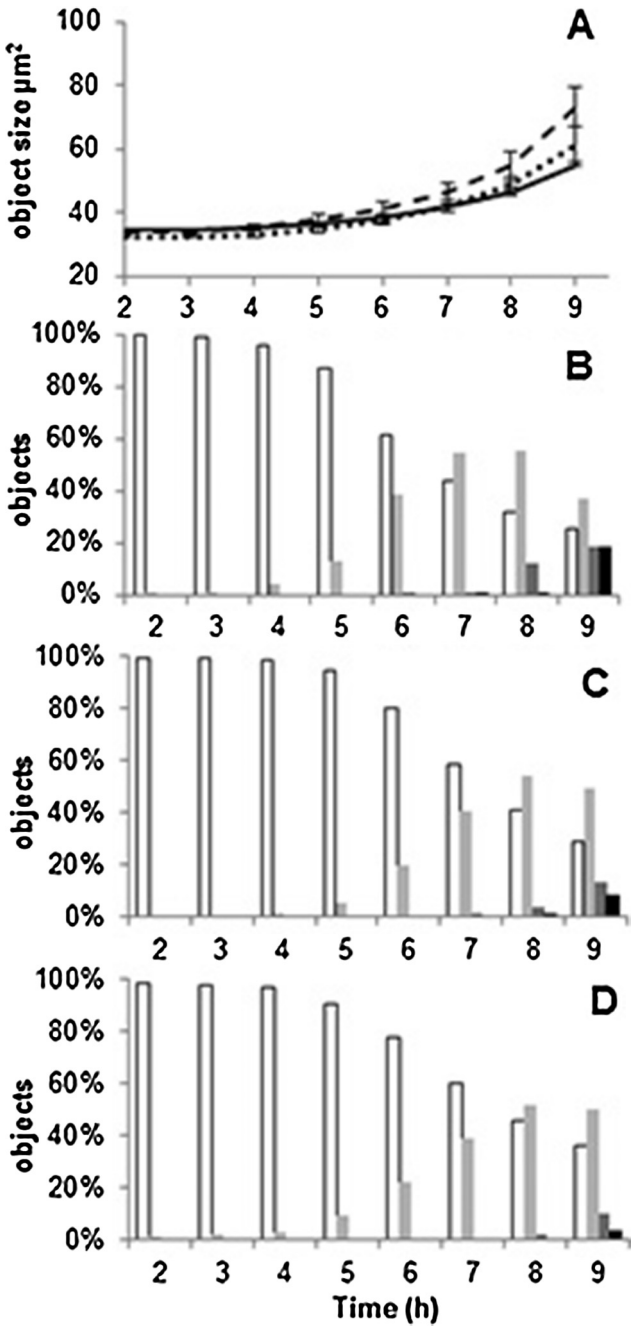

Fig. 6. Size distribution of 2- (A), 5- (B), and 8- (C) day-old spores that had been heat-stressed for 5 min at 55 °C and incubated for 2–9 h in medium. Size classes represent resting conidia (21–42 µm^2, non-shaded bars), swollen conidia (42–76 µm^2, light grey bars), germinating conidia (76–106 µm^2, dark grey bars), and hyphal growth (106–212 µm^2, black bars). Average size of all objects (D) resulting from 2- (striped line), 5- (dotted line) and 8- (black line) day-old conidia. Error bars represent standard deviation of biological duplicates.

source differ with respect to gene expression (Levin et al., 2007a; de Bekker et al., 2011; Vinck et al., 2005, 2011) and protein secretion (Wösten et al., 1991; Levin et al., 2007b; Krijgsheld et al., 2012). The zonal differences in gene expression are caused by local depletion of nutrients as well as a differentiation program (Levin et al., 2007a) and results in conidia with different composition (Bleichrodt et al., 2013). In nature, colonies are exposed to more heterogeneous substrates like citrus peel and onion scales when compared to defined agar media. Using sugar beet pulp as a model of a heterogeneous substrate it was shown that also in this case differential gene expression was observed across the colony (Benoit et al., 2015). Thus, also in this case conidia from different zones may differ in composition.

Apart from a spatial component there is also a temporal component of differentiation. Conidiophore formation starts in the colony center spreading to the outer parts of the mycelium. Thus, conidia at conidiophores within the colony center are older than those at the periphery. On top of that, age differences are observed within a single conidiophore. The oldest conidium is located at the end of the spore chain, while the youngest conidium contacts the phialide. In our study, spore chain formation started between day 1 and 2 and had completed at day 3 implying that there is an age difference of about 1.5 days between the youngest and oldest conidia of the spore chain. This would agree with a cell cycle of 95 min (Bergen and Morris, 1983) and a spore chain length of 20 conidia (our results, unpublished). To be able to study the impact of the temporal component of conidial heterogeneity the spatial component of differentiation had to be eliminated. To this end, we used synchronized colonies by inoculating spores in a confluent layer on agar medium. By doing so, it was shown that conidia mature on the conidiophore; a process accompanied with changes in RNA and compatible solute composition, melanin extractability, water dispersal efficiency, and germination rate. These results are in line with previous studies that showed that culture age affects properties of fungal spores (Hallsworth and Magan, 1996; Conner et al., 1987; Dijksterhuis and Teunissen, 2004). It should be noted that our experimental set-up did not take into account the temporal component of differentiation within a spore chain.

A total number of 645–2421 genes showed a $\geqslant$2-fold change in expression when conidia were compared that had matured for 2, 5, and 8 days on conidiophores. Notably, up to 39% of the up-regulated genes had no predicted function according to PFAM or KEGG annotation, while this was only up to 18% within the down regulated genes. These genes also comprised 60% of the top 25 of up-regulated genes and 36% of the top 25 down-regulated genes. The fact that asexual reproduction has not been studied in detail may explain why so many differentially expressed genes in maturing conidia have unknown functions. GO analysis showed that gene classes involved in regulation were also over-represented in the up-regulated genes. Their encoding proteins may be involved in the major changes that occur in the mRNA profiles during germination of *A. niger* conidia (van Leeuwen et al., 2013; Novodvorska et al., 2013). On the other hand, RNA levels of gene classes involved in metabolism and biosynthetic processes were over-represented in the down-regulated genes. This would be in agreement with cells that prepare themselves to go into a dormant state.

Melanin was more easy to extract from 2-day-old conidia when compared to 5- and 8-day-old conidia. Older spores may have higher levels of cross-linking of melanin to polysaccharide components of the cell wall, especially those containing mannose (Zhong et al., 2008). The functional consequences of the reduced extractability of melanin in older conidia is not yet clear. Mannitol levels were also shown to decrease upon prolonged maturation on the conidiophore. They dropped by about half from day 2 to day 5. Mannitol functions in stress resistance of *A. niger* conidia (Ruijter et al., 2003). A 3-fold reduction in mannitol content in a Δ*mpdA* strain resulted in extreme sensitivity to high temperature, oxidative stress and, to a lesser extent, freezing and lyophilization. Mannitol supplied to the medium complemented the phenotype. These data suggest that 5- and 8-day-old conidia would be less stress resistant when compared to 2-day-old conidia. However, this could not be shown for heat stress, which may be explained by increased levels of trehalose, although differences in this compatible solute were not statistically significant.

Conidia that had matured on the conidiophore for 2–8 days were metabolically active in aqueous solution as shown by PDT

reduction and CO_2 release. This is in agreement with a recent study that showed conidial metabolism using Warburg manometry (Novodvorska et al., 2016). Although metabolic differences could not be observed, the 2-day-old conidia germinated faster when compared to 5- and 8-day-old conidia. Dispersal efficiency in wind and insect assays was similar between 2-, 5-, and 8-day-old conidia. Yet, 8-day-old conidia were less effectively dispersed by water droplets when compared. In all cases only a fraction of the conidia were dispersed. These data imply that conidia of different age are released by each dispersal effect. The heterogeneity caused by these age differences can have an ecological advantage. For instance, only part of the conidia that are dispersed to an aqueous micro-environment with sugars or amino acids (Hayer et al., 2013, 2014) will immediately start to germinate, allowing rapid colonization of the substrate. However, the germlings will be killed when the water in the aqueous environment evaporates during the day. The population of slow germinating conidia would serve as a backup during such highly dynamic conditions.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2016.12.005.

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