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Relation between germination and mycelium growth of individual fungal spores

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ABSTRACT

The relation between germination time and lag time of mycelium growth of individual spores was studied by combining microscopic and macroscopic techniques. The radial growth of a large number (100–200) of *Penicillium expansum* and *Aspergillus niger* mycelia originating from single spores was monitored macroscopically at isothermal conditions ranging from 0 to 30 °C and 10 to 41.5 °C, respectively. The radial growth curve for each mycelium was fitted to a linear model for the estimation of mycelium lag time. The results showed that the lag time varied significantly among single spores. The cumulative frequency distributions of the lag times were fitted to the modified Gompertz model and compared with the respective distributions for the germination time, which were obtained microscopically. The distributions of the measured mycelium lag time were found to be similar to the germination time distributions under the same conditions but shifted in time with the lag times showing a significant delay compared to germination times. A numerical comparison was also performed based on the distribution parameters λ_m and λ_g, which indicate the time required from the spores to start the germination process and the completion of the lag phase, respectively. The relative differences $\%(\lambda_m-\lambda_g)/\lambda_m$ were not found to be significantly affected by temperatures tested with mean values of 72.5 ± 5.1 and 60.7 ± 2.1 for *P. expansum* for *A. niger*, respectively. In order to investigate the source of the above difference, a time-lapse microscopy method was developed providing videos with the behavior of single fungal spore from germination until mycelium formation. The distances of the apexes of the first germ tubes that emerged from the swollen spore were measured in each frame of the videos and these data were expressed as a function of time. The results showed that in the early hyphal development, the measured radii appear to increase exponentially, until a certain time, where growth becomes linear. The two phases of hyphal development can explain the difference between germination and lag time. Since the lag time is estimated from the extrapolation of the regression line of the linear part of the graph only, its value is significantly higher than the germination time, t_G. The relation of germination and lag time was further investigated by comparing their temperature dependence using the Cardinal Model with Inflection. The estimated values of the cardinal parameters (T_{min}, T_{opt}, and T_{max}) for $1/\lambda_g$ were found to be very close to the respective values for $1/\lambda_m$, indicating similar temperature dependence between them.

1. Introduction

Fungi are ubiquitous in nature and have evolved over time to colonize a wide range of ecosystems including foods. Airborne transfer of fungal spores is now seen as a significant route for contamination in many sectors of the food industry. If environmental conditions allow growth, the colonization of foods results in spoiled products and subsequent significant economic losses (Burfoot et al., 2000; McCartney and West, 2007). Although industrial standards have been greatly improved in the last years, food spoilage by fungi is still a major concern for the food industry (Dantigny et al., 2005a; Garcia et al., 2009; van Egmond et al., 2007). Fungal presence in food may adversely affect not only the organoleptic value of the commodity, but most importantly its nutritional value.

The concept of predictive mycology has been used to forecast the behavior of spoilage and mycotoxigenic fungi (germination or inactivation, growth, and mycotoxin production) in order to study the shelf life of food products (Dantigny, 2004; Dantigny et al., 2005a, 2005b). Although this approach has, in many cases (Dantigny et al., 2005b; Garcia et al., 2009; Gougouli et al., 2011; Membré et al., 2001), plugged the gap in knowledge on prevention and control of fungi presence and reduced challenge tests applied from the food industry, further research in understanding the dynamics of fungal spores is necessary. More specifically, one area that needs improvement is the modeling of mycelium lag phase, since lag time duration can be a significant part of the total shelf life of foods (Gougouli et al.,

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2011; Gougouli and Koutsoumanis, in press). Within the domain of quantitative mycology, a series of reports have been based, up until now, on empirical descriptions of population kinetics (deterministic approaches), without taking into account realistic events of contamination of foods with low number of fungal spores. However, in order to improve mechanistic descriptions of mycelium lag time, it is important to study the distributions of the lag time of mycelia that originate from individual spores, since that would potentially provide important information with regards to the ability of a single spore to germinate, grow and spoil a food product (Garcia et al., 2010; Samapundo et al., 2007).

As soon as a fungal spore is exposed to favourable conditions it transits from a dormant to an actively metabolizing cell, which gives rise after an ordered sequence of steps to the formation of a visible mycelium. In particular, in the stage of germination, the conversion of the resting spore into an active one occurs and a period of gradual swelling of the spore, including increase in both diameter and weight, follows (Gervais et al., 1988; Nielsen, 1992; Osherov and May, 2001). After a given time the growth polarity is established and a germ tube emerges from the enlarged spore (d'Enfert, 1997; Nielsen, 1992). When the germ tube reaches a certain length, the spore is considered germinated. Eventually, in the next steps, germ tube elongation and branching take place until the mycelium forms a colony (Gougouli and Koutsoumanis, in press). From the early stages of expansion, the peripheral zone of mycelium grows linearly, and from the plot of the mycelium radius versus time, the lag time can be obtained by extrapolating the linear part of the graph to a zero increase in radius. It is obvious here, that the lag time is a geometrically estimated kinetic parameter, which measures the period required for a spore to adjust to the new environment and form into a growing colony (Dantigny et al., 2005a). One significant component of lag time is the germination time, but, currently, very little is known about the relationship between them (Dantigny et al., 2002).

Studies on germination of fungal spores have shown that spores with the same historic treatment (physiological state) and the same germination conditions are very heterogeneous in terms of individual germination time (Gougouli and Koutsoumanis, 2012; Judet et al., 2008; Nanguy et al., 2010). This observed variability in germination time is likely to result from multiple biological sources, such as the existence of self-inhibitors and auto-stimulators that prevent premature and rapid germination of all spores at the same time (Barrios-González et al., 1989; Chitarra and Dijksterhuis, 2007; Chitarra et al., 2004; Feofilova et al., 2012). Thus, the germination time of a spore is not a fixed value and can be characterized better by a probability distribution. Considering the variation characterising the germination time of individual spores, the formation and growth of mycelium colonies originating from these spores is expected to be dependent on this variation in terms of lag time measurements. Up until now, however, kinetic studies of single spores have focused on examining only germination distributions (Dantigny and Nanguy, 2009; Gougouli and Koutsoumanis, 2012; Judet et al., 2008; Marín et al., 1998; Nanguy et al., 2010; Pardo et al., 2006; Schubert et al., 2010), while data on the link between germination and mycelium growth at a single spore level are missing.

In the present study, the relation between germination time and lag time of mycelial growth of individual spores was studied by combining macroscopic and microscopic techniques. The objectives of the study were: i) to determine the lag time of a large number (100–200) of *Penicillium expansum* and *Aspergillus niger* mycelia originating from single spores, ii) to compare the distributions of the lag times with the respective distributions for the germination time of single spores and evaluate the relationship between them, iii) to confirm the above relation using a time-lapse microscopy method which provides videos with the behavior of single fungal spore from germination until mycelium formation, and iv) to evaluate and compare the temperature dependence of lag time and germination time.

2. Materials and methods

2.1. Fungal strains

P. expansum (strain PE-YV1) and *A. niger* (strain AN-YV7) examined in this study were isolated from the environment of a yogurt production unit (Gougouli et al., 2011). The above isolates, which are deposited in the strain collection of the Laboratory of Food Microbiology and Hygiene of Aristotle University of Thessaloniki, were maintained on sterile distilled water containing 0.1% (vol/vol) wetting agent (Tween 80; Merck, Darmstadt, Germany) at 5 °C and were subcultured bimonthly.

2.2. Preparation of inocula

P. expansum and *A. niger* were routinely grown at 25 °C on malt extract agar (MEA; LAB M Limited, Lancashire, United Kingdom) for 7 and 5 days, respectively, to obtain heavily sporulating cultures. Spores were then suspended in sterile distilled water containing 0.1% (vol/vol) Tween 80 by gently scraping the surface of the medium with a sterile spatula. After filtering the spore suspensions through four layers of sterile medical tissue (Aseptica, Athens, Greece) in order to remove any debris (mostly mycelial fragments), their final concentration was determined using a Neubauer counting chamber (Precicolor, HBG, Germany). Immediately after preparation, each spore suspension of each of the fungal strains tested was diluted in Ringer's solution (Lab M Limited) to yield an inoculum count of approximately 10^7 spores/ml and used as quickly as possible. Appropriately diluted spore suspensions were then used to inoculate the samples. For all experiments time zero was defined the time at which the suspension was made.

2.3. Preparation of medium

The standard growth medium used in all experiments was MEA, which was acidified with 10% (vol/vol) lactic acid (Fluka, Buchs, Germany) to pH 4.2. Water activity measurements of uninoculated agar samples at initial and final treatment time of each experiment were made using an AquaLab water activity meter (Model series 3; Decagon Devices, Inc., Pullman, Washington, United States). The water activity values for each treatment remained constant during the storage period, being 0.997 at 25 °C. The pH of the agar before inoculation was also determined at 25 °C by using a pH meter with a glass electrode (pH 211 Microprocessor, Hanna Instruments BV, Ijsselstein, the Netherlands).

2.4. Assessment of germination

The germination kinetic data used in this work were generated in a previous study undertaken in our laboratory (Gougouli and Koutsoumanis, 2012). To evaluate the effect of storage temperature on the germination kinetics of the tested isolates, the germination kinetic behavior of *P. expansum* and *A. niger* was assessed on MEA at isothermal conditions ranging from 0 to 33 °C and 5 to 41.5 °C, respectively. Germination time was defined as the time at which the length of the germ tube was equal to the diameter of the swollen spore. The percentage of germinated spores was calculated as $P(\%) = (N_{\text{germinated spores}}/N_{\text{total spores}}) \cdot 100$ and expressed as percentage germination ($\%P$) versus time at each tested temperature.

2.5. Assessment of growth

Large Petri plates (diameter = 145 mm) containing 50 ml of solidified MEA were inoculated (0.1- ml) by surface plating from the appropriate dilution of spore suspensions in order to obtain approximately 10 mycelia in each Petri plate. After inoculation plates were sealed with

Parafilm to avoid dehydration and stored under controlled isothermal storage conditions (20 Petri plates at each temperature) in high-precision (±0.2 °C) low-temperature programmable incubators (model MIR 153, Sanyo Electric Co., Ora-Gun, Gunma, Japan), which were set at 0, 5, 10, 15, 20, 25, 27.5 and 30 °C for *P. expansum* and at 10, 12.5, 15, 20, 25, 30, 35, 37, 40 and 41.5 °C for *A. niger*. The temperatures tested for each fungal species at the present study were selected considering the findings of previous studies undertaken to characterize the effect of temperature on their growth (Gougouli and Koutsoumanis, 2010) and germination (Gougouli and Koutsoumanis, 2012), to cover the growth region of the species to the greatest possible extent. The temperature during the experiments was monitored using electronic data loggers (Cox tracer, Cox Technologies, Belmont N.C., United States) with the internal and external sensors monitoring temperature of the incubator and MEA, respectively.

The inoculated Petri dishes were examined at appropriate time intervals during storage depending on the tested temperature and fungal isolate, for a maximum of 90 days. Perpendicular diameters (mm) of the developed mycelia were measured using a ruler without opening the dishes, to enable efficient kinetic analysis. The measurements were made at the early stages of growth to assess the linear part of growth and to avoid interactions of the growing colonies. Based on the above inoculation procedure, each mycelium that appeared was assumed to originate from a single spore. In total, the growth of 100–200 mycelia was monitored for each isolate and temperature condition.

2.6. Preparation of an experimental device for microscopic observation

Aiming at monitoring the kinetic behavior of the same fungal spore for prolonged periods (from germination to mycelium formation) an experimental device was developed. The device was prepared in a biological safety cabinet by placing a portion (0.1- ml) of the appropriate dilution of *P. expansum* spore suspension in a defined area (diameter = 1 mm) on the surface of air-permeable polyethylene plastic film in order to obtain one spore on this area. After 30 min drying time, the inoculated area was covered with 1 ml of molten MEA and left to solidify for 1 min. Then, the film was turned upside down and was placed aseptically stretching it over a lid of a Petri plate (diameter = 35 mm), which contained peripherally hydrated cotton. In this way, the desiccation of the medium was minimized for the period of the measurements, as shown from a_w measurements of preliminary experiments. In order for the microscopic observation to be facilitated, the inoculated area was positioned in the center of the lid. Once prepared, the sealed device was quickly transferred from the cabinet to the microscope stage and measurement was started. Measurement began between 10 and 20 min after addition of MEA, due to the difficulty of manually finding the spore in the field of view.

2.7. Microscopy

Spore kinetic behavior was monitored by phase contrast microscopy at magnification of ×40 (Olympus, Tokyo, Japan) initially, and then at ×4 (Olympus) with an Olympus (BX61) optical microscope at room temperature. The experimental device was maintained at 25 °C using a temperature controlled stage (Linkam PE60, Linkam Scientific Instruments, Surrey, United Kingdom). The microscope was equipped with a Z stage with a motor controller, which was controlled by the ScopePro module of the Image-Pro Plus image analysis software version 6.3 (MediaCybernetics Inc., Bethesda, United States) and an auto-focus system, so that the stage could be moved to multiple positions in order to find the plane of the spore during each experiment. Images of the field of view were acquired every 5 min for 48 h using a high resolution device camera (Olympus DP71) and the lamp of the microscope was turned on automatically during the capture of the image. The auto-focus procedure in conjunction with an Extended Depth of Focus system allowed for multiple serial images in different Z-axis planes to be captured, and then combined the best focal areas of the serial images into a single in-focus image (z-stack) providing high quality images. Individual images were compiled to give a sequence of frames for the field of view, allowing the same spore to be monitored throughout the entire experiment. In total, spore's kinetic behavior data were collected from 3 replicate experiments.

The compiled image sequence allowed the individual spore to be followed during dormancy, germination, germ tube elongation, and finally mycelium formation. From the point at which the first tube started emerging, the distance of the apex hyphae from the swollen spore (Distance 1) was measured using the Image-Pro Plus image analysis software until it reached the edge of the optical view. The same procedure was followed for the next three emerged germ tubes from the enlarged spore. The kinetics of each distance were assessed via the use of Image-Pro Plus, which automatically can convert pixels into μm by setting the resolution of the image and the magnification at which the image was captured. After measuring the distances (Distance 1, Distance 2, Distance 3, and Distance 4), data was plotted versus time. Germination time was defined, as in the case of the germination study (Gougouli and Koutsoumanis, 2012), as the time at which the length of the first germ tube was greater than equal to the greatest dimension of the swollen spore.

2.8. Data analysis

For the germination study, for each fungal strain and storage temperature, data of $P(\%)$ over time were fitted to the modified Gompertz equation (Eq. (1)) (Zwietering et al., 1990) for the estimation of the germination kinetic parameters (μ_g and λ_g):

$$P_t = P_{\max} \exp\left(-\exp\left[\frac{\mu_g e(1)}{P_{\max}}\left(\lambda_g - t\right) + 1\right]\right) \quad (1)$$

where t (h) is the time, P_t (%) is the percentage of germinated spores at time t, $P_{\max}$ (%) is the asymptotic P_t value at $t \to +\infty$, μ_g (1/h) is the slope of tangent line through the inflection point, and λ_g (h) is the geometrical lag time (t-axis intercept of the tangent through the inflection point). In this study, all spores (100%) were germinated in all the treatments examined. By replacing t with λ_g in Eq. (1) it can be estimated that the geometrical lag time λ_g corresponds to the time at which 6.6% of spores have been germinated ($P_{\lambda g} = 100 \cdot \exp(-\exp(0+1)) = 100 \cdot 0.066 = 6.6\%$). The inflection point t_{ig} is determined as: $t_{ig} = \lambda_g + P_{\max}/(\mu_g\, e(1))$.

For the growth study, the growth responses of the formed mycelia of each tested fungus, at each storage temperature were plotted against time and fitted to a linear model (Eq. (2)) for the estimation of their growth rate (μ) (mm/h) and their apparent lag time for growth (λ) (h) using Microsoft Excel.

$$D_{(t)} = \mu(t - \lambda) \quad (2)$$

where t (h) is time and $D_{(t)}$ is the diameter at time t. The apparent lag (λ) can be estimated by extrapolating the regression line to $D_{(t)} = 0$.

The obtained distributions of λ were transformed to % cumulative frequencies and fitted to the Gompertz equation as follows:

$$\lambda_t = 100 \exp\left(-\exp\left[\frac{\mu_m e(1)}{100}\left(\lambda_m - t\right) + 1\right]\right) \quad (3)$$

where t (h) is the time, λ_t (%) is the percentage of mycelia which have completed their lag for growth at time t, μ_m (1/h) is the slope of tangent line through the inflection point, and λ_m (h) corresponds to the time at which 6.6% of mycelia have been completed their lag time for growth. The inflection point was determined as follows: $t_{im} = \lambda_m + 100/(\mu_m\, e(1))$.

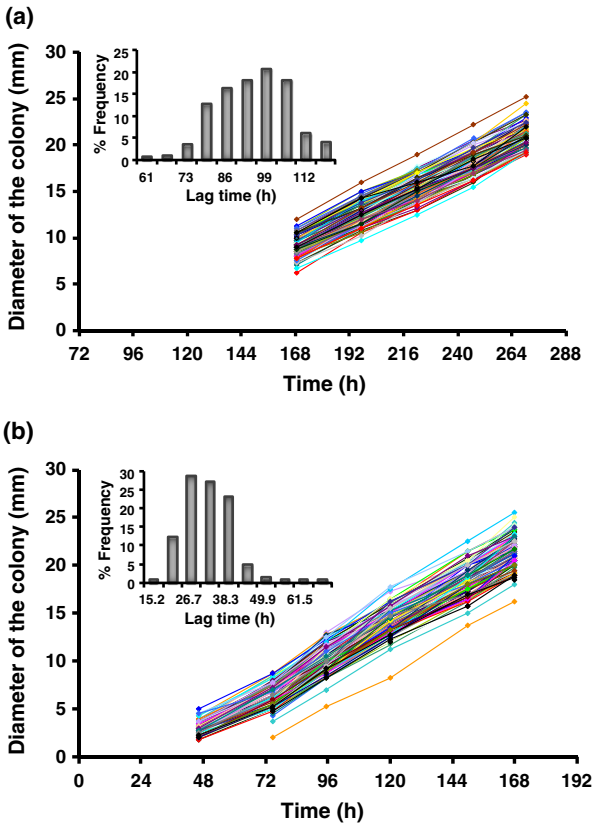

Fig. 1. Representative growth curves of *Penicillium expansum* (a) and *Aspergillus niger* (b) colonies at 10.5 °C and 41.4 °C, respectively, when growth initiates from individual spores. Inset: % Probability distributions of the lag time as estimated from the linear model for each colony.

The effect of temperature on x ($x = 1/\lambda_g, 1/\lambda_m$) was modeled using the Cardinal Model with Inflection (CMI) originally developed by Rosso et al. (1993).

$$x = \frac{x_{opt}(T - T_{max})(T - T_{min})^2}{(T_{opt} - T_{min})\left[(T_{opt} - T_{min})(T - T_{opt}) - (T_{opt} - T_{max})(T_{opt} + T_{min} - 2T)\right]} \quad (4)$$

where T_{opt}, T_{min} and T_{max} (°C) are the theoretical optimum, minimum and maximum temperature, respectively, for germination or growth depending on the case, and x_{opt} is the x at the optimum temperature.

The Table Curve 2D software (Systat Software Inc., San Jose, CA, United States) was used for fitting the data to the models. The goodness of fit of the developed models was evaluated graphically and also by the coefficient of determination r^2 and the root mean square error (RMSE) (Ratkowsky, 2004).

3. Results and discussion

The radial growth of a large number (100–200) of *P. expansum* and *A. niger* mycelia which originated from single spores was monitored at isothermal conditions ranging from 0 to 33 °C and 10 to 41.5 °C, respectively. Representative plots for mycelium growth of single spores of *P. expansum* at 10.5 °C and *A. niger* at 41.4 °C are presented in Fig. 1. The radial growth curves were fitted to a linear model (Eq. (2)) for the estimation of mycelium lag time (λ). The statistics and the % frequency distributions of the estimated lag times for all tested temperatures are presented in Table 1 and Fig. 2, where it can be seen that λ varied significantly among single spores. In addition, the results showed that the distributions of lag time of single spores were shifted to higher values and became more variable as temperature conditions became more stressful. The same trend of increased variance in lag time at stressful conditions was also reported in the study of Samapundo et al. (2007) as well as in investigations conducted with mycelia derived from high number of spores (Baert et al., 2008; Gougouli and Koutsoumanis, 2010). Cumulative (%) frequency distributions were reconstructed from % frequency distributions of the lag time of single spores for each isolate and temperature tested and further fitted to the modified Gompertz model (Eq. (3)). The fitted model described satisfactorily the percentage of spores that completed their lag phase over time for both fungi tested, with coefficients of determination (r^2) ranging from 0.994 to 0.998 for *P. expansum* and from 0.987 to 0.998 for *A. niger*. The estimated kinetic parameters μ_m and λ_m from Eq. (3) are shown in Table 2. The parameter μ_m determines the slope of the cumulative frequency distribution of lag time for single spores. This parameter is related in a reverse manner with the standard deviation of the distribution and constitutes an indication for the spread of the distribution (Dantigny et al., 2007; Gougouli and Koutsoumanis, in press; Judet et al., 2008). The parameter λ_m represents the time at which 6.6% of mycelia from single spores have completed their lag and, furthermore, specifies the position of the distribution.

The main objective of the present study was to investigate how the germination time of individual spores is linked with their imminent mycelium lag time. Dantigny et al. (2002), aiming at substituting a microscopic observation by a macroscopic one, showed that the lag time of *Mucor racemosus* mycelia originating from an inoculum of 225 spores coincided with the completion of the germination process of this inoculum, where more than 99% of spores were germinated. The above

Table 1
Descriptive statistics for lag time for growth of *Penicillium expansum* and *Aspergillus niger* mycelia originating from single spores.

Fungus	T (°C)[a]	Mean	Median	St. dev.	Kurtosis	Skewness	Min.	Max.	*n*
Penicillium expansum	0.1	800.0	803.1	71.2	−0.25	−0.20	627.9	946.6	102
	5.2	182.1	185.5	25.9	−0.50	−0.16	125.7	244.0	197
	10.5	91.4	92.3	11.1	−0.47	−0.09	60.6	116.2	205
	15	55.5	55.6	6.4	−0.72	0.12	41.7	68.8	101
	19.5	37.7	37.2	4.2	−0.17	0.14	27.1	50.2	158
	25.5	30.1	30.1	2.7	−0.48	−0.09	23.2	35.8	153
	27.5	35.9	35.9	3.9	−0.36	−0.03	26.2	46.5	132
	30.5	89.1	89.9	18.8	0.16	0.03	38.2	140.7	114
Aspergillus niger	15	93.1	93.2	14.4	−0.78	0.03	62.0	122.5	126
	19.5	48.5	48.2	3.0	1.37	0.27	40.8	60.5	160
	25.5	27.2	27.3	1.5	1.28	0.05	23.3	32.7	117
	30	18.1	18.1	1.2	−0.15	−0.18	15.0	20.8	157
	35	16.4	16.8	1.2	−0.73	−0.52	13.2	18.4	103
	37	17.2	16.9	1.6	−0.98	0.17	13.4	20.6	149
	40.1	19.3	19.2	1.6	−0.65	0.03	15.8	22.7	102
	41.4	29.2	28.3	7.7	2.61	1.05	15.2	62.6	147

[a] Temperature in the agar during storage as recorded by the temperature monitoring devices.

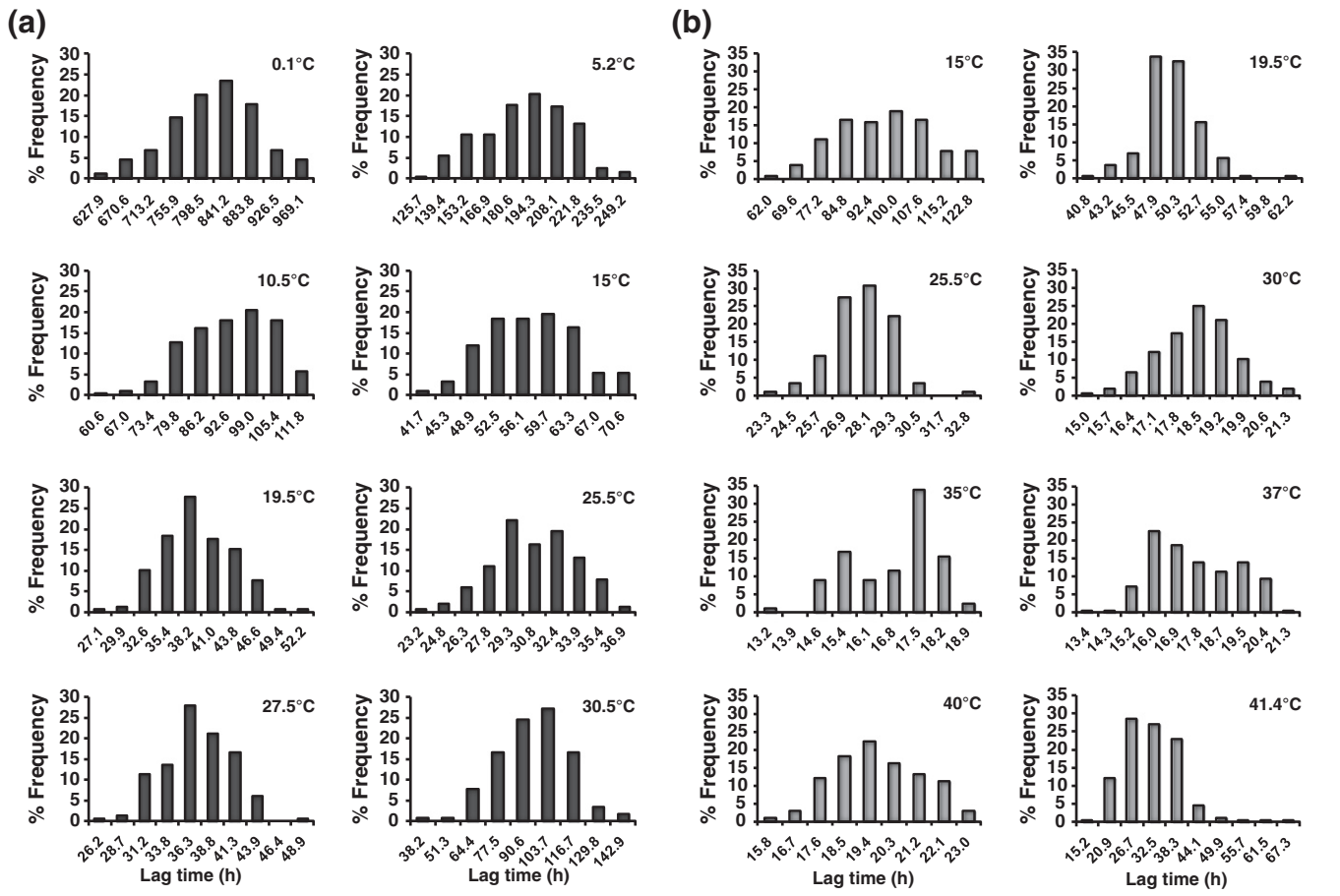

Fig. 2. % Frequency distributions of *Penicillium expansum* (a) and *Aspergillus niger* (b) single spores lag time estimated from the linear model at different temperature conditions.

observation however, is not applicable in the case of single spores. In order to investigate the relation of germination time with the mycelium lag times of single spores, a comparison between the distributions of germination times for single spores of *P. expansum* and *A. niger* produced in a previous work (Gougouli and Koutsoumanis, 2012) with the distributions of mycelium lag times derived from single spores produced in the present study was performed. The germination data were compared graphically (Fig. 3) and numerically with the mycelium lag time data at the different tested temperature conditions. Fig. 3 shows that the % cumulative distributions of the measured mycelium lag time are similar to the germination time distributions under the same conditions but shifted in time with the lag times showing a significant delay compared to germination times. In the case of stressful environments (e.g., 0.1 and 30.5 °C for *P. expansum*, and 15 and 41.4 °C for *A. niger*) the slope of the cumulative distributions for the lag times were lower indicating a more spread distribution compared to germination times. This can be attributed to the fact that the total variance of the lag time includes not only the biological variability of the single spores, but also the uncertainty that arose from the measurement technique used for the assessment of mycelium growth of single spores, as well as from the fitting to the linear model. The above uncertainty is generally higher at conditions close to the boundary of growth (Gougouli and Koutsoumanis, 2010; Marín et al., 2008).

Table 2
Estimated parameters for Gompertz equation (Eq. (3)).

T (°C) [a]	*Penicillium expansum*		T (°C) [a]	*Aspergillus niger*	
	μ_m (1/h) [b,d]	λ_m (h) [c,d]		μ_m [b,d]	λ_m [c,d]
0.1	0.6 ± 0.04	707.6 ± 5.7	15	3.4 ± 0.3	76.4 ± 1.4
5.2	1.7 ± 0.1	149.0 ± 2.3	19.5	15.0 ± 1.9	44.1 ± 0.4
10.5	3.8 ± 0.3	75.7 ± 1.1	25.5	28.2 ± 2.1	25.2 ± 0.1
15	6.4 ± 0.4	49.5 ± 0.4	30	38.4 ± 2.2	16.7 ± 0.1
19.5	10.1 ± 0.5	31.7 ± 0.3	35	36.4 ± 4.6	15.0 ± 0.2
25.5	16.0 ± 0.8	26.5 ± 0.2	37	29.2 ± 1.9	15.1 ± 0.1
27.5	11.1 ± 0.8	30.4 ± 0.3	40.1	24.3 ± 1.1	17.0 ± 0.1
30.5	2.0 ± 0.1	59.2 ± 1.7	41.4	7.5 ± 0.5	21.2 ± 0.5

[a] Temperature in the agar during storage as recorded by the temperature monitoring devices.
[b] The slope term of tangent line through the inflection point.
[c] Corresponds to the time at which 6.6% of mycelia have been completed their lag time for growth.
[d] ±: Standard error of fit.

The delay of the mycelium lag times compared to germination times shown in Fig. 3 was analyzed by comparing the λ_g and λ_m derived from Eqs. (1) and (3), respectively. As demonstrated in Fig. 4, where the relative difference $\%(\lambda_m-\lambda_g)/\lambda_m$ is presented for both isolates as a function of temperature, the environment did not significantly affect the relation of these two variables. In particular, it was found that the difference $\%(\lambda_m-\lambda_g)/\lambda_m$ was relatively constant for all temperatures tested with mean values of 72.5 ± 5.1 (mean ± st.dev) and 60.7 ± 2.1 for *P. expansum* for *A. niger*, respectively. A similar relative difference was also observed for the inflection points t_{ig} and t_{im} with $\%(t_{im}-t_{ig})/t_{im}$ being 72.5 ± 5.5 and 60.6 ± 2.9, for *P. expansum* and *A. niger*, respectively.

The question that arises here is why the lag times of single spores estimated macroscopically from the mycelium growth curve are significantly higher than the germination times. Considering that the lag time is estimated by extrapolating the mycelium radial growth curve to radius = 0 it would be expected to coincide with the germination time which is the start point of the mycelium growth. The

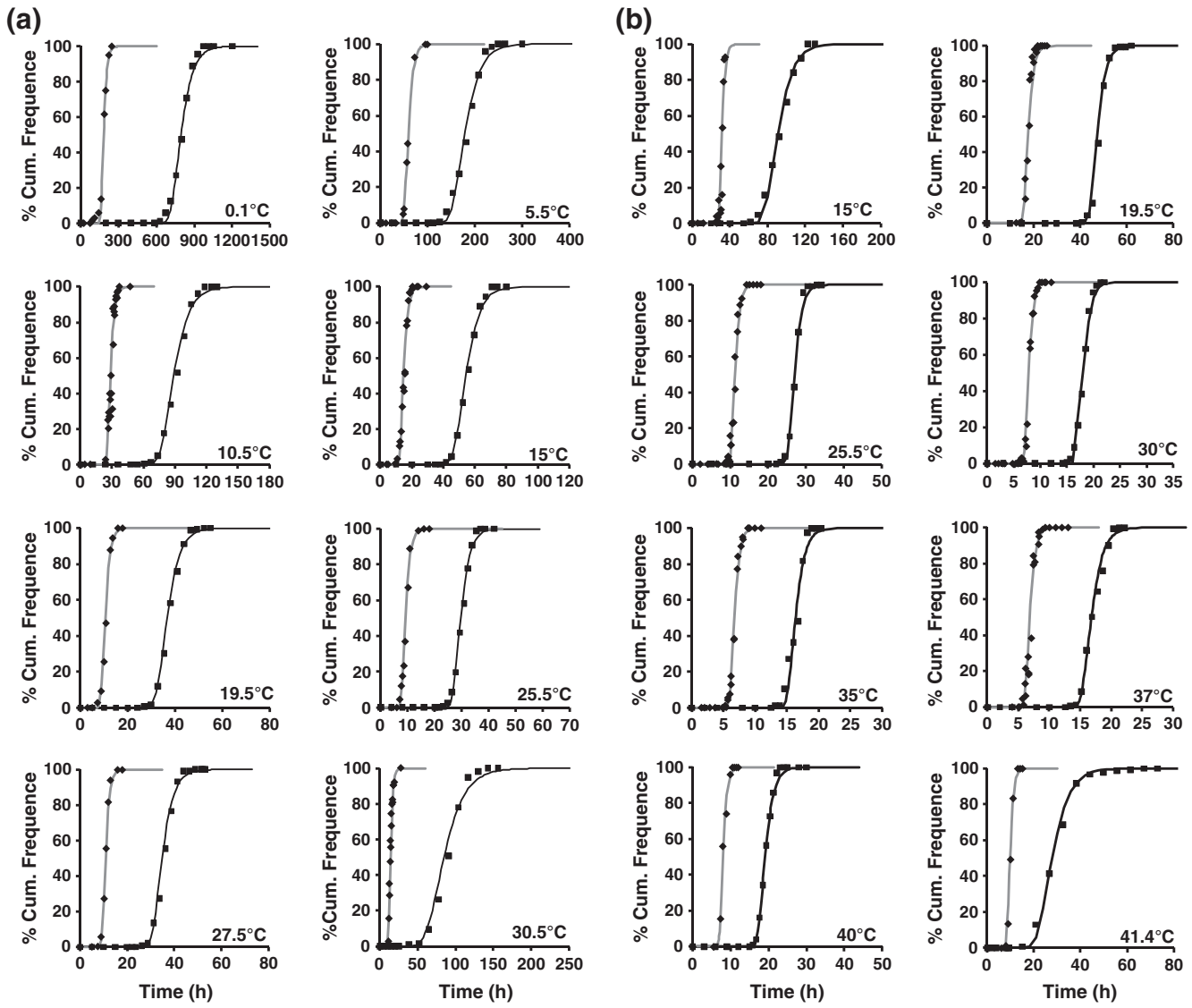

Fig. 3. % Cumulative frequency distributions of the germination times (♦) and lag times (■) of *Penicillium expansum* (a) and *Aspergillus niger* (b) single spores at different temperatures. The lines depict the fitting of the Gompertz model to the germination (gray lines) and lag time data (black lines).

research on the relation between germination and mycelium growth at a single spore level is, in general, limited to the outstanding early studies of Trinci published 40 years ago (Trinci, 1969, 1971a, 1971b, 1974). This can be partly explained by the technical challenge in monitoring the same spore continuously, from germination to complete mycelium development.

In the present study we developed a time-lapse microscopy method for monitoring the behavior of single fungal spore from germination

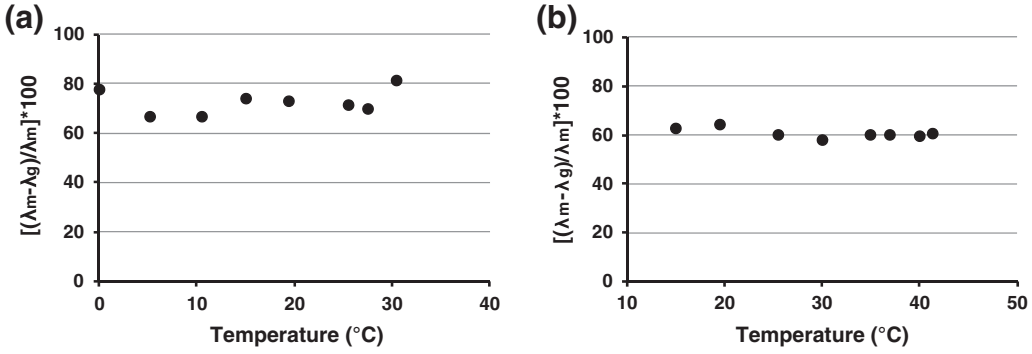

Fig. 4. % Relative difference $(\lambda_m - \lambda_g)/\lambda_m$ for *Penicillium expansum* and *Aspergillus niger* at different temperatures. The λ_g and λ_m values were estimated from the fitting of the germination cumulative frequency distributions of single spores' germination time and lag time, respectively, on the Gompertz model.

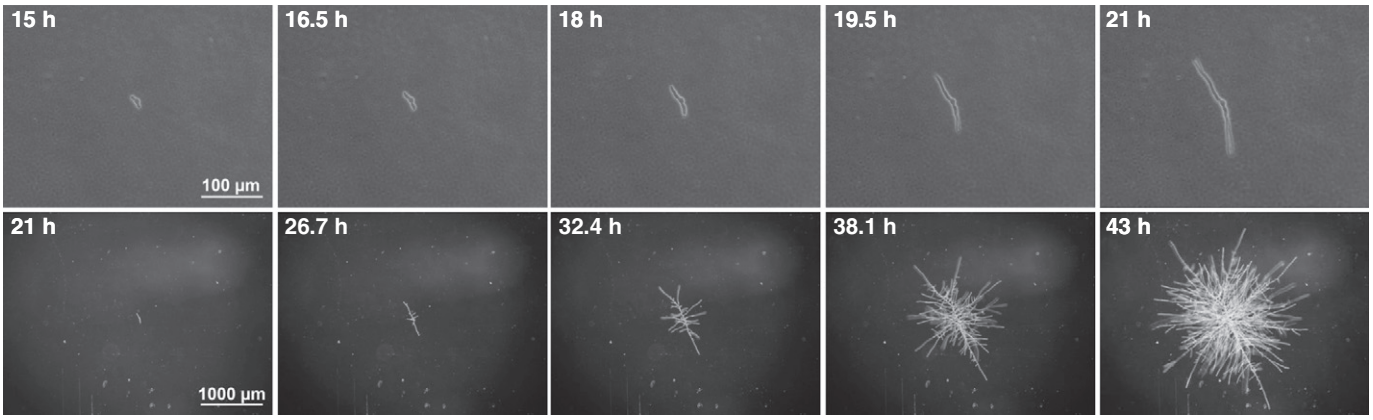

Fig. 5. Selected frames from a time-lapse video of growth of *Penicillium expansum* spore on malt extract agar at 25 °C (first line: magnification ×400, second line: magnification ×40).

until mycelium formation in order to directly relate the germination time with the lag time of mycelium growth. The method is based on an auto-focus procedure in conjunction with an Extended Depth of Focus system (EDF). This procedure allowed for multiple serial images in different z-axis planes to be captured and then combine the best focal areas of the serial images into a single in-focus image (z-stack) providing high quality images. Using the time-lapse microscopy, the behavior of individual spores of *P. expansum* was monitored at 25 °C. Images were captured periodically (Fig. 5) from inoculation to mycelium formation and then compiled to create time-lapse videos (Video 1). The distances of the apexes of the first germ tubes emerged from the swollen spore were measured in each frame, and these data were expressed as a function of time (Fig. 6). As demonstrated in Fig. 6, in the phase of germ tubes emergence and in the early hyphal development, the measured radii appear to increase exponentially, until a certain time (*ca.* 40 h), where growth becomes linear. The above results are in agreement with the finding of Trinci (1969, 1971b) who examined the kinetics of hyphal extension of several fungi via the use of time lapse photography. The increasing radial growth rate in the early stages of growth has been associated with the depletion of the spore's internal storage components and the biosynthesis of new materials during the expansion of the hyphae (Bizukojc and Ledakowicz, 2006; Papagianni, 2004; Trinci, 1971b). Furthermore, considering that fungal hyphae increase in length only at their apices when a mycelium is formed (Trinci, 1971a), the observed transition from exponential to linear growth can be attributed to the weakness of the hyphal tips to incorporate the increasing material that is being supplied or the deficiency of transporting material from distal hyphal regions, probably because of the formation of septa (Prosser, 1994; Prosser and Trinci, 1979).

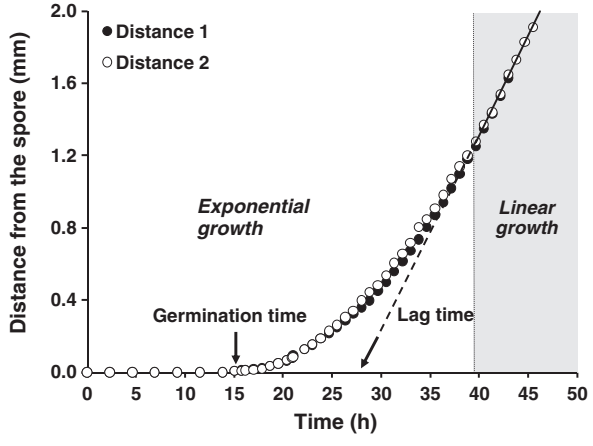

Fig. 6. Resultant graph of distances, which have been measured with the aid of Image-Pro Plus software in a series of images monitoring the growth of a *Penicillium expansum* spore on malt extract agar at 25 °C, against time. Distances 1 and 2 are the distances of the growth zone from the spore at the directions of emergence of the first and the second germ tube, respectively. Germination time was defined as the time at which the first emerged tube has equal length with the greatest dimension of the swollen spore. The lag time for mycelium formation was estimated via the extrapolation of the regression line (from the data observed in linear growth) to x-axis.

As it is shown in Fig. 6, the two phases of hyphal development can explain the difference between germination and lag time. Since the lag time λ_1 is estimated from the extrapolation of the regression line of the linear part of the graph only, its value is significantly higher than the germination time t_G. The relative difference $\%(\lambda_1 - t_G)/\lambda_1$ for all replicates estimated from data generated by the time-lapse microscopy method was found to be 52.3 ± 4.0 (mean ± st.dev). This value is not far to the one obtained in the first part of the study (72.5 ± 5.1) considering that due to the low number of spores tested it is calculated from single values of λ_1 and t_G and not from the distribution parameters λ_m and λ_g. It should be noted that the mycelium growth kinetics from the time-lapse microscopy are in agreement with those obtained macroscopically for *P. expansum* in the first part of the study. In particular, the mean estimated radius growth rate and lag time obtained from the time-lapse microscopy at 25 °C were 0.114 mm/h and 27.4 h, respectively, while the respective values for the mycelia observed macroscopically at the same temperature were 0.105 mm/h and 30.1 h.

The relation of germination and lag time was further investigated by comparing their temperature dependence. Since both germination and lag time are considered as major factors affecting the level of risk for spoilage and mycotoxin production (Dantigny et al., 2002; Pardo et al., 2004, 2005a, 2005b, 2006; Samapundo et al., 2007), the knowledge of their temperature dependence in terms of $T_{min} - T_{max}$ range is considered of great importance. The CMI (Eq. (4)) as chosen to describe this effect, since it incorporates parameters (T_{min}, T_{opt}, T_{max}) which are regarded as biologically interpretable (Cuppers et al., 1997; Ratkowsky, 2004). The r^2 and RMSE values (Table 3), as well as the graphical evaluation from the fittings curves (Fig. 7) indicate the satisfactory performance of the CMI in describing the effect of temperature on $1/\lambda_g$ and $1/\lambda_m$ for both isolates. The estimated values of the cardinal parameters for $1/\lambda_g$ were found to be close to the respective values for $1/\lambda_m$ (Table 3), indicating a similar temperature dependence between them (Fig. 7). Only in the case of T_{max} for *P. expansum* germination the estimated value was found to be slightly higher compared to mycelium growth and this can be attributed to the fact that at conditions close to the germination boundaries, the spores can germinate but are not always able to form a mycelium (Pardo et al., 2004, 2006).

Table 3
Cardinal parameter values and statistics of Cardinal Model with Inflection (Eq. (4)) describing the temperature dependence of the parameters λ_g and λ_m estimated from the Gompertz model (Eqs. (1) and (3)).

Fungus	λ_g						λ_m					
	Parameter	Estimated value	Lower 95% CI	Upper 95% CI	r^2	RMSE	Parameter	Estimated value	Lower 95% CI	Upper 95% CI	r^2	RMSE
Penicillium expansum	T_{min} (°C)	−4.0	−13.7	5.7	0.975	0.010	T_{min} (°C)	−4.0	−7.2	−0.8	0.997	0.000001
	T_{max} (°C)	33.5	29.1	37.9			T_{max} (°C)	31.8	31.2	32.3		
	T_{opt} (°C)	25.1	22.5	27.7			T_{opt} (°C)	24.8	24.0	25.7		
	$1/\lambda_{g\ opt}$ (1/h)	0.126	0.108	0.144			$1/\lambda_{m\ opt}$ (1/h)	0.037	0.035	0.039		
Aspergillus niger	T_{min} (°C)	4.0	1.5	5.7	0.997	0.004	T_{min} (°C)	4.6	−0.7	9.9	0.993	0.000005
	T_{max} (°C)	44.5	43	46			T_{max} (°C)	44.3	42.1	46.5		
	T_{opt} (°C)	36.2	35.4	36.9			T_{opt} (°C)	36.1	34.8	37.4		
	$1/\lambda_{g\ opt}$ (1/h)	0.167	0.160	0.174			$1/\lambda_{m\ opt}$ (1/h)	0.068	0.064	0.072		

In conclusion, the results of the present study provide detailed information on the relation between germination time and mycelium lag time at a single spore level for *P. expansum* and *A. niger*. The observed mirror relationship between germination and lag time indicates that the variability observed in the lag time of mycelia originating from single spores is mainly a result of the biological variability in the stage of germination and, thus, it is feasible to predict the duration of lag time from early growth events, such as germination. In addition, the study provides a quantitative description of the variability of single spore behavior as affected by storage temperature, which can be used as the basis for risk assessment of mould spoilage.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2012.12.006.

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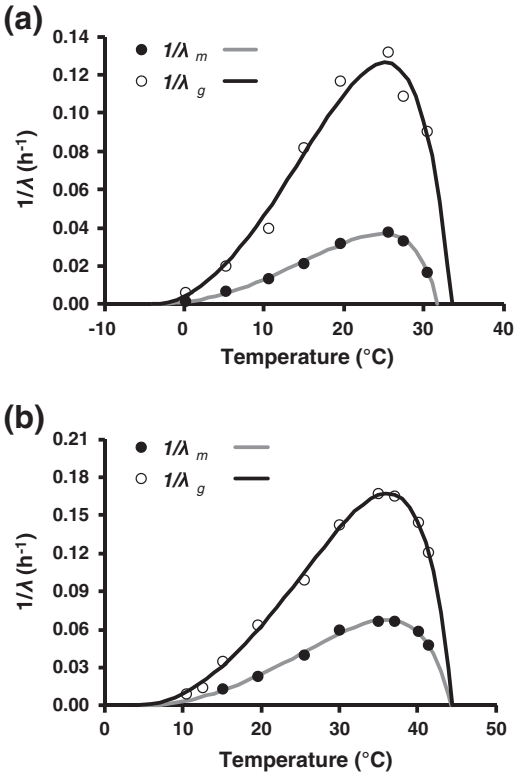

Fig. 7. Modeling the effect of temperature on λ_g (black lines) and λ_m (gray lines) with the Cardinal Model with Inflection for *Penicillium expansum* (a) and *Aspergillus niger* (b). Points represent the obtained values of λ_g and λ_m.

References

Baert, K., Devlieghere, F., Bo, L., Debevere, J., De Meulenaer, B., 2008. The effect of inoculum size on the growth of *Penicillium expansum* in apples. Food Microbiology 119, 170–181.
Barrios-González, J., Martínez, C., Aguilera, A., Raimbault, M., 1989. Germination of concentrated suspensions of spores from *Aspergillus niger*. Biotechnology Letters 11, 551–554.
Bizukojc, M., Ledakowicz, S., 2006. A kinetic model to predict biomass content for *Aspergillus niger* germinating spores in the submerged culture. Process Biochemistry 41, 1063–1071.
Burfoot, D., Brown, K., Xu, Y., Reavell, S.V., Hall, K., 2000. Localised air delivery systems in the food industry. Trends in Food Science and Technology 11, 410–418.
Chitarra, G.S., Dijksterhuis, J., 2007. The germinating spore as contaminating vehicle. In: Dijksterhuis, J., Samson, R.A. (Eds.), Food Mycology — a Multifaceted Approach to Fungi and Food. CPR Press, Boca Raton, FL, pp. 83–100.
Chitarra, G.S., Abee, T., Rombouts, F.M., Posthumus, M.A., Dijksterhuis, J., 2004. Germination of *Penicillium paneum* conidia is regulated by 1-octen-3-ol, a volatile self-inhibitor. Applied and Environmental Microbiology 70, 2823–2829.
Cuppers, H.G.A.M., Oomes, S., Brul, S., 1997. A model for the combined effects of temperature and salt concentration on growth rate of food spoilage molds. Applied and Environmental Microbiology 63, 3764–3769.
Dantigny, P., 2004. Predictive mycology. In: McKellar, R.C., Lu, X. (Eds.), Modelling Microbial Responses in Food. CRC Press, Boca Raton, FL, pp. 313–320.
Dantigny, P., Nanguy, S.P.-M., 2009. Significance of physiological state of fungal spores. International Journal of Food Microbiology 134, 16–20.
Dantigny, P., Soares Mansur, C., Sautour, M., Tchobanov, I., Bensoussan, M., 2002. Relationship between spore germination kinetics and lag time during growth of *Mucor racemosus*. Letters in Applied Microbiology 35, 395–398.
Dantigny, P., Guilmart, A., Bensoussan, M., 2005a. Basis of predictive mycology. International Journal of Food Microbiology 100, 187–196.
Dantigny, P., Guilmart, A., Radoi, F., Bensoussan, M., Zwietering, M., 2005b. Modelling the effect of ethanol on growth rate of food spoilage moulds. International Journal of Food Microbiology 98, 261–269.
Dantigny, P., Marín, S., Beyer, M., Magan, N., 2007. Mould germination: data treatment and modelling. International Journal of Food Microbiology 114, 17–24.
d'Enfert, C., 1997. Fungal spore germination: insights from the molecular genetics of *Aspergillus nidulans* and *Neurospora crassa*. Fungal Genetics and Biology 21, 163–172.
Feofilova, E.P., Ivashechkinb, A.A., Alekhinc, A.I., Sergeeva, Y.E., 2012. Fungal spores: dormancy, germination, chemical composition, and role in biotechnology (review). Applied Biochemistry and Microbiology 48, 1–11.
Garcia, D., Ramos, A.J., Sanchis, V., Marín, S., 2009. Predicting mycotoxins in foods: a review. Food Microbiology 26, 757–769.
Garcia, D., Ramos, A.J., Sanchis, V., Marín, S., 2010. Modelling mould growth under suboptimal environmental conditions and inoculum size. Food Microbiology 27, 909–927.
Gervais, P., Fasquel, J.-P., Molin, P., 1988. Water relations of fungal spore germination. Applied Microbiology and Biotechnology 29, 586–592.
Gougouli, M., Koutsoumanis, K.P., 2010. Modelling growth of *Penicillium expansum* and *Aspergillus niger* at constant and fluctuating temperature conditions. International Journal of Food Microbiology 140, 254–262.
Gougouli, M., Koutsoumanis, K.P., 2012. Modeling germination of fungal spores at constant and fluctuating temperature conditions. International Journal of Food Microbiology 152, 153–161.
Gougouli, M., Koutsoumanis, K.P., in press. Primary models for fungal growth, in: Dantigny, P., Panagou, E.Z., (Eds.), Predictive Mycology. Nova Science Publishers, Inc. New York, USA.
Gougouli, M., Kalantzi, K., Beletsiotis, E., Koutsoumanis, K.P., 2011. Development and application of predictive models for fungal growth at tolls to improve quality control in yogurt production. Food Microbiology 28, 1453–1462.

Judet, D., Bensoussan, M., Perrier-Cornet, J.M., Dantigny, P., 2008. Distributions of the growth rate of the germ tubes and germination time of *Penicillium chrysogenum* conidia depend on water activity. Food Microbiology 25, 902–907.

Marín, S., Sanchis, V., Sáenz, R., Ramos, A.J., Vinas, I., Magan, N., 1998. Ecological determinants for germination and growth of some *Aspergillus* and *Penicillium* spp. from maize grain. Journal of Applied Microbiology 84, 25–36.

Marín, S., Cuevas, D., Ramos, A.J., Sanchos, V., 2008. Fitting of colony diameter and ergosterol as indicators of food borne mould growth to known growth models in solid medium. International Journal of Food Microbiology 121, 139–149.

McCartney, A., West, J., 2007. Dispersal of fungal spores through the air. In: Dijksterhuis, J., Samson, R.A. (Eds.), Food Mycology — A Multifaceted Approach to Fungi and Food. CPR Press, Boca Raton, FL, pp. 65–81.

Membré, J.-M., Kubaczka, M., Chèné, C., 2001. Growth rate and growth-no-growth interface of *Penicillium brevicompactum* as functions of pH and preservative acids. Food Microbiology 18, 531–538.

Nanguy, S.P.-M., Perrier-Cornet, J.-M., Bensoussan, M., Dantigny, P., 2010. Impact of water activity of diverse media on spore germination of *Aspergillus* and *Penicillium* species. International Journal of Food Microbiology 142, 273–276.

Nielsen, J., 1992. Modeling the growth of filamentous fungi. Advances in Biochemical Engineering/Biotechnology 46, 187–223.

Osherov, N., May, G., 2001. The molecular mechanisms of conidial germination. FEMS Microbiology Letters 199, 153–160.

Papagianni, M., 2004. Fungal morphology and metabolite production in submerged mycelial processes. Biotechnology Advances 22, 189–259.

Pardo, E., Marín, S., Solsona, A., Sanchis, V., Ramos, A.J., 2004. Modeling of germination and growth of ochratoxigenic isolates of *Aspergillus ochraceus* as affected by water activity and temperature on a barley-based medium. Food Microbiology 21, 267–274.

Pardo, E., Lagunas, U., Sanchis, V., Ramos, A.J., Marín, S., 2005a. Influence of water activity and temperature on conidial germination and mycelial growth of ochratoxigenic isolates of *Aspergillus ochraceus* on grape juice synthetic medium. Predictive models. Journal of the Science of Food and Agriculture 85, 1681–1686.

Pardo, E., Ramos, A.J., Sanchis, V., Marín, S., 2005b. Modelling of effects of water activity and temperature on germination and growth of ochratoxigenic isolates of *Aspergillus ochraceus* on a green coffee-based medium. International Journal of Food Microbiology 98, 1–9.

Pardo, E., Malet, M., Marín, S., Sanchis, V., Ramos, A.J., 2006. Effects of water activity and temperature on germination and growth profiles of ochratoxigenic *Penicillium verrucosum* isolates on barley meal extract agar. International Journal of Food Microbiology 106, 25–31.

Prosser, J.I., 1994. Kinetics of filamentous growth and branching. In: Gow, N.A.R., Gadd, G.M. (Eds.), The growing fungus. Chapman and Hall, London, UK, pp. 301–318.

Prosser, J.I., Trinci, A.P.J., 1979. A model for hyphal growth and branching. Journal of General Microbiology 111, 153–164.

Ratkowsky, D.A., 2004. Model fitting and uncertaintly. In: McKellar, R.C., Lu, X. (Eds.), Modelling microbial responses in food. CRC Press, Boca Raton, FL, pp. 151–196.

Rosso, L., Lobry, J.R., Flandrois, J.P., 1993. An unexpected correlation between cardinal temperatures of microbial growth highlighted by a new model. Journal of Theoretical Biology 162, 447–463.

Samapundo, S., Devlieghere, F., De Meulenaer, B., Debevere, J., 2007. Growth kinetics of cultures from single spores of *Aspergillus flavus* and *Fusarium verticillioides* on yellow dent corn meal. Food Microbiology 24, 336–345.

Schubert, M., Mourad, S., Schwarze, F.W.M.R., 2010. Statistical approach to determine the effect of combined environmental parameters on conidial development of *Trichoderma atroviride* (T-15603.1). Journal of Basic Microbiology 50, 1–11.

Trinci, A.P.J., 1969. A kinetic study of the growth of *Aspergillus nidulans* and other fungi. Journal of General Microbiology 57, 11–24.

Trinci, A.P.J., 1971a. Influence of the width of the peripheral growth zone on the radial growth rate of fungal colonies on solid media. Journal of General Microbiology 67, 325–344.

Trinci, A.P.J., 1971b. Exponential growth of the germ tubes of fungal spores. Journal of General Microbiology 67, 345–348.

Trinci, A.P.J., 1974. A study of the kinetics of hyphal extension and branch initiation of fungal mycelia. Journal of General Microbiology 81, 225–236.

van Egmond, H.P., Schothorst, R.C., Jonker, M.A., 2007. Regulations relating to mycotoxins in food - Perspectives in a global and European context. Analytical and Bioanalytical Chemistry 389, 147–157.

Zwietering, M.H., Jonkenburger, I., Rombouts, F.M., Van 'T Riet, K., 1990. Modeling of bacterial growth curve. Applied and Environmental Microbiology 56, 1875–1881.