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ORIGINAL ARTICLE

# *Aspergillus niger* mutants affected in conidial pigmentation do not have an increased susceptibility to water stress during growth at low water activity

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**Significance and Impact of the Study:** *Aspergillus niger*, a cosmopolitan fungus with melanized conidia, is used here as a model system for fungal growth at low water activity ($a_w$) and humidity dynamics. From this study it becomes clear that melanin, contrary to what has been suggested before, is not a key factor in survival and growth during situations that mimic indoor conditions. Indoor fungal growth can lead to cosmetic damage to building materials and health problems. This knowledge makes clear that novel ways to limit indoor fungal growth have to be based on interference with other cellular traits of fungi.

**Keywords**
*Aspergillus niger*, fungal growth, germination, melanin, relative humidity, water activity.

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## Abstract

*Aspergillus niger* forms conidia that contain melanin in their cell wall. This black pigment has been shown to protect fungi against UV radiation, and experimental evidence has indicated that it also protects against drought and high salt concentrations. In this study, growth of *A. niger* was evaluated at low water activity ($a_w$) and after changes in relative humidity (RH). In addition, deletion strains of *A. niger* affected in the melanin synthesis pathway were compared. Germination of conidia of the wild-type and deletion strains was observed at 0·81 $a_w$ and germ tubes continued growth at $a_w \geq 0{\cdot}83$. Conidia and microcolonies of the different strains were incubated for 1 week at lowered RH (33–84%). Conidia of all strains germinated and formed colonies after exposure to RH ≥33% when transferred back to malt extract medium at $a_w$ 0·98. Conidia germinated and showed limited growth at 84% RH. Microcolonies of all strains did not survive an incubation of 1 week at RH ≤75%, but continued growth after exposure to 84% RH. Together, this is the first genetic evidence that melanin does not play a role during germination and radial extension of fungi at low water conditions.

## Introduction

Melanin is a family of dark pigments consisting of carbon ring structures such as naphthalene and indoles (Bell and Wheeler 1986; Butler and Day 1998). Fungal melanin can be produced from different precursors such as L-3,4-dihydroxyphenylalanine (L-DOPA), 1,8-dihydroxynaphthalene (DHN) and L-tyrosine (Butler and Day 1998; Langfelder *et al.* 2003; Schmaler-Ripcke *et al.* 2009; Eisenman and Casadevall 2012; Gessler *et al.* 2014; Nosanchuk *et al.* 2015). Melanin in the fungal cell wall plays an important role in ultraviolet radiation resistance and also during the infection process of plant and human (Langfelder *et al.* 2003; Jackson *et al.* 2009; Esbelin *et al.* 2013; Gessler *et al.* 2014; Braga *et al.* 2015). Melanin has also been implicated in resistance to drought and high salt conditions. For instance, the DHN pathway is induced in black halotolerant yeasts under hypersaline conditions (Kogej *et al.* 2004, 2006), while tricyclazole-mediated repression of melanin synthesis in the ectomycorrhizal fungus *Cenococcum geophilum* is accompanied by a reduced thickness of the cell wall and increased vulnerability to water stress

and desiccation (Fernandez and Koide 2013). However, genetic evidence for a role of melanin in drought and hypersalinity is lacking.

*Aspergillus niger* is a cosmopolitan fungus with melanized conidia that is found in many habitats including the indoor environment (Samson *et al.* 2010). It causes food spoilage, but is also a cell factory for enzymes and organic acids (Punt *et al.* 2002; Andersen *et al.* 2011). Melanin in *A. niger* was thought to be formed by the DHN pathway (Jørgensen *et al.* 2011), although it also has been proposed that the L-DOPA pathway is responsible for melanin synthesis (Pal *et al.* 2014). The *Aspergillus* strain used in the latter study was identified using the ITS locus sequence, which often is not sufficient for identification to species level (Samson *et al.* 2014). Deletion strains are available that are affected during melanin synthesis in *A. niger*. The *ΔpptA* strain has a deletion in the 4′phosphopantetheinyl transferase gene. It produces white conidia and has a pleiotropic phenotype. Gene *fwnA* encodes a putative polyketide synthase, while *brnA* and *olvA* encode a pigment biosynthesis oxidase and a hydrolase involved in pigment production. These deletion strains produce fawn, brown and olive conidia respectively. Using these deletion strains, it is here shown that melanin does not play a role in germination of conidia and hyphal growth of *A. niger* at low water availability.

## Results and discussion

Malt extract agar (MEA) supplemented with 0–50% glycerol was used to assess growth of *A. niger* strains at steady-state water activity ($a_w$) of 0·98–0·78. Growth beyond initial germination of the *A. niger* strains (Table 1) in this experiment was not observed within 3 weeks upon inoculation at $a_w$ = 0·81 (Table 2) although earlier studies showed very restricted growth (Segers *et al.* 2015). All pigmentation deletion strains (*ΔpptA*, *ΔbrnA*, *ΔolvA* and *ΔfwnA*) and the wild-type strain had formed colonies at $a_w \geq 0{\cdot}83$ (Table 2). Growth rate (as judged by the increase in diameter size of the colony) was similar in all cases with a maximal growth rate between 13 and 14 mm day$^{-1}$ at 0·97 $a_w$.

Germination of conidia was assessed in more detail using stereo microscopy at steady-state $a_w$ of 0·84, 0·81 and 0·78 using MEA plates supplemented with 35, 40 and 45% glycerol respectively. Germination was followed for 32 days using eight replicates. After 7 days, all strains formed extended germ tubes and aerial hyphae at $a_w$ 0·84, except for *ΔpptA* that started to germinate with some extension in the agar medium. After 10 days all strains had formed a colony with aerial hyphae (Fig. 1). Surprisingly, tips of leading hyphae growing at $a_w$ 0·84 were occasionally ruptured, showing loss of cytoplasm indicating an unknown turgor stress in an apparently homogenous medium. Germination of conidia but no colony growth was observed at $a_w$ 0·81 in case of the wild-type strain and the mutant strains except for *ΔpptA* after 10 days (Fig. 1). Remarkably, germination was most extensive in the case of *ΔolvA*, while N402, *ΔfwnA* and *ΔbrnA* showed a comparable response. After 13·5 weeks all strains had formed small colonies with *ΔolvA* being slightly larger than N402, *ΔfwnA* and *ΔbrnA*, and all being larger than *ΔpptA*. Perhaps the relatively hydrophylic conidia of strain *ΔolvA* (Van Veluw *et al.* 2013) compared to the other strains is related to faster germination. The *ΔpptA* strain was markedly slower and did not develop into a colony in five of eight cases, which may be explained by the pleiotropic phenotype of this strain as several biochemical pathways seem to be affected (Jørgensen *et al.* 2011; Van Veluw *et al.* 2013). After 13·5 weeks no germination was observed at $a_w$ 0·78 in all cases.

Conidia and microcolonies without aerial hyphae were exposed to relative humidity (RH) 33–84% in the absence of agar medium. After 1 week, they were retransferred to MEA with $a_w$ 0·98. Survival was evaluated 1–7 days post-transfer. Conidia of all strains survived exposure to RH ≥33%, as shown by germination and outgrowth. However, microcolonies (without aerial hyphae and conidia-forming structures) of all strains did not survive a 1-week period at ≤75% RH, but they continued growth during and after exposure to 84% RH (Fig. 1k). Despite hydrophilicity of the conidia, *ΔolvA* does not behave differently to osmotic stress. Apparently, hydrophobicity of the surface layer does not contribute to drought resistance. This is in line with the finding that the hydrophobic rodlet layer that coats fungal aerial structures does not prevent evaporation (Wösten 2001). This was explained by the porosity of the rodlet layer (Wang *et al.*

**Table 1** Strains used in this study

| Strain | Genotype | ProteinID | Phenotype | Reference |
|---|---|---|---|---|
| N402 | *cspA1* | | Black | Bos *et al.* (1988) |
| JP1.1, *ΔpptA* | *cspA1, pptA::AopyrG* in AB4.1 | An12g03950 | White | Jørgensen *et al.* (2011) |
| AW6.1, *ΔbrnA* | *cspA1, brnA::AopyrG* in MA169.4 | An14g05370 | Brown | Jørgensen *et al.* (2011) |
| AW8.4, *ΔolvA* | *cspA1, olvA::AopyrG* in MA169.4 | An14g05350 | Olive | Jørgensen *et al.* (2011) |
| MA93.1, *ΔfwnA* | *cspA1, fwnA::hygB* in N402 | An09g05730 | Fawn | Jørgensen *et al.* (2011) |

**Table 2** Average growth in colony diameter in mm day$^{-1}$ at $a_w$ 0·81–0·98. Growth rate was determined from the regression coefficient of graphs of each colony using Microsoft Office Excel Professional Plus 2010. One-way ANOVA ($\alpha = 0{\cdot}05$) followed by Tukey's multiple comparisons test was performed using GRAPHPAD PRISM ver. 7.0a (GraphPad Software, La Jolla, CA; www.graphpad.com)

| $a_w$ | N402 | *ΔpptA* | *ΔfwnA* | *ΔolvA* | *ΔbrnA* |
|---|---|---|---|---|---|
| 0·98 | 6·3 ± 0·1 | 4·7 ± 1·5 | 5·6 ± 1 | 5·0 ± 0·2 | 5·2 ± 0·2 |
| 0·97 | 14·2 ± 0·8 | 13·6 ± 0·7 | 13·2 ± 0·6 | 13·4 ± 0·3 | 12·7 ± 0·2 |
| 0·96 | 12·7 ± 0·6 | 12·2 ± 0·3 | 13·3 ± 0·2 | 12·3 ± 0·6 | 13 ± 0·7 |
| 0·95 | 10 ± 0·5 | 10·4 ± 0·6 | 9·9 ± 0·9 | 10·7 ± 0·8 | 10·2 ± 0·4 |
| 0·92 | 6·4 ± 0·2 | 7·1 ± 0·7 | 7 ± 0·3 | 7·2 ± 0·5 | 6·9 ± 0·6 |
| 0·90 | 4·9 ± 0·1 | 5·5 ± 0·5 | 5·6 ± 0·6 | 5·7 ± 0·7 | 5·1 ± 0·2 |
| 0·88 | 3·6 ± 0·1 | 3·8 ± 0·3 | 4·3 ± 0·1 | 4·4 ± 0·3 | 4·0 ± 0·2 |
| 0·85 | 1·9 ± 0·0 | 1·7 ± 0·4 | 2·7 ± 0·4 | 2·4 ± 0·6 | 2·1 ± 0·4 |
| 0·83 | 0·7 ± 0·0 | 0·9 ± 0·1 | 1·0 ± 0·2 | 1·1 ± 0·2 | 1·0 ± 0·2 |
| 0·81 | Germination | Germination | Germination | Germination | Germination |

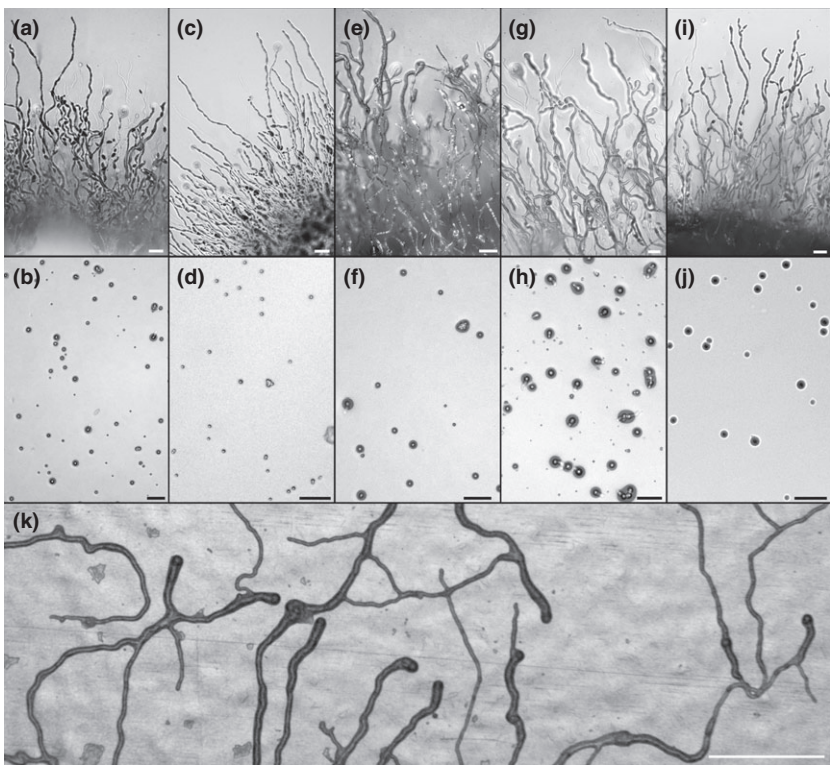


**Figure 1** Germination and outgrowth of wild-type N402 (a, b, k), *ΔpptA* (c, d), *ΔfwnA* (e, f), *ΔolvA* (g, h) and *ΔbrnA* (i, j) on MEA at $a_w$ 0·84 (a, c, e, g, i), and $a_w$ 0·81 (b, d, f, h, j) after 10 days and 84% RH (k) after 7 days. Scale bars (a, c, e, g, i, h) = 50 $\mu$m. Scale bars (b, d, f, j) = 35 $\mu$m. Scale bar (*k*) = 100 $\mu$m. Pictures were taken using a Nikon Zoom AZ-100 stereomicroscope linked to a Nikon DS-Ri2 camera (Nikon Instruments, Amsterdam, the Netherlands).

2005), which may also hold for the melanin coating on conidia of *A. niger*.

All mutant strains of *A. niger* affected in conidial pigmentation were able to grow on media with steady-state $a_w$ lowered to 0·81. The growth is equal to the wild-type N402. A slightly faster germination was seen for the hydrophilic conidia of *ΔolvA*, while the conidia of the pleiotropic strain *ΔpptA* were slower in germination. Eventually, these strains showed similar growth and survival after germination. All strains grew at ≥84% RH in the absence of agar medium. The slow extension was similar to that on malt extract agar at $a_w$ 0·83 and 0·84. Germination and growth was not observed at ≤75% RH in the absence of agar medium. Conidia, but not microcolonies, survived this condition at 33–75% RH. This strengthens our findings that melanin has no role in drought resistance.

A role of melanin in turgor pressure and fungal response to water and salt stress has been reported (Kogej *et al.* 2006; Gachomo *et al.* 2010; Fernandez and Koide

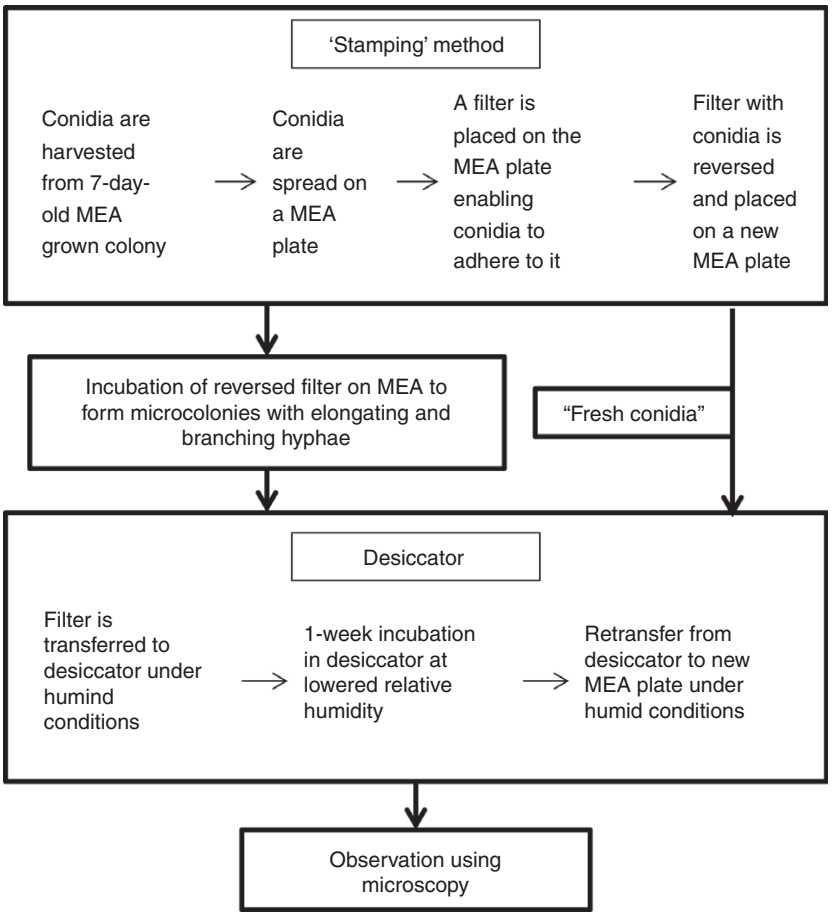


**Figure 2** Flow chart of experimental setup of survival at dynamic $a_w$. Adjusted from Segers *et al.* (2016).

2013; Kejžar *et al.* 2013; Gessler *et al.* 2014). This study presents the first genetic evidence showing that melanin does not have a role in germination and growth under low steady-state $a_w$ and humidity dynamics in *A. niger*. A possible role of melanin under water stress for other species of fungi cannot be excluded. Together it is concluded that survival, germination and growth of *A. niger* at low $a_w$ and humidity dynamics are not affected in the absence of melanin or one of its precursors.

## Materials and methods

### Fungal strains and culture condition

Strains of *A. niger* (Table 1) were grown for 7 days at 25°C on MEA (Oxoid, Landsmeer, The Netherlands) ($a_w$ = 0·98) for the production of conidia. Conidia were harvested from 7-day-old cultures with a T-spatula (VWR, Amsterdam, the Netherlands) using ice-cold sterile 10 mmol $l^{-1}$ *N*-(2-acetamido)-2-aminoethanesulfonic acid, 0·02% Tween 80 (ACES; pH 6·8) as described by Segers *et al.* (2015, 2016) and diluted to 1 × $10^6$ conidia per ml.

### Growth at steady-state water activity

MEA supplemented with 0–50% glycerol (v/v) was three-point inoculated with 3 $\mu$l spore suspension (Segers *et al.* 2015). The $a_w$ of the growth medium was determined using a Novasina labmaster-$a_w$ water activity meter (Novasina, Lachen, Switzerland). Growth in diameter was determined three times a week for each strain in triplicate in two separate experiments for 3 weeks at steady-state $a_w$ of 0·98–0·78 at 25°C (Table 2).

### Germination at steady-state water activity

Germination of conidia was assessed in more detail using stereo microscopy at steady-state $a_w$ of 0·84, 0·81 and 0·78 using MEA plates supplemented with 35, 40 and 45% glycerol respectively. They were inoculated in the centre with 3 $\mu$l spore solution. Germination at 25°C was followed for 32 days using eight replicates. Pictures were taken using a Nikon Zoom AZ-100 stereomicroscope linked to a Nikon DS-Ri2 camera (Nikon Instruments, Amsterdam, the Netherlands).

### Growth after a defined period of lowered $a_w$

In addition, the response of the *A. niger* strains on a period of defined and lowered $a_w$ was evaluated. MEA plates were inoculated with a 50-$\mu$l spore suspension. A polycarbonate filter (Ø 47 mm, pore size, 0·1 $\mu$m; GE Water & Process Technologies, Trevose, PA) was placed on top of the medium and inoculation spot and immediately transferred to a new MEA plate with the side with 100–500 adhering conidia oriented upwards (stamping method; Fig. 2). The filters (≥2 technical replicates and three biological replicates) were removed from the agar medium immediately (containing dormant conidia) or after 36 h (when microcolonies without aerial hyphae were formed) and transferred to Nalgene 150*150 mm (1·5 l) desiccators (VWR) containing 100 ml saturated KCl (RH 84%), NaCl (RH 75%), NaBr (RH 58%) or $MgCl_2$ (RH 33%) solutions (Winston and Bates 1960; Greenspan 1977; Segers *et al.* 2016). RH was confirmed using a Testo 174H hygrometer (Testo, Lenzkirch, Germany). After 1 week of incubation at 25°C, filters were transferred to MEA plates ($a_w$ 0·98) and incubation was continued for 7 days at 25°C (desiccator; Fig. 2). Regrowth of mycelium indicated survival of the colony. Survival was evaluated using a stereomicroscope (Nikon Zoom AZ-100) after 30 min and 1–7 days post-transfer.

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## Conflict of Interest

None.

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