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Minimal nutrient requirements for induction of germination of *Aspergillus niger* conidia

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ABSTRACT

Aspergillus niger reproduces asexually by forming conidia. Here, the minimal nutrient requirements were studied that activate germination of *A. niger* conidia. To this end, germination was monitored in time using an oCelloScope imager. Data was used as input in an asymmetric model to describe the process of swelling and germ tube formation. The maximum number of spores (P_{max}) that were activated to swell and to form germ tubes was 32.54% and 20.51%, respectively, in minimal medium with 50 mM glucose. In contrast, P_{max} of swelling and germ tube formation was <1% in water or 50 mM glucose. Combining 50 mM glucose with either $NaNO_3$, KH_2PO4, or MgSO4 increased P_{max} of swelling and germination up to 15.25% and 5.4%, respectively, while combining glucose with two of these inorganic components further increased these P_{max} values up to 25.85% and 10.99%. Next, 10 mM amino acid was combined with a phosphate buffer and $MgSO_4$. High (e.g. proline), intermediate and low (e.g. cysteine) inducing amino acids were distinguished. Together, a combination of an inducing carbon source with either inorganic phosphate, inorganic nitrogen or magnesium sulphate is the minimum requirement for *A. niger* conidia to germinate.

1. Introduction

The genus *Aspergillus* (Galagan et al., 2005; Hawksworth, 2011) includes species that are among the most abundant fungi worldwide. Aspergilli can grow as saprotrophs on a wide variety of dead organic material, can be pathogens of plants, animals and human, and can act as endophytes (Bennett, 2010; Krijgsheld et al., 2013). Aspergilli also grow in a wide range of abiotic conditions (Bennett, 2010; Krijgsheld et al., 2013). For instance, *Aspergillus penicillioides* can grow at a water activity as low as 0.585 (Stevenson et al., 2017), while *A. niger* grows at a pH as low as 1.5 (Krijgsheld et al., 2013). The production and dispersion of enormous numbers of conidia also explains the abundance of aspergilli (Taha et al., 2005; Teertstra et al., 2017). Their asexual spores are among the most dominant fungal structures in the air (Abdel Hameed et al., 2004; Bennett, 2010). For instance, more than 10 *Aspergillus fumigatus* conidia were found per cubic meter air (Mullins et al., 1984), implying that humans inhale hundreds of these spores at a single day.

Conidia are formed on conidiophores after a period of vegetative growth. The conidiophores of *A. niger* are formed 1–2 days after inoculation on minimal medium at 30 °C, while conidia are formed up to and including day 4 (Teertstra et al., 2017). A single conidiophore produces about 10,000 conidia. Consequently, a colony in a Petri dish can easily form more than a billion of these spores. Prolonged incubation results in maturation of the conidia on the conidiophores. The conidia of 10-day-old colonies are still metabolically active (Novodvorska et al., 2016; Teertstra et al., 2017). Yet, these matured conidia germinate slower than young conidia. In addition, dispersal efficiency of the matured conidia by water is lower than that of young immature conidia, while dispersal by wind and a hydrophobic object is not affected (Teertstra et al., 2017). Data also showed that a minor fraction of the spores of an *A. niger* colony is released by a single exposure to a vector. From these data and reporter studies performed by Bleichrodt et al. (2013) it was concluded that a colony of *A. niger* releases a

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population of conidia that is heterogeneous in age, composition and germination ability. This heterogeneity was proposed to provide a selective advantage in environments with rapidly changing conditions such as availability of water (Teertstra et al., 2017).

Conidia have to decide when to germinate. Resting conidia are relative stress resistant, while germlings can colonize a substrate thus preventing competitors to take this niche. Immediate germination, i.e. without sensing the environmental conditions, will have a selective advantage by allowing the fungus to rapidly colonize a substrate. However, the germling will die when nutrients are limiting. Unravelling the signals that initiate germination will help us to understand how conidia cope with the dilemma when to give up the stress resistant state of dormancy. The signals that induce germination also impact the ecological niche of fungi and also partly explain why certain fungi spoil certain food sources or infect certain hosts.

Activation of germination of conidia results in swelling by water uptake. The swollen conidia form germ tubes by switching from isotropic to polarized growth. When sufficient nutrients are available these germ tubes grow out into hyphae that grow at their apices and that branch sub-apically. Sugars that trigger germination and outgrowth of *A. niger* conidia include glucose, mannose, and xylose (Hayer et al., 2013). On the other hand, tagatose, lyxose, and 2-deoxy-glucose activate the conidia to swell but do not support subsequent outgrowth. Galactose and arabinose are among the non-triggering sugars but the former does support outgrowth when a triggering sugar is also present. Together, particular sugars activate germination and/or support outgrowth. Hayer et al. (2014) studied N-sources that trigger germination and/or support outgrowth. To this end, a non-triggering sugar was also added in the medium to support outgrowth of the hyphae. A total of 14 out of 20 proteinogenic L-amino acids triggered germination and supported outgrowth. Inorganic nitrogen compounds, urea, alanine, arginine, glycine, histidine, lysine, and methionine did not activate spores but did support outgrowth. It was shown that both the triggering sugars and amino acids are initially not taken up by the conidia, implying that the nutrient sensors reside in the plasma membrane (Hayer et al., 2013, 2014).

Here, we studied the minimum nutrient requirements that activate swelling and germtube formation of *A. niger* conidia. To this end, oCelloScope imaging was performed enabling high throughput analysis of germination of conidia. Data show that the presence of an inducing carbon source combined with either inorganic phosphate, inorganic nitrogen or magnesium sulphate is sufficient to trigger germination of >5% of the conidia.

2. Material and methods

2.1. Strains and culture conditions

A. niger strain N402 (Bos et al., 1988) was routinely grown at 30 °C on minimal medium (MM; 1% glucose, 70.6 mM $NaNO_3$, 11 mM KH_2PO_4, 6.7 mM KCl, 2 mM $MgSO_4.7H_2O$, trace elements according to Vishniac and Santer (1957)) supplemented or not with 1.5% agar. Plates (20 ml MM) were inoculated by spreading 10^7 conidia over the agar surface and 5-day-old conidia were harvested with water after colonies had grown for 7 days. Miracloth (Millipore, www.merckmillipore.com) was used to filter the conidia and the spores were washed twice with water with intermittent centrifugation for 10 min at 4 °C and 1700 g.

2.2. Germination analysis

Swelling of conidia and germ tube formation was monitored in MMG or (mixtures of) its components (Table 1). In the latter case, concentrations of these components were used that are present in MM, unless stated otherwise. Moreover, the impact of replacing KH_2PO_4 for K_2HPO_4, NaH_2PO_4, or Na_2HPO_4 was assessed (Table 2) as well as the effect of using amino acids as carbon source (Table 3). Conidia were used directly after harvesting. As a control, conidia were used that had been heat inactivated by exposure to 85 °C for 15 min. Wells of a 24 wells suspension culture plate (Greiner bio-one, Cellstar 662102, www.gbo.com) were seeded with 5 10^4 conidia contained in 750 μl MM or solutions containing one or more of its components. Alternatively, 4 10^4 conidia were seeded in 96 wells suspension culture plates (Greiner bio-one, Cellstar 655185) in 150 μl medium. These different densities allowed imaging of at least 200 spores per well, while 683–5325 spores were analysed per experimental condition (i.e adding up the spores of the biological replicates) (see Tables 1–3).

Germination of conidia was monitored on line at 30 °C using an oCelloScope imager (Biosense Solutions, www.biosensesolutions.dk) (Fredborg et al., 2013) with UniExplorer software version 8.1.0.7682-RL2. Measurements (using at least biological triplicates) were started after 1 h of incubation, enabling settling of the conidia at the bottom of the well. Objects were scanned every hour during the first 10 h and every 2 h during the next 14 h. The scan area length was set at 405 (96-wells plates) or 2205 (24-wells plate) μm, the object area (min–max) at 70–700 pixels, and the maximum number of objects at 1200. Individual objects were followed over time using oCelloScope XY coordinates and a custom R script. Conidial aggregates and non-conidial objects at t = 1 h were manually removed from the data set. Conidia were followed in time based on their X and Y coordinates using the fast k-nearest neighbor (KNN) searching algorithm from the R package 'FNN' (Beygelzimer et al., 2019). This was done from t = x to t = x+1 and vice versa. In addition, neighbor distance of an object was not allowed to exceed 27.5 μm (i.e. 50 pixels) between 2 adjacent time points. The lineage was discontinued if these conditions were no longer met. The objects were classified into 4 groups based on surface area and circularity (Teertstra et al., 2017). Resting conidia had a surface area ≤39 μm^2 and a circularity >0.97, swollen conidia had a surface area >39 μm^2 and a circularity >0.97; conidia with germ tubes had a surface area >39 μm^2 and a circularity >0.75 and ≤0.97, while hyphae had a surface area >39 μm^2 and a circularity ≤0.75. The latter circularity equals a length of the germ tube/hypha ≥ the length of the swollen spore.

2.3. Modelling of germination kinetics

The asymmetric model (Dantigny et al., 2011) was used to describe swelling and germ tube formation (P) as a function of time, τ (h) (Eq. (1)).

$$P = P_{max}\left(1 - \frac{1}{1 + \left(\frac{t}{\tau}\right)^{d}}\right) \quad (1)$$

P_{max} is the maximal percentage of swollen conidia or spores that had formed a germ tube (the asymptotic value of P at $t \to +\infty$), τ (h) is the time at which P = 0.5 P_{max} and d is a shape parameter that can be correlated to heterogeneity of the population. A low d reflects a population where conidia have more variable individual germination times. To estimate the model parameters, the biological triplicates were fitted per tested condition with the R package GrowthRates (Petzoldt, 2019) using the Levenberg–Marquardt algorithm. Parameters were limited to P ≥ 0 and ≤120%, τ ≥ 1 and ≤ 18, d ≥ 1 and ≤ 30 when fitting the model. Confidence intervals (95%) were determined using the standard error of the

Table 1
Parameter estimates of the asymmetrical model used to describe swelling and germ tube formation of spores in MMG_{50} and components thereof. MMG_{50} consists of 50 mM glucose (G_{50}), KCl (K), $NaNO_3$ (N), KH_2PO_4 (P), $MgSO_4$ (S) and Vishniac trace elements (V). Confidence intervals are indicated between brackets, N represents the number of objects at t = 1 h, while M represents the number of objects that were no longer detected between 2 and 14 h because the hypha had become too long or the object was obscured by hyphae of other objects. RMSE represents the root mean square error of the modelled data and is a measure for the goodness of fit (Dantigny et al., 2011; Ratkowsky, 2004).

Component	Parameter estimates							Objects	
	P_{max} (%)		τ (h)		d (−)		RMSE	N	M
Swelling									
MMG_{50}	32.54	[29.08; 36.01]	5.03	[4.25; 5.82]	3.59	[1.69; 5.49]	0.90	3822	293
MMG_{50}-KV	46.56	[43.76; 49.36]	4.82	[4.38; 5.26]	3.94	[2.63; 5.24]	0.80	2312	284
G_{50}PS	25.85	[22.17; 29.54]	4.67	[3.62; 5.73]	3.13	[1.01; 5.25]	0.92	4389	100
G_{50}NS	23.47	[20.03; 26.92]	5.50	[4.39; 6.61]	3.59	[1.09; 6.09]	0.86	3970	130
G_{50}NP	25.50	[21.40; 29.60]	5.12	[3.90; 6.35]	2.85	[0.96; 4.73]	0.88	3863	557
G_{50}N	12.86	[6.19; 19.52]	5.12	[1.14; 9.11]	2.32	[-1.73; 6.37]	1.09	3051	19
G_{50}P	15.25	[12.64; 17.85]	5.87	[4.53; 7.21]	2.84	[1.07; 4.62]	0.48	3289	22
G_{50}S	9.34	[8.72; 9.97]	6.07	[5.54; 6.60]	2.93	[2.21; 3.65]	0.12	5325	47
Germ tube formation									
MMG_{50}	20.51	[16.92; 24.10]	7.26	[6.03; 8.49]	6.28	[-0.03; 12.59]	1.01	3822	293
MMG_{50}-KV	29.33	[25.61; 33.04]	7.21	[6.35; 8.08]	7.07	[1.47; 12.67]	1.10	2312	284
G_{50}PS	5.02	[3.90; 6.14]	7.58	[5.79; 9.38]	4.46	[0.05; 8.87]	0.25	4389	100
G_{50}NS	10.99	[8.83; 13.14]	8.75	[7.18; 10.33]	6.59	[-0.26; 13.43]	0.54	3970	130
G_{50}NP	3.78	[3.10; 4.46]	5.58	[4.19; 6.97]	3.01	[0.81; 5.22]	0.14	3863	557
G_{50}N	2.08	[1.57; 2.60]	7.18	[5.16; 9.20]	3.34	[0.43; 6.25]	0.09	3051	19
G_{50}P	5.40	[1.88; 8.91]	7.48	[0.36; 14.60]	1.81	[-0.34; 3.95]	0.25	3289	22
G_{50}S	1.90	[1.23; 2.57]	7.27	[4.14; 10.39]	2.64	[0.07; 5.22]	0.09	5325	47

Table 2
Parameter estimates of the asymmetrical model used to describe swelling and germ tube formation of spores in 11 mM KH_2PO_4 (K), K_2HPO_4 (K_2), NaH_2PO_4 (Na), Na_2HPO_4 (Na_2), 2 mM $MgSO_4$ (S) and 10 mM glucose (Glu) as carbon source (C) or 10 mM proline (Pro) as carbon and nitrogen source (C–N). Confidence intervals are indicated between brackets, N represents the number of objects at t = 1 h, while M represents the number of objects that were no longer detected between 2 and 16 h because the hypha had become too long or the object was obscured by hyphae of other objects. RMSE represents the root mean square error of the modelled data and is a measure for the goodness of fit (Dantigny et al., 2011; Ratkowsky, 2004).

Salts	C/C–N	pH	Parameter estimates							Objects	
			P_{max} (%)		τ (h)		d (−)		RMSE	N	M
Swelling											
KS	Glu	4.5	22.79	[14.42; 31.16]	5.50	[2.46; 8.54]	2.00	[-0.01; 4.01]	0.44	729	51
K_2S	Glu	8.5	20.12	[13.73; 26.51]	6.86	[3.8; 9.92]	2.00	[0.60; 3.40]	4.10	745	2
NaS	Glu	4.5	36.23	[28.32; 44.14]	4.82	[3.14; 6.49]	2.00	[0.66; 3.34]	1.17	722	121
Na_2S	Glu	8.5	27.04	[17.02; 37.05]	7.31	[3.57; 11.05]	2.00	[0.47; 3.53]	0.51	753	12
KS	Pro	4.5	81.87	[79.37; 84.38]	3.74	[3.52; 3.96]	2.83	[2.38; 3.27]	0.73	725	23
K_2S	Pro	8.5	53.13	[50.14; 56.11]	7.50	[7.07; 7.94]	4.84	[3.55; 6.13]	13.04	811	9
NaS	Pro	4.5	91.04	[89.39; 92.69]	4.01	[3.88; 4.14]	3.56	[3.18; 3.93]	2.02	692	26
Na_2S	Pro	8.0	54.85	[47.84; 61.87]	7.65	[6.60; 8.69]	3.87	[1.97; 5.77]	0.85	786	5
Germ tube formation											
KS	Glu	4.5	10.84	[5.09; 16.59]	9.99	[4.46; 15.53]	2.84	[-0.12; 5.81]	6.64	729	51
K_2S	Glu	8.5	3.94	[-2.22; 10.09]	15.00	[-8.38; 38.38]	2.31	[-0.87; 5.49]	5.91	745	2
NaS	Glu	4.5	15.21	[8.21; 22.21]	10.89	[5.23; 16.55]	2.33	[0.79; 3.87]	4.15	722	121
Na_2S	Glu	8.5	4.58	[1.01; 8.15]	15.00	[4.39; 25.61]	2.60	[0.67; 4.53]	0.34	753	12
KS	Pro	4.5	17.53	[10.18; 24.88]	14.98	[12.06; 17.9]	7.8	[-1.84; 17.45]	1.12	725	23
K_2S	Pro	8.5	0.63	[0.44; 0.81]	7.39	[5.16; 9.62]	5.54	[-3.27; 14.36]	7.79	811	9
NaS	Pro	4.5	47.61	[36.70; 59.15]	15.00	[13.17; 16.83]	6.30	[2.76; 9.84]	0.58	692	26
Na_2S	Pro	8.0	4.87	[2.03; 7.17]	15.00	[7.15; 22.58]	2.64	[1.16; 4.12]	5.47	786	5

parameter estimates, were Y represents the estimated P, τ or d value; SEy is the standard error obtained for P, τ or d; α is 0.05; df is the degrees of freedom; *qt* represents the Students T distribution function (Eq (2)).

$$CI_y = SE_y \pm qt\left(1 - \frac{\alpha}{2},\ df\right) \quad (2)$$

Objects that had an object area >300 pixels (90 μm^2) and that decreased in size were excluded from the data set. Missing objects represent resting spores (R) that are lost during the analysis without moving to the swelling (S) or germination (G) stage. Size and circularity data of all objects were used for the parameter estimation until a time point when hyphal growth had obscured resting spores.

3. Results

Minimal medium with glucose (MMG) is routinely used to grow *A. niger*. It consists of glucose (G), KCl (K), $NaNO_3$ (N), KH_2PO_4 (P), $MgSO_4$ (S) and Vishniac trace elements (V) (for concentrations see Material and Methods). Here, it was assessed which components of MM are required to activate swelling and germ tube formation of conidia. In addition, it was assessed whether mono- or di-potassium phosphate salts or mono- or di-sodium phosphate salts impact activation of germination and which amino acids can replace glucose.

Table 3
Asymmetric modelling of swelling and germination of spores in 25 mM $NaPO_4$ buffer pH 6.0 (NaP), 2 mM $MgSO_4$ (S), and 10 mM L-amino acid (AA), the latter described by their three letter code (IUPAC). The pH was 6 throughout the experiment, except for tyrosine that had a pH between 1 and 2. Confidence intervals are indicated between brackets, N represents the number of objects at t = 1 h, while M represents the number of objects that were no longer detected between 2 and 16 h because the hypha had become too long or the object was obscured by hyphae of other objects. RMSE represents the root mean square error of the modelled data and is a measure for the goodness of fit (Dantigny et al., 2011; Ratkowsky, 2004). Grey shading indicates amino acids that are highly inducing in swelling or germ tube formation, while light grey and no shading indicates amino acids that are intermediate or lowly inducing.

Salts	AA	Parameter estimates							Objects	
		SWELLING								
		P_{max} (%)		τ (h)		d (-)		RMSE	N	M
NaPS	Ala	83.84	[80.74;86.94]	5.57	[5.28;5.87]	3.85	[3.11;4.58]	1,44	683	88
NaPS	Arg	20.71	[14.44;26.97]	11.89	[8.83;14.95]	3.23	[1.61;4.86]	0,20	786	75
NaPS	Asn	5.46	[-3.52;14.43]	15.00	[-8.89;38.89]	2.32	[-1.02;5.67]	1,03	840	49
NaPS	Asp	7.78	[-1.70;17.27]	15.00	[-4.82;34.82]	2.03	[0.01;4.05]	0,19	832	83
NaPS	Cys	0.55	[0.33;0.76]	2.46	[-0.08;5.01]	2.00	[-2.21;6.21]	1,72	940	5
NaPS	Gln	3.65	[1.49;5.80]	9.48	[2.39;16.56]	2.00	[0.22;3.78]	0,23	909	42
NaPS	Glu	8.11	[2.50;13.73]	9.11	[0.84;17.37]	2.00	[-0.22;4.22]	0,26	956	13
NaPS	Gly	6.79	[0.26;13.33]	15.00	[0.82;29.18]	2.28	[0.38;4.19]	1,41	945	29
NaPS	His	2.48	[1.08;3.88]	8.20	[2.11;14.29]	2.00	[-0.03;4.03]	1,38	856	11
NaPS	Ile	5.52	[1.00; 10.03]	12.28	[0.47;24.10]	2.00	[0.24;3.76]	0,30	842	8
NaPS	Leu	1.65	[1.24;2.07]	3.02	[1.33;4.72]	2.00	[-0.30;4.30]	1,83	855	14
NaPS	Lys	1.97	[1.13;2.82]	6.86	[2.79;10.94]	2.00	[0.12;3.88]	0,93	946	10
NaPS	Met	3.15	[-3.58;9.89]	15.00	[-20.19;50.19]	2.00	[-1.47;5.47]	0,41	874	6
NaPS	Phe	6.26	[3.76;8.75]	3.25	[0.50;6.00]	2.00	[-1.47;5.47]	0,46	818	79
NaPS	Pro	96.95	[95.90;98.01]	3.18	[3.10;3.26]	4.14	[3.76;4.51]	0,35	934	28
NaPS	Ser	7.61	[4.77;10.46]	8.83	[5.66;12.01]	3.76	[-0.59;8.11]	0,61	810	32
NaPS	Thr	1.76	[1.01;2.50]	7.76	[3.36;12.16]	2.00	[0.37;3.63]	1,44	1016	14
NaPS	Trp	8.82	[3.04;14.60]	15.00	[4.58;25.42]	2.08	[0.96;3.21]	0,36	972	10
NaPS	Tyr	2.95	[1.46;4.44]	8.96	[3.23;14.7]	2.02	[0.36;3.69]	1,26	737	9
NaPS	Val	3.31	[1.68;4.93]	5.18	[1.23;9.12]	2.00	[-0.87;4.87]	0,52	750	9
NaPS	no	1,66	[1.43;1.90]	1.26	[0.37;2.14]	2.00	[0.75;4.75]	0,54	706	7
		GERM TUBE FORMATION								
NaPS	Ala	37.21	[33.99;40.44]	12.05	[11.38;12.71]	6.3	[4.52;8.07]	0,19	683	88
NaPS	Arg	15.27	[11.04;19.49]	14.84	[12.19;17.49]	3.96	[2.50;5.42]	0,48	786	75
NaPS	Asn	2.82	[-0.13;5.77]	15.00	[4.73;25.27]	3.84	[-1.24;8.92]	0,24	840	49
NaPS	Asp	3.92	[1.65;6.19]	11.35	[5.70;17.00]	3.33	[-0.22;6.88]	0,64	832	83
NaPS	Cys	0.17	[0.01;0.33]	4.31	[-2.89;11.50]	2.00	[-4.66;8.66]	1,25	940	5
NaPS	Gln	2.02	[-0.25;4.29]	13.22	[-3.83;30.27]	2.00	[-0.18;4.18]	0,59	909	42
NaPS	Glu	7.30	[-1.66;16.25]	15.00	[-5.61;35.61]	2.00	[-0.01;4.01]	1,37	956	13
NaPS	Gly	2.75	[1.55;3.95]	15.00	[11.49;18.51]	5.12	[1.39;8.85]	0,54	945	29
NaPS	His	1.65	[-0.24;3.54]	15.00	[0.70;29.30]	2.80	[-0.35;5.95]	3,44	856	11
NaPS	Ile	1.64	[0.70;2.57]	11.49	[6.05;16.93]	3.50	[-0.29;7.30]	0,45	842	8
NaPS	Leu	0.17	[0.00;0.34]	4.37	[-3.15;11.89]	2.00	[-4.84;8.84]	3,88	855	14
NaPS	Lys	0.83	[-0.24;1.91]	13.46	[-6.33;33.25]	2.00	[-0.44;4.44]	0,21	946	10
NaPS	Met	0.68	[0.33;1.04]	15.00	[11.26;18.74]	6.28	[-0.53;13.09]	0,20	874	6
NaPS	Phe	2.53	[1.87;3.20]	3.66	[1.94;5.38]	6.90	[-12.92;26.72]	1,58	818	79
NaPS	Pro	54.95	[52.75;57.14]	11.25	[10.95;11.56]	7.24	[6.04;8.43]	1,24	934	28
NaPS	Ser	3.37	[1.97;4.77]	11.21	[7.89;14.53]	5.87	[-2.33;14.07]	0,28	810	32
NaPS	Thr	0.69	[-0.11;1.49]	15.00	[0.05;29.95]	2.71	[-0.32;5.74]	0,44	1016	14
NaPS	Trp	2.80	[0.71;4.89]	15.00	[5.99;24.01]	2.93	[0.71;5.16]	0,56	972	10
NaPS	Tyr	1.33	[0.40;2.26]	8.52	[0.81;16.22]	2.00	[-0.40;4.40]	0,23	737	9
NaPS	Val	0.97	[0.62;1.32]	1.06	[-1.09;3.21]	2.00	[-6.08;10.08]	2,15	750	9
NaPS	no	1.02	[0.71;3.11]	15.00	[-10.96;40.96]	2.01	[-0.59;4.62]	0.50	706	7

3.1. Effect of glucose and inorganic salts on germination

Swelling and germ tube formation of 5-day-old conidia was monitored for 24 h with an oCelloScope imager after inoculation in liquid minimal medium with 50 mM glucose (MMG_{50}). Monitoring of swelling and germ tube formation started after 1 h to enable conidia to settle at the bottom of the 24 wells plate. After 1 h incubation, ≥99% of the spores were still in the resting stage (Fig. 1A,D), while after 8 h both resting and swollen spores were observed as well as germ tubes and hyphae (Fig. 1B,D). More

conidia had germinated after 16 h and germ tubes and short hyphae had extended into long filaments (Fig. 1C,D). The asymmetric model (Dantigny et al., 2011) was used with P_{max}, τ and d as outcome to describe the process of swelling and germ tube formation. The former is the maximal percentage of swollen spores or conidia that had formed a germ tube, τ is the time when $P = 0.5\ P_{max}$ and d is a shape parameter showing the heterogeneity within the spore population. After 14 h, too many objects had formed long hyphae that could not be traced back to one of the objects or that obscured other objects. Therefore, data of 14 h were also used in the modelling for time points 16–24 h. Visual inspection confirmed that no additional spores germinated after 14 h, thereby legitimizing this approach. P_{max} of swelling and germ tube formation was 32.54% and 20.51%, respectively, while τ was 5.03 h and 7.26 h (Fig. 2A; Table 1).

To assess which constituents of MMG_{50} determine germination of conidia, 5-day-old spores were inoculated in solutions containing one or more of these components. P_{max} of swelling and germ tube formation was <1.5% when glucose was excluded from MM (Supplementary Table 1). This low percentage is indicative of the absence of germination since heat killed spores showed a similar P_{max} (Supplementary Table 1). In contrast, P_{max} of swelling and germination was 46.56% and 29.33%, respectively, while τ was 4.82 and 7.21 h (Supplemental Table 1) when both K and V were excluded from MMG_{50}. Thus, external K and V are not needed to induce germination of conidia. Therefore, these components were not included in further analysis.

Spores did not settle at the bottom of the well plates in water or water containing G_{50} only, disabling automated monitoring of germination with the oCelloScope. Mathematical analysis was also not possible when spores were directly dry-tipped from plates onto siliconized glass cover slides (Teertstra et al., 2017). Although these spores remained attached to the slide in water or water containing G_{50} only, focusing was impaired due to slight drifting of the slides. Visual inspection showed that spores did not swell and did not show outgrowth during 24 h (data not shown). Spores did settle in the presence of only N, P, or S, thus allowing oCelloScope analysis. Presence of N, P or S showed a P_{max} and τ of swelling $\leq$1.45% and $\geq$1.00 h, respectively (see rows G_0N, G_0P and G_0S in Supplementary Table 1). These values were $\leq$0.88% and $\geq$1,00 h for germ tube formation. Together, presence of a single medium component hardly, if at all, induces swelling and germination of spores.

In the next set of experiments G_{50} was combined with N, P or S. P_{max} of swelling and germination of these two-component media were between 9.34 and 15.25% and 1.90 and 5.40%, respectively, while τ was between 5.12 and 6.07 h and 7.18 and 7.48 h (Table 1; Fig. 2C, F). Next, swelling and germination in $G_{50}NP$, $G_{50}NS$ and $G_{50}PS$ were analysed. P_{max} of swelling and germination of these three-component media were between 23.47 and 25.85% and 3.78% and 10.99%, respectively, while τ was between 4.67 and 5.50 h and 5.58 and 8.75 (Table 1; Fig. 2B, E). These data imply that swelling and germination incidence is increased about 2-fold in the three-component media when compared to the two-component media. Moreover, τ of swelling is reached at least 30 min earlier.

The effect of glucose concentration was assessed by growing spores in MM, MM-KV and in the single, double and triple component media (see above) in the presence of 10 or 50 mM glucose. P_{max} and τ of swelling were 11.74 and 32.54% and 6.00 and 5.03 h, respectively, for MMG_{10} and MMG_{50} (Supplementary Table 1, Table 1). These values were 5.11 and 20.51% and 8.21 and 7.26 h for germination. Thus, reducing the glucose concentration slows down and reduces the number of spores that swell and form germ tubes. Similar results were obtained in MM-KV, and the double and triple media (Supplementary Table 1).

3.2. The effect of sodium and potassium phosphate on germination

The effect of KH_2PO_4, K_2HPO_4, NaH_2PO_4 or Na_2HPO_4 on germination was assessed by combining either phosphate source with 2 mM $MgSO_4$ and either 10 mM glucose or proline. The pH of the K_2HPO_4 and Na_2HPO_4 solutions ranged between 8.0 and 8.5, while the pH of the solutions containing KH_2PO_4 and NaH_2PO_4 was 4.5 (Table 2). P_{max} of swelling in the presence of glucose was between 20.12 and 36.23%, while τ was between 4.82 and 7.31 h. The values for germ tube formation were 3.94–15.21% and 9.99–15.00 h (Table 2). No significant differences were observed in swelling and germ tube formation between the different phosphate salts.

Incidence of swelling was higher when proline was used as a carbon source instead of glucose, either combined with KH_2PO_4, K_2HPO_4, NaH_2PO_4 or Na_2HPO_4. In contrast, incidence of germ tube formation was only significantly higher in the case of proline compared to glucose when combined with NaH_2PO_4. P_{max} of swelling in the presence of proline was between 53.13 and 91.04%, while τ was between 3.74 and 7.65 h (Table 2). These values were between 0.63% and 47.61% and 7.39 h and 15.00 h for germ tube formation. P_{max} of swelling and germ tube formation were significantly different between KH_2PO_4 and K_2HPO_4 and between NaH_2PO_4 and Na_2HPO_4. In both cases the $H_2PO_4^-$ salt was superior in promoting swelling and germ tube formation when compared to the HPO_4^{2-} salts, probably explained by the pH difference of these solutions (Table 2). Notably, P_{max} of swelling and germ tube formation were significantly higher in the case of NaH_2PO_4 when compared to KH_2PO_4 (Table 2). Moreover, no significant difference in τ of swelling was detected while using glucose, but τ of swelling with proline was significantly lower with the $H_2PO_4^-$ salt compared to the HPO_4^{2-} salts. Together, NaH_2PO_4 and KH_2PO_4 show highest germination incidence in the case of proline, while germination is not affected by the phosphate salt in the case of glucose.

3.3. The effect of amino acids on germination

Conidia were exposed to 10 mm of one of the proteogenic amino acids (as C- and N-source) in Na-phosphate buffer (pH 6) containing $MgSO_4$. The pH of all media was 6 at the start and after 24 h of incubation, except for incubations with L-tyrosine where the pH at the start and the end of the incubation was between 1 and 2. High, intermediate, and low inducing amino acids were distinguished that had a P_{max} of swelling <5%, $\geq$5% - < 25%, and $\geq$25%, respectively (Fig. 3; Table 3). Alanine and proline highly induced swelling with a P_{max} of swelling of 83.84% and 96.95%, respectively, and a τ of 5.57 h and 3.18 h. P_{max} of germ tube formation of these amino acids was 37.21% and 54.95%, while τ was 12.05 and 11.25 h respectively. Cysteine, glutamine, histidine, leucine, lysine, methionine, threonine, tyrosine and valine were classified as low inducing amino acids with a P_{max} of swelling between 0.55 and 3.65%, while τ was between 2.46 and 15.00 h (modeling only allowed a maximum for τ of 15 h, see Material and Methods, therefore 15 h means $\geq$ 15 h). Their P_{max} of germ tube formation was between 0.17 and 2.02%, and 4.31 and 15.00 h respectively.

4. Discussion

Conidia are dispersed by wind, water or other vectors such as insects. At a certain moment, these spores will settle in an environment of unknown composition. The main decision for a conidium to make is when to germinate. Presence of water is the main prerequisite to sustain germination and outgrowth of spores. Conidia of *Cladosporium halotolerans* and *Penicillium rubens* germinate in pure water (Segers et al., 2017). However, germination of conidia of most other fungi, including *A. niger*, are only triggered

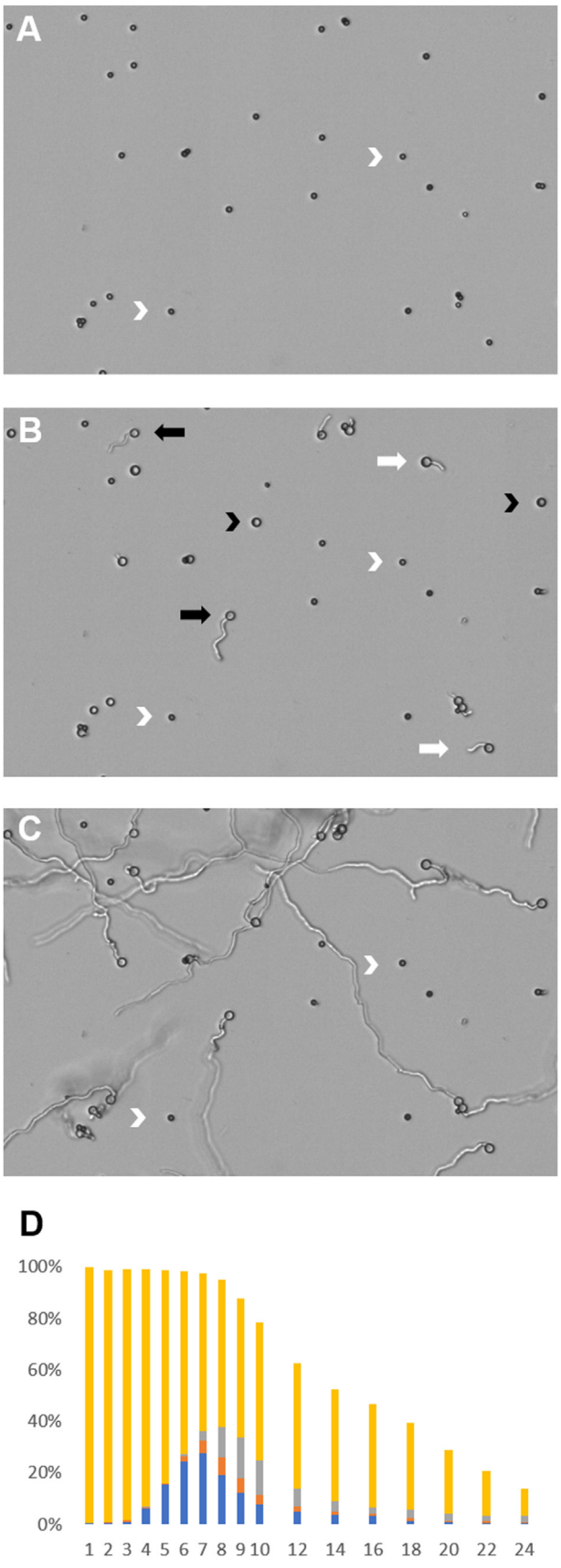

Fig. 1. Germination of *A. niger* conidia in MMG_{50} monitored by oCelloScope. Imaging started after 1 h of settling (t = 1). Selected area of oCelloScope images after 1 (A), 8 (B) and 16 (C) hours. Resting and swollen conidia and germ tubes are indicated by white arrowheads, black arrowheads, and white arrows, respectively, while hyphae are indicated by black arrows. Circularity and surface area were used to classify 489 objects in time as resting (yellow) and swollen (blue) spores and as spores forming germ tubes (orange) and hyphae (grey) (D). Resting conidia had a surface area ≤39 μm² and a circularity >0.97, swollen conidia had a surface area >39 μm² and a circularity >0.97; conidia with germ tubes had a surface area >39 μm² and a circularity >0.75 and ≤0.97, while hyphae had a surface area >39 μm² and a circularity ≤0.75. After 7 h, part of the objects are no longer included in the analysis because the object was obscured by hyphae of other objects or the hypha of the object itself had become too long to be able to trace it back to the original object by the oCelloScope. This explains the decrease in total percentage of the objects.

in the presence of water and certain nutrients. We here assessed the minimal nutrient requirements that activate swelling and/or germ tube formation of *A. niger* conidia. A combination of an inducing carbon source with either inorganic phosphate, inorganic nitrogen or magnesium sulphate was sufficient to activate germination up to 6% of the conidia. This shows that a fraction of the conidia germinate in environments that do not support full outgrowth into a colony. Apparently, conidia, at least a fraction of them, take a chance by germinating without signalling the presence of all nutrients needed to sustain establishment of a colony. Minimal sensing of the environment would allow fast colonization of a substrate in the case all these nutrients are present and would thus give a competitive advantage. The fact that the time needed to reach half P_{max} was similar in nutrient poor and nutrient rich media supports this hypothesis.

Initiation of germination without scanning the presence of all nutrients that are needed to sustain growth can be a successful strategy considering the enormous numbers of conidia that are released from colonies of *A. niger*. The opposite is also observed; even when all nutrients were present did only a fraction of the conidia germinate within 24 h. Only 20% of the conidia produced germ tubes in a minimal medium with 50 mM glucose. Thus, the majority of the conidia remained in their resting state despite the presence of a medium that can fully support establishment of a colony. Activation of germination is probably a bet hedging strategy to prevent that all individuals die when environmental conditions become unfavourable. For instance, temperature may exceed the cardinal temperature of 47 °C during daytime (Krijgsheld et al., 2013), which would kill the germlings but not the conidia. An important question to address is what makes conidia heterogeneous with respect to their activation. Previously, we showed that conidia are heterogeneous in composition of proteins and RNA (Bleichrodt et al., 2013; Teertstra et al., 2017). Possibly, conidia differ with respect to the number of sensors in or near their plasma membrane such as G- or Ras-proteins (Fortwendel et al., 2004, 2008; Lafon et al., 2005).

Particular carbon and nitrogen sources can either activate or support outgrowth of conidia, while others can do neither or both (Hayer et al., 2013, 2014). Inorganic nitrogen compounds, urea, alanine, arginine, glycine, histidine, lysine, and methionine did not activate spores to germinate but could support outgrowth (Hayer et al., 2014). In our case alanine, arginine, glycine were classified as highly or intermediate inducing amino acids, while cysteine, glutamic acid, leucine, threonine and tyrosine and valine lowly induced germination. What could explain the differences between our study and that of Hayer et al. (2014)? Our study did not include alternative organic compounds or an alternative nitrogen source. In contrast, Hayer et al. included the non-inducing sugar galactose as well as nitrate. In addition, Hayer et al. determined swelling after 1 h incubation in a liquid shaken culture. In our set up, swelling and outgrowth were monitored on line for 24 h. Up to 20.71% of the conidia were activated to swell by the group of 9 intermediate inducing amino acids. The time needed to reach half P_{max} could be as high as 15 h explaining why part of these amino acids were

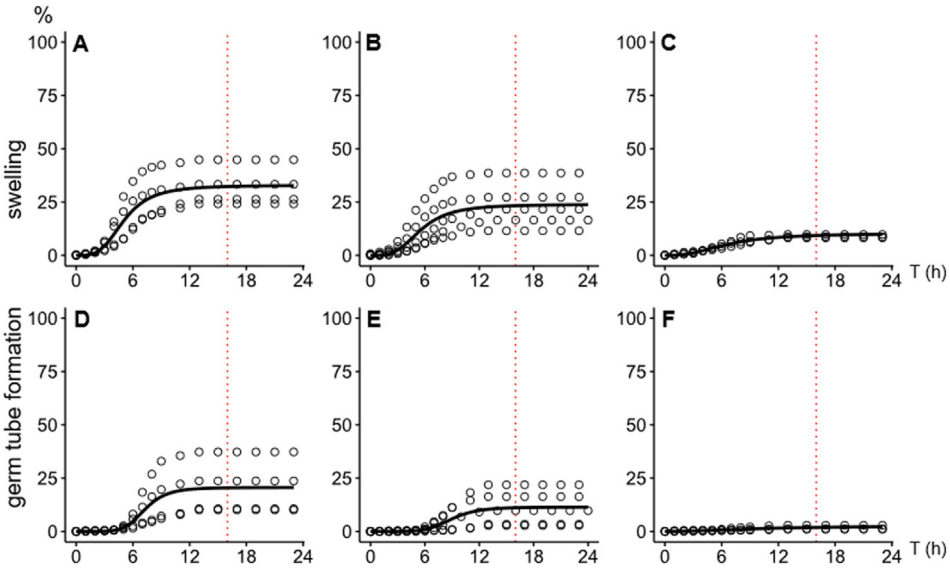

Fig. 2. Parameter estimates of the asymmetrical model used to describe swelling (A–C) and germ tube formation (D–F) of spores in MMG_{50} (A, D), NSG_{50} (B, E) and SG_{50} (C, F). MM consists of $NaNO_3$ (N), $MgSO_4$ (S), KH_2PO_4 (P), KCl (K), and Vishniac trace elements (V). NSG medium contains glucose, $NaNO_3$ and $MgSO_4$, while SG medium contains glucose and $MgSO_4$. Open dots represent data points of individual measurements. Experimental data of 16–24 h were not used in the modelling. Instead, those of 14 h were used (for explanation see Results section). Dotted line indicates this time limit for data used in the model.

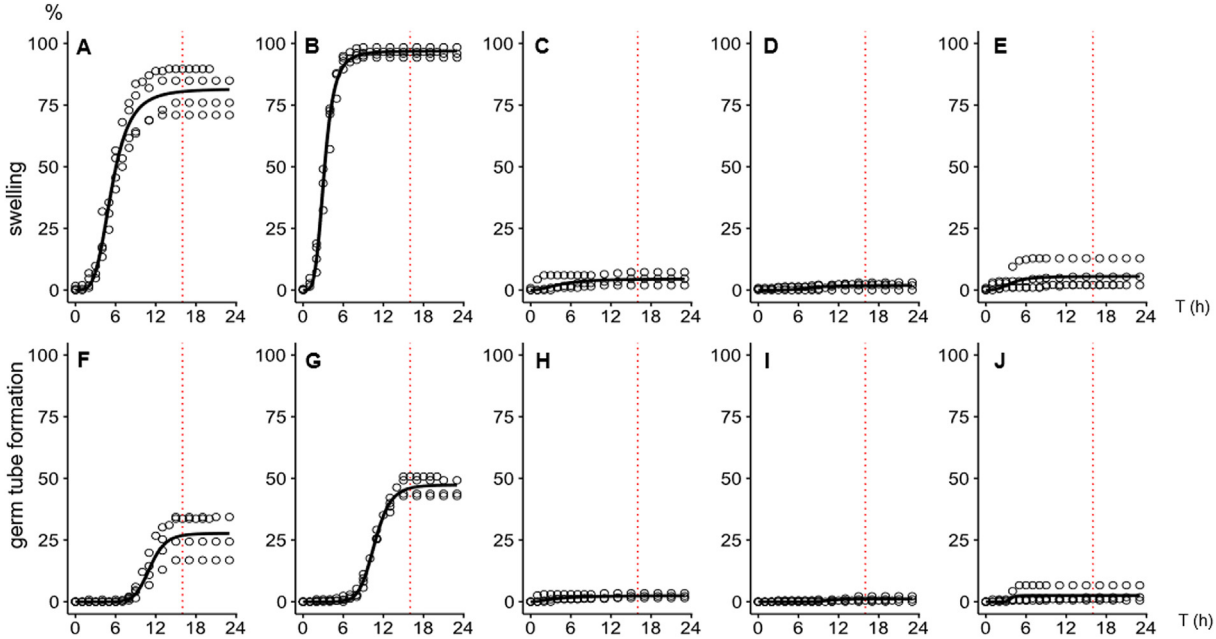

Fig. 3. Parameter estimates of the asymmetrical model used to describe swelling (A–E) and germ tube formation (F–J) of spores in Na-phosphate buffer containing $MgSO_4$ and 10 mM of the nonpolar amino acids alanine (A, F) and proline (B, G), the negatively charged amino acid glutamate (C, H), the positively charged amino acid histidine (D, I), and phenylalanine that has an aromatic side chain (E, J). Open dots represent data points of individual measurements. Experimental data of 16–24 h were not used in the modelling. Instead, those of 14 h were used (for explanation see Results section). Dotted line indicates this time limit for data used in the model.

considered non-activating by Hayer et al. (2014). Our study also used a different analysis system but this does not seem to play a role since we obtained similar results as Hayer et al. (2014) when we tested their medium composition with the oCelloScope imager (our unpublished data).

Proline and alanine were classified as highly inducing by activating >80% of the conidia to swell. In contrast, P_{max} of germ tube formation was only about 50% and 35%, respectively. Notably, 80% of the conidia form germ tubes within 24 h when they are incubated in a mixture of amino acids and even 100% in the combined presence with glucose (data not shown). This implies that spores have different sensors that each contribute to the activation of germination of spores. A question is why certain amino acids are stronger inducers than others. Our study does not indicate that certain classes of amino acids are strictly low inducing amino acids. Another question is why proline and alanine are such strong activators of germination. It is tempting to speculate that *A. niger* senses these amino acids because they accumulate in plants exposed to stress (Meena et al., 2019; Ricoult et al., 2005). For instance, proline accumulates during drought, saline and high

temperature exposure, while alanine accumulates in plants that experience hypoxia. Such plants may be weaker to prevent fungal colonization (Chojak-Koźniewska et al., 2018) and would thereby be an easy target for *A. niger*. Onions are known to be infected by this fungus and this plant indeed accumulates proline under salt and drought stress and during cold storage (Hanci and Cebeci, 2015; Romo-Pérez et al., 2020).

Declaration of competing interest

The authors do not have a declaration of interest. Moreover, the funding organization had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2020.11.004.

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