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Distributions of the growth rate of the germ tubes and germination time of *Penicillium chrysogenum* conidia depend on water activity

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ABSTRACT

The effects of water activities for sporulation (a_{wsp}) and germination (a_{wge}) on the distributions of the growth rate of the germ tubes (μ) and the germination time (t_G) of *Penicillium chrysogenum* conidia were determined by monitoring the length of the same germ tubes throughout the experiments automatically. No relationship between the individual t_G's and μ's could be established. Irrespective of the water activity for germination, μ was greater and t_G was less for conidia produced at $0.95a_{wsp}$ than that at $0.99a_{wsp}$. At 0.99 a_{wge} the mean and the standard deviation of t_G were smaller than those obtained at $0.95a_{wge}$. At $0.99a_{wge}$, normal distributions for μ and t_G were exhibited, but not at $0.95a_{wge}$. The cumulative frequencies were used to reconstruct the germination curves. Great differences in the percentage of spores capable of germination (P_G) and in the mean germination times between conidia produced at $0.95a_{wsp}$ and at $0.99a_{wsp}$ were clearly exhibited at $0.95a_{wge}$, thus demonstrating the paramount influence of sporulation conditions on germination kinetics.

1. Introduction

Most of the experiments that concerned growth of bacteria and moulds were carried out using a large inoculum. However, in most cases, products are contaminated by a low initial level of organisms. In some cases this level maybe as low as a single spore. Therefore, for improving the prediction of microbial growth, the distribution of kinetic parameters such as the lag time should be determined (Dantigny et al., 2007). The effects of water activity (a_w) and temperature (T) on the distribution of radial growth rate (g) and lag time (λ) for growth of single spores of *Aspergillus flavus* and *Fusarium verticillioides* were assessed (Samapundo et al., 2006). All the distributions were normal. The standard deviations were increased by decreasing the a_w, but the effect of T on the distributions was less clear.

Germination can be considered as the main step to be focused on, because a product is spoiled as soon as visible hyphae can be observed (Dantigny et al., 2005). For a single spore, the germination time is defined as the time at which the length of the germ tube was equal to the greatest dimension of the swollen spore (Dantigny et al., 2006). It is well known that spores did not germinate at the same time, but the distribution of the growth rate of germ tubes (μ) and the germination time (t_G) amongst a population of spores has not been examined yet.

Water activity (a_w) was the main factor for explaining the variability of the time to obtain 90% germinated spores (Sautour et al., 2001b). Reduced a_w has the effect of reducing the mean rate of extension of the germ tube, in addition to increasing the mean germination time (Tomkins, 1929; Heintzeler, 1939; Snow, 1949; Charlang and Horowitz, 1971; Pascual, 1998). It is suggested that a_w may have a great influence on the distributions on the germination parameters, μ and t_G.

This study aimed at assessing, not only the effects of the water activity for germination (a_{wge}), but also those of the water activity for sporulation (a_{wsp}) on the distributions of μ and t_G. It was shown that germination plots generated for normal distributions were symmetrical with respect to the inflection point and should be fitted by the logistic model (Dantigny et al., 2007). These authors have also demonstrated that asymmetric germination curves can be simulated assuming skewed distributions. Therefore, the distributions described in this study were tested for normality, the skewness coefficients were evaluated and the germination curves were redrawn from the cumulative frequencies. It was the purpose of this study to analyse the frequency distributions of the parameters germ tube growth rate and germination time in *Penicillium chrysogenum* and to derive

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hypothesis about the biological and mechanistic reasons resulting in different frequency distributions.

2. Material and methods

2.1. Mould and media

P. chrysogenum was maintained on potato dextrose agar (PDA) medium (bioMérieux, Marcy l'Etoile, France) at room temperature (18–25 °C). The media for spore production and spore germination was PDA. The initial pH for all experiments was 5.7 ± 0.1. Water activity (a_w) in these media was adjusted by substituting part of the water with an equal weight of glycerol (Gervais et al., 1988).

2.2. Spore production

The PDA medium was adjusted to $0.95a_{wsp}$ and $0.99a_{wsp}$ and was incubated at 25 °C for 7 d. Spores were collected by flooding the surface of the plates with 4.5 ml of sterile saline solution (NaCl, 9 g l^{-1} of water) containing Tween 80 (0.05% vol/vol; Prolabo, Paris, France) and glycerol for keeping a_w at the set value. After counting the spores on a Malassez cell, the spore suspensions were standardised to 1×10^6 spores ml^{-1} for further examination of about 20–50 spores per microscopic field.

2.3. Spore germination

The device used in this study was made from a Petri dish and was described previously (Sautour et al., 2001a). The PDA medium was adjusted to $0.95a_{wge}$ and $0.99a_{wge}$ and was poured on the internal side of the lid of the Petri dish. In order to equilibrate the relative humidity inside each device after inoculation, an appropriate water/glycerol solution (15 ml) was poured into the Petri dish. The water activity of this solution was identical to that of the culture medium tacked to the lid. The devices sealed with Parafilm® constituted the closed incubation chambers. After solidification, the medium was inoculated with 10 µl of the standardized suspensions.

2.4. Germ tubes monitoring

Without opening the devices, spores (20–50 per microscopic field) were examined through the Petri dish lid every 15 min for a maximum duration of 3 d. In order to examine about 100 spores per experimental condition, experiments were repeated twice to four times depending on the experiment. The temperature was 20 ± 1 °C. The length of the germ tube was measured by means of a Leica DMLB (× 400) (Leica, Rueil-Malmaison, France) connected to a Kodak Mega Plus E.S. 1.0 (Kodak, San Diego, CA) camera. Pictures were analysed using Matrox Inspector 2.2 (Matrox Electronics Systems Ltd, Dorval, Canada). The length of the germ tube (l) was plotted against time. Prior to pooling the data, a Student t-test was performed for comparing the variance and the mean. The growth rate of the germ tube (μ) was evaluated from the slope of the straight line. The germination time (t_G) was the time at which the length of the germ tube equalled one spore diameter and was determined as the intercept between the straight line and the spore diameter (approximately 10 µm).

2.5. Model fitting

Germination data were obtained by calculating the cumulative frequencies. Two models for fitting the germination data were used:

The Gompertz equation :

$$P = A \exp\left(-\exp\left[\frac{\mu_m e(1)}{A}(\delta - t) + 1\right]\right) \quad (1)$$

where A (%) was the asymptotic P value at $t \to +\infty$, μ_m (% h^{-1}) was the slope term of the tangent line through the inflection point (t_i) as defined further, δ (h) was the t-axis intercept of the tangent through the inflection point and t was the time (h). The inflection point was determined as follows (Dantigny et al., 2003):

$$t_i = \delta + A/(\mu_m e(1)) \quad (2)$$

The logistic function : $$P = \frac{P_{max}}{1 + \exp[k(\tau - t)]} \quad (3)$$

where P_{max} (%) was the asymptotic P value at $t \to +\infty$, τ (h) was the inflection point where P equals half of the P_{max}, t was the time (h) and the slope of the tangent line through the inflection point was $kP_{max}/4$, k (h^{-1}) (Dantigny et al., 2006).

2.6. Statistics

The distributions of μ and t_G were analysed using Analyse it® software that is based on the Shapiro–Wilkinson test. Non-linear regressions were performed by SlideWrite 5.0 (Advanced Graphics Software, Inc., Carlsbad, CA, USA). This software was based upon the Levenberg–Marquardt Algorithm. The goodness of fit was evaluated by means of the root mean square error (RMSE) and extracted from ANOVA tables.

3. Results

3.1. Germ tubes elongation

The growth rate of the germ tube was plotted against the germination time for each spore, Fig. 1. At $0.99a_{wge}$, the germination time did not depend on the water activity for sporulation, 9.9 ± 0.8 h and 11.4 ± 1.0 h at $0.95a_{wsp}$ and at $0.99a_{wsp}$ respectively, Table 1. But, at $0.95a_{wsp}$, the variance was 3-fold that one obtained at $0.99a_{wsp}$, Table 1. An increase of the mean growth rate of germ tube can also be observed with decreasing the mean germination time. The same observation was made at $0.95a_{wge}$. The mean growth rate at $0.95a_{wsp}$, 4.19 h, was greater than that one at $0.99a_{wsp}$, 2.23 h, whereas the mean germination time at $0.95a_{wsp}$, 35.1 h, was smaller than that one obtained at $0.99a_{wsp}$, 45.9 h, Table 1.

However, the increase of the mean growth rate of the germ tubes with decreasing the mean germination time that was observed at $0.99a_{wge}$ and $0.95a_{wge}$ was no longer true if the experiments carried out at the same water activities for sporulation and for germination were compared. At $0.95a_{wsp}$ and $0.95a_{wge}$, the mean growth rate of germ tubes was not significantly different than that one obtained at $0.99a_{wsp}$ and $0.99a_{wge}$, Table 1. But at $0.99a_{wsp}$ and $0.99a_{wge}$, the mean germination time was much smaller, 11.4 h, than that one calculated at $0.95a_{wsp}$ and $0.95a_{wge}$, 35.1 h. Moreover, no correlation between individual growth rate of the germ tubes and the germination time was found.

3.2. Distributions of the growth rates of the germ tubes

At $0.95a_{wsp}$ and $0.95a_{wge}$, the W_{value}, 0.974, was not significant at the significance level $\alpha = 0.05$, Table 1. In contrast, the skewness coefficient, $S = 0.554$, was significant. A positive

skewness coefficient means a longer right tail (more density of data on the left hand of the distribution), Fig. 2a.

At $0.99a_{wsp}$ and $0.95a_{wge}$, the positive skewness value, 1.279, was even higher and highly significant, $P<0.001$, and clearly showed that the distribution was not centred, Fig. 2b. As compared to the mean growth rate, $\mu = 2.23\,\mu m\,h^{-1}$, the density of data on the left hand of the distribution (cumulative frequency of about 47) was greater than that on the right hand (cumulative frequency of about 17). In these conditions, the W_{value}, 0.886, was significantly less than 0.947, thus demonstrating that μ was not normally distributed, Table 1.

At $0.99a_{wge}$, whatever a_{wsp} was, W values were greater than 0.947 and significant at the significance level $\alpha = 0.05$, Table 1. In these conditions, μ values were normally distributed, Fig. 2c and d. None of the skewness coefficients was significant.

3.3. Distributions of the germination time

At $0.95a_{wsp}$ and $0.95a_{wge}$, the positive skewness value, 0.987, was highly significant, $P<0.001$, and demonstrated clearly that the distribution was not centred. The smoothing curve exhibited a maximum frequency approximately at 32.5 h, Fig. 3a. More density was observed in the left-hand side of the distribution, thus the skewness coefficient was positive. The W_{value}, 0.923, was significantly less than 0.947, thus demonstrating that t_G was not normally distributed, Table 1.

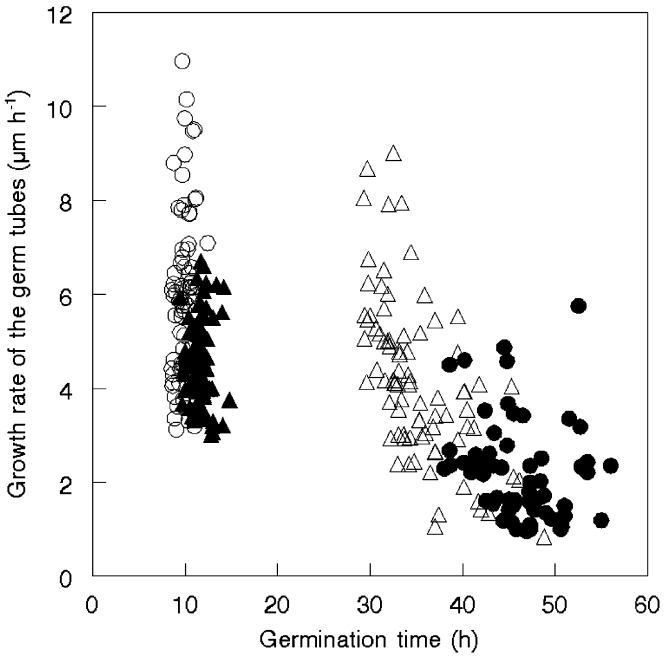

Fig. 1. Growth rate of the germ tubes vs germination time of *Penicillium chrysogenum* conidia at (△) $0.95a_{wsp}/0.95a_{wge}$; (●) $0.99a_{wsp}/0.95a_{wge}$; (▲) $0.95a_{wsp}/0.99a_{wge}$; (○) $0.99a_{wsp}/0.99a_{wge}$.

At $0.99a_{wsp}$ and $0.95a_{wge}$, none of the W and skewness values was significant, Table 1. The density distribution was shown in Fig. 3b.

At $0.95a_{wsp}$ and $0.99a_{wge}$, the mean germination time was 9.9 h, Table 1. The skewness coefficient was not found significant, Table 1. In contrast, the W_{value} was significantly greater than 0.947, thus suggesting that t_G values were normally distributed, Fig. 3c.

At $0.99a_{wsp}$ and $0.99a_{wge}$, both the W and the skewness values were significant, Table 1. In these conditions, t_G values were normally distributed, Fig. 3d.

3.4. Modelling of the germination curves

The germination curves were reconstructed from the cumulative frequencies of the germination time as described Fig. 3. In all cases, but for the Gompertz equation at $0.99a_{wsp}$; $0.95a_{wge}$, the ratio, n_{ger}/n_{obs}, was within the 95% confidence intervals for A or P_{max}. Normal distributions were obtained at $0.99a_{wge}$. It was shown from the RMSE values that the logistic model performed better than the Gompertz equation, Table 2. At $0.99a_{wsp}$; $0.99a_{wge}$, the normal distribution of t_G was skewed, $S = 0.585$ ($P = 0.026$). For a strictly normal, centred distribution, t_G should be equal to the value of τ at the inflection point of the logistic model. Due to skewness, in addition to non-strictly normal distribution, the mean germination time, $t_G = 11.4$ h, was greater than $\tau = 10.6$ h, Tables 1 and 2.

In contrast, at $0.95a_{wge}$, the non-normal distributions were fitted better by the Gompertz equation as illustrated by the RMSE values, Table 2. The variance of t_G were greater at $0.95a_{wge}$ than at $0.99a_{wge}$, Table 1. Accordingly, the slopes of the germination curves at $0.95a_{wge}$ were less than those obtained at $0.99a_{wge}$, Fig. 4.

4. Discussion

From the data of Snow (1949), a non-linear relationship between the mean growth rate of the germ tubes and the mean germination time was established (Dantigny et al., 2007). It was also demonstrated that the non-linear coefficient depended upon the medium. An increase of the mean radial growth rate with decreasing the mean germination time was also shown in this study. However, the mean growth rate of the germ tubes was not significantly different at $0.95a_{wsp}$; $0.95a_{wge}$ and at $0.99a_{wsp}$; $0.99a_{wge}$ thus suggesting that such a relationship should also be

Table 1
Influence of the water activities for sporulation (a_{wsp}) and for germination (a_{wge}) on the distributions of growth rate of the germ tubes (μ) and germination time (t_G) of *Penicillium chrysogenum*

Experimental conditions	n_{obs}	n_{ger}	P_G (%)	μ						t_G					
				Mean ($\mu m\,h^{-1}$)	Var ($\mu m^2\,h^{-2}$)	Normality		Skewness		Mean (h)	Var (h^2)	Normality		Skewness	
						W	P-value	S	P-value			W	P-value	S	P-value
$0.95a_{wsp}$; $0.95a_{wge}$	172	82	47.7	4.19	3.01	0.974	0.089	0.554	0.040	35.1	18.8	0.923	<0.001	0.987	<0.001
$0.99a_{wsp}$; $0.95a_{wge}$	99	64	64.6	2.23	1.09	0.886	<0.001	1.279	<0.001	45.9	18.6	0.981	0.408	0.298	0.306
$0.95a_{wsp}$; $0.99a_{wge}$	74	71	95.9	6.07	3.17	0.964	0.037	0.552	0.055	9.9	0.6	0.966	0.048	0.426	0.131
$0.99a_{wsp}$; $0.99a_{wge}$	88	88	100	4.55	0.80	0.971	0.045	0.416	0.104	11.4	1.0	0.967	0.024	0.585	0.026

$W_{table} = 0.947$ for $n>50$ at $\alpha = 5\%$. n_{obs}: number of spores observed; n_{ger}: number of spores germinated used for constructing the distributions; $P_G = n_{ger}/n_{obs}$: maximum percentage of spores capable of germination.

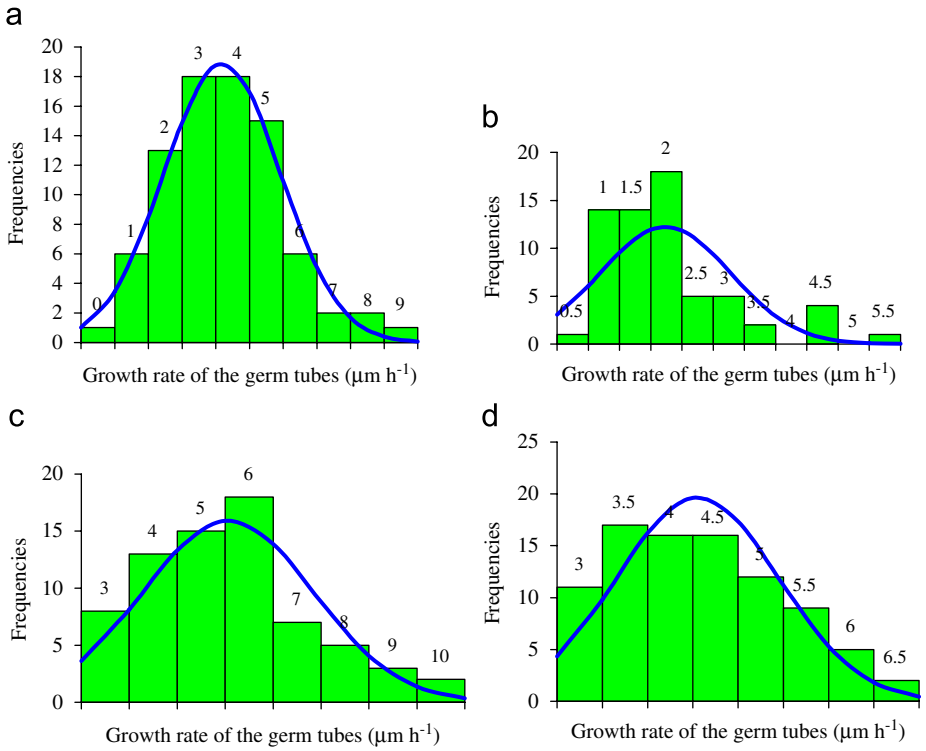

Fig. 2. Observed and smoothed (continuous curve) density distributions of the growth rate of the germ tubes of *Penicillium chrysogenum* at (a) $0.95a_{wsp}/0.95a_{wge}$; (b) $0.99a_{wsp}/0.95a_{wge}$; (c) $0.95a_{wsp}/0.99a_{wge}$; (d) $0.99a_{wsp}/0.99a_{wge}$.

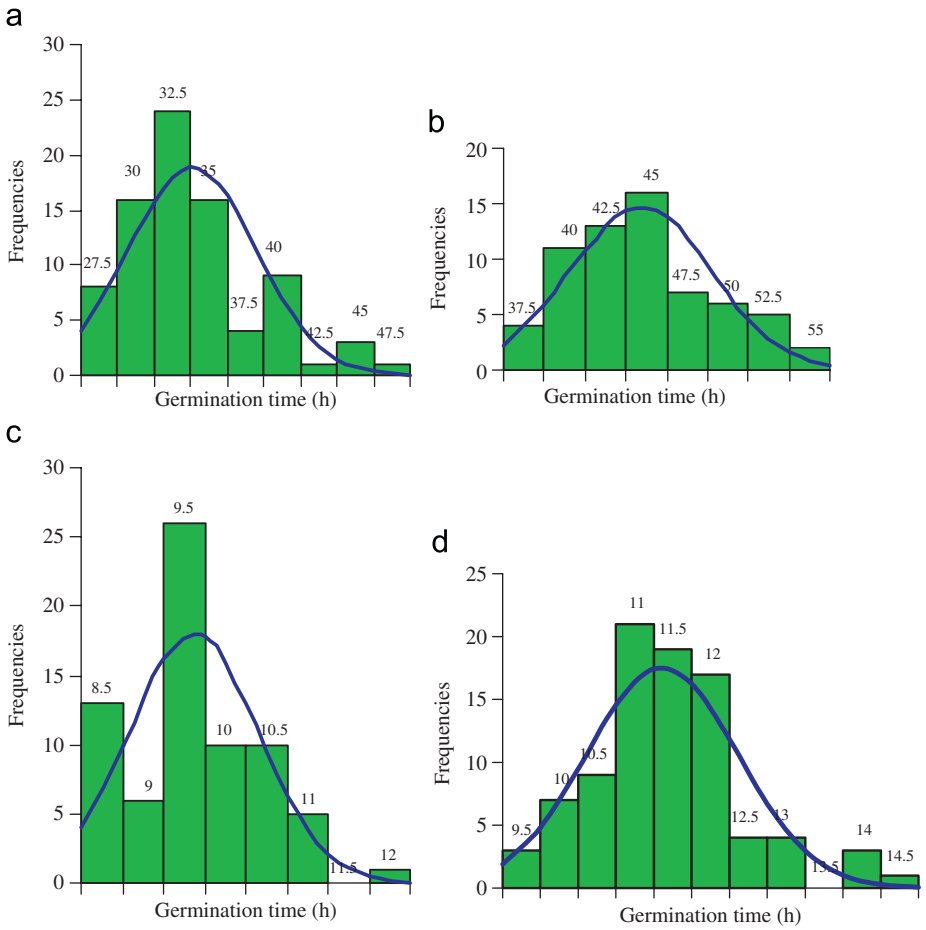

Fig. 3. Observed and smoothed (continuous curve) density distributions of the germination time of *Penicillium chrysogenum* at (a) $0.95a_{wsp}/0.95a_{wge}$; (b) $0.99a_{wsp}/0.95a_{wge}$; (c) $0.95a_{wsp}/0.99a_{wge}$ and (d) $0.99a_{wsp}/0.99a_{wge}$.

Table 2
Parameter estimate [95% confidence interval] and RMSE values obtained by fitting *Penicillium chrysogenum* germination plots at different a_w for sporulation and for germination with the Gompertz and the logistic models

Experimental conditions	Model							
	Gompertz				Logistic			
	A (%)	μ_m (% h^{-1})	δ (h)	RMSE	P_{max} (%)	k (h^{-1})	τ (h)	RMSE
$0.95a_{wsp}$; $0.95a_{wge}$	47.6 [46.2, 49.0]	4.96 [4.55,5.37]	28.6 [28.2, 29.0]	**1.114**	46.2 [44.4, 48.0]	0.415 [0.351, 0.479]	33.5 [33.1, 33.9]	2.858
$0.99a_{wsp}$; $0.95a_{wge}$	69.3 [66.9, 71.6]	5.68 [5.35, 6.02]	38.9 [38.6, 39.2]	**1.076**	64.8 [63.0, 66.7]	0.361 [0.330, 0.393]	44.8 [44.5, 45.1]	1.580
$0.95a_{wsp}$; $0.99a_{wge}$	97.5 [92.7, 102.4]	47.9 [39.2, 56.7]	7.67 [7.48, 7.85]	1.750	95.6 [93.2, 97.9]	2.007 [1.754, 2.260]	8.73 [8.66, 8.80]	**0.594**
$0.99a_{wsp}$; $0.99a_{wge}$	99.8 [94.0, 105.6]	49.2 [38.1, 60.4]	9.57 [9.34, 9.79]	4.754	97.9 [94.1, 101.8]	1.989 [1.615, 2.364]	10.6 [10.5, 10.7]	**3.032**

Bold values indicated a better fit.

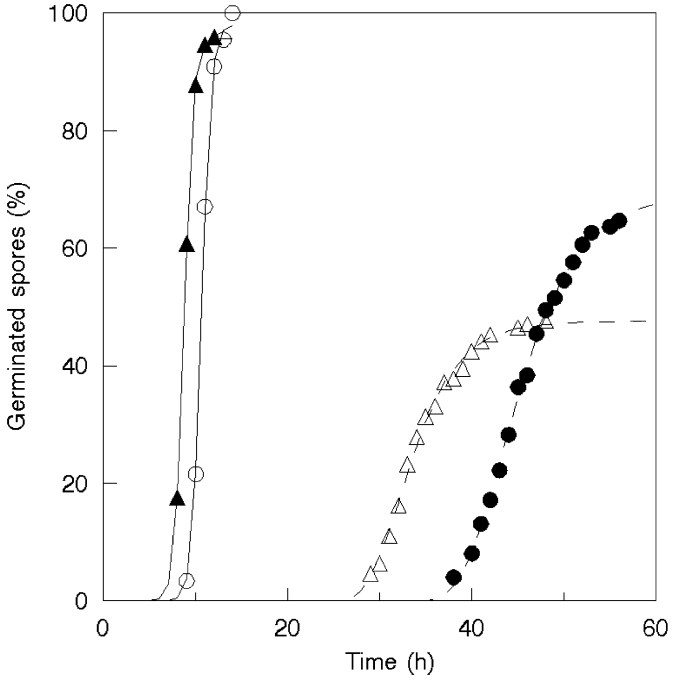

Fig. 4. Germination curves obtained for *P. chrysogenum* at (△) $0.95a_{wsp}/0.95a_{wge}$; (●) $0.99a_{wsp}/0.95a_{wge}$; (▲) $0.95a_{wsp}/0.99a_{wge}$; (○) $0.99a_{wsp}/0.99a_{wge}$. Fitting models: (—) logistic model; (- - -) Gompertz equation.

dependent on the water activity for germination. But, no relationship between the individual growth rate of germ tube and germination time could be determined.

It was shown that, by carefully standardizing the inoculum size, the mean germination time can be substituted for the mean lag time for growth (González et al., 1987; Dantigny et al., 2002). In the objective of substituting a microscopic observation from a macroscopic one, the distributions of the lag time for growth and colony growth rates from single spores of *A. flavus* and *F. verticillioides* were assessed (Samapundo et al., 2006). The colony growth rate depends on the rate of elongation of the leading hyphae spanning the colony's peripheral zone (Trinci, 1971a). In addition, the growth rate of the germ tubes, that is initially constant, subsequently decreases (Trinci, 1971b). Therefore, the distribution of the growth rate of the colony does not necessarily mirror that of the germ tubes. In the objective of assessing the distributions of the germination time indirectly through macroscopic measurements of the radius of the colony, it would be very interesting to measure the growth rate of the germ tube and the resulting colony growth rate of the same spores.

In this study, it was shown that the standard deviation in the germination time at $0.95a_w$ was greater than at $0.99a_w$. It was reported that the standard deviations of the lag time for growth in *A. flavus* were greater at $0.88a_w$ than at $0.98a_w$ and were greater at $0.92a_w$ than at $0.98a_w$ in *F. verticillioides* (Samapundo et al., 2006). The increase of the standard deviation of the germination time is synonymous with a decrease of the slope of the germination curve that has been observed for example by decreasing a_w (Marín et al., 1996). A positive skewness coefficient for the distribution of t_G was obtained at $0.95a_{wsp}$ and $0.95a_{wge}$. It was shown previously through simulation that cumulative frequencies calculated from such a skewed distribution could be better fitted by the Gompertz equation. Our study demonstrated that skewed distributions of the germination time can be observed experimentally at $0.95a_{wge}$. The skewed distributions were not characterised, this could be done once more non-normal distributions will be accumulated.

A decrease in the slope of the germination curve with increasing the age of conidia was reported (Kawanabe, 1986; Araujo and Gonçalves Rodrigues, 2004). A greater standard deviation in the germination time was shown at a low water activity for sporulation. But this could not be explained entirely by the effect of the age of the conidia. In this study, all conidia were harvested from mycelium aged 7 d. Therefore the age distribution amongst the conidia was not greatly affected by differences in the water activity for sporulation. The production of the first conidia at $0.95a_w$ was delayed for about 1d as compared to that at $0.99a_w$. Below $0.95a_w$, mature mycelium did not produce conidia within 7 d. Accordingly water activities less than 0.95 were not tested. This could be done in a next step, when the influence of the age of the conidia on the distributions of the germination and the growth rate of the germ tube would have been assessed.

Inoculum concentration was also proved to be a critical factor in controlling the rate of germination (Ryan, 1948; Araujo and Gonçalves Rodrigues, 2004). Germination was inhibited when spores are present in high densities, an effect observed for example in *Aspergillus niger* and the zygomycete *Syncephalastrum racemosum* (Hobot and Gull, 1980; Barrios-Gonzáles et al., 1989). Self-inhibitors inhibit spore germination reversibly. The major function of self-inhibitors is stated as prevention of premature germination of spores (Chitarra et al., 2004). Highly active extracellular siderophores, that are important in conidial germination, were detected in young cultures of *P. chrysogenum* (Charlang et al., 1982). When exposed to solutions at low water

activity, conidia lose a fraction of their cellular siderophores and subsequent germination failed, or was greatly delayed. The inoculum size density was standardized in this study. The important loss of germinability, that was pointed out at $0.95a_{wge}$ maybe due to the excretion of this self-inhibitor, although no analytical analysis was performed to detect such molecules.

The water activity for germination had a greater influence on germination kinetics than the water activity for sporulation. However, the impact of water activity during sporogenesis was illustrated more clearly at low water activity. At $0.99a_w$, conidia obtained at $0.95a_w$ germinated marginally faster than that at $0.99a_w$. In contrast at $0.95a_w$, the germination time was greatly shortened for conidia produced at a lower water activity. *Penicillium roqueforti* spores produced at $0.88a_w$ with either glycerol or NaCl exhibited shorter germination times than those obtained at $0.99a_w$ by 5.5 and 4 h, respectively (Blaszyk et al., 1998). However, this effect was not shown for *Penicillium aurantiogriseum* and *Penicillium viridicatum*. Recent studies of spoilage fungi such as *Aspergillus flavus* and *Aspergillus ochraceus* have demonstrated that when exposed to osmotic stress, they accumulate significantly elevated amounts of glycerol and sometimes erythritol in their conidia when compared to those present under freely available conditions (Nesci et al., 2004). A lower internal water activity of the former conidia as compared to the latter may be deduced from this observation. It is therefore suggested that the enhancement of the germination of the conidia obtained under osmotic stress may be due to a greater gradient of water across the cell membrane. Some more studies that include monitoring the water activity of the spore during swelling should be carried out to verify this hypothesis.

5. Conclusions

In many studies, conidia were obtained by growing mycelium under optimum conditions. But, in real conditions, spores are rather produced under osmotic or other stresses. In order to adapt to these unfavourable conditions, fungi have developed strategies that allowed fast germination of some conidia, while others were self-inhibited. As a result, an increased variability of the germination time and the growth rate of the germ tubes were observed for adapted conidia as compared to non-adapted ones. The differences between the distributions of these two kinds of conidia were more clearly illustrated at low water activities for germination. The adaptation to stress of the organisms is of paramount importance for explaining the observed kinetics. The development of more accurate and also more realistic models would require a better evaluation of the physiological state of organisms. This could be achieved by assessing either changes in gene expression or intracellular composition with the environmental conditions. The real challenge for the future will be how to integrate mathematically all this knowledge into stochastic or probabilistic models.

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