Standardisation of methods for assessing mould germination: A workshop report

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Conference report

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Abstract

The first workshop on predictive mycology was held in Marseille, France, 2–4 February 2005 under the auspices of the French Microbiological Society. The purpose of the workshop was to list the different techniques and definitions used by scientists for assessing mould germination and to evaluate the influence of the different techniques on the experimental results. Recommendations were made when a large consensus was obtained. In order to facilitate the study of germination, alternative methods to microscopic examination were examined.

Keywords: Mould; Fungi; Germination; Standardisation; Methods

1. Introduction

Predictive modelling of filamentous fungi growth has not received the same level of attention as that of bacterial growth (Gibson and Hocking, 1997). Although this observation is true, to date the number of studies devoted to fungal development had increased significantly. Fungal development involves germination followed by growth. Whereas, growth is usually reported as the measurement of the radial growth rate in millimeter per day, techniques and definitions for assessing mould germination vary greatly between authors. Therefore germination data obtained in the literature are difficult to compare. This is of major concern because a lot of time is required for obtaining these experimental data.

Germination can be considered as the main step to be focused on. In fact, visible mycelium appears shortly after germination is completed. Germination requires microscopic observation for evaluating the length of the germ tube (Dantigny et al., 2005a). Moreover, observations and measurements should be carried out without opening the dishes (Magan and Lacey, 1984) and experimental devices should be developed for this purpose. In order to provide reproducible, accurate data, there is an urgent need for standardising methods for assessing mould germination.

Spores, which are responsible for mould dissemination, are always used to study germination. Therefore, the way that spores are obtained, stored, prepared, inoculated and grown may have an influence on the germination kinetics. Two important definitions

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were discussed, these being when is a spore considered germinated and what is the most suitable definition of the germination time? In order to accurately determine biological parameters such as the germination time, models should be used such as those applied to determining bacterial growth rate. The type of model chosen, as compared to another one, may affect the parameter values. A model may also be based either implicitly or explicitly, on assumptions about the physiology of the germination. Eventually, the key step of the modelling procedure is the validation of the model on food products and raw materials. Because the products are often opaque, it is clear that any germination model will be difficult to validate. Alternative methods to microscopic observations should be developed.

Predictive mycology aims at predicting mould development and mycotoxin production in food and raw products (Dantigny, 2004). For this purpose, models that take into account mould specificities are developed. However, this approach is not restricted to moulds responsible for food spoilage and mycotoxin production. As a primary step in mould development, germination is also of great concern in biotechnology for producing starters or fungal metabolites.

Every two years, a meeting dedicated to moulds is organised by the French Microbiological Society. In 2005, two sessions of the 4th meeting were devoted to predictive mycology. These sessions were followed by a workshop on mould germination. The workshop was articulated into five major sections (i.e., techniques, definitions, models, physiology of the germination, and alternative methods to microscopic observations).

About 30 scientists from different countries, working in different fields (either food mycology or biotechnology) and belonging to academic institutions or industry, participated in the workshop. They are studying different kind of moulds (i.e., Zygomycetes, Ascomycetes and Deuteromycetes) that are grown on different substrates under various environmental conditions. Due to the diversity of the participants, we believe that this workshop report may be useful to the whole community of mycologists.

2. Techniques

2.1. Spore preparation

Fresh spores are obtained after mycelium was grown on various semi-synthetic media (a_w 0.99) such as Potato Dextrose Agar (PDA) or Malt Extract Agar (MEA). However, media characterised by lower a_w are required for growing xerophilic fungi such as *Wallemia sebi*. With the objective of producing fresh spores, the environmental conditions are usually set at the optimum for growth in terms of water activity (a_w) and temperature (T). Depending on the radial growth rate of the colony, the incubation time for obtaining conidia produced by most of the Deuteromycetes ranged from 5–12 days, but longer incubation times may be necessary for particular spores. For example, 19–98 days were necessary to produce macroconidia from *Fusarium graminearum* (Beyer et al., 2004) whereas 67±11 days were necessary to produce ascospores from *Gibberella zeae*, (teleomorph *F. graminearum*) Beyer et al. (2005).

The surface of aerial fungal cultures is flooded either with distilled water, saline water or tryptone salt. Very often a wetting agent, Tween80 at a concentration 0.01–0.05% (vol/vol), is added to the solution. The normalisation protocol NF V 18–301 for determining the number of mould spores in cattle food recommended 0.033% (vol/vol) Tween80 (AFNOR, 1983). At these concentrations, the effect of the diluting solution on the lag time for growth, that can be related under certain conditions to the germination time (see Section 6), was not significant (Sautour et al., 2003). Therefore no particular recommendation was made on choice of suspending solution.

Whatever the use of the spore suspension, all participants agreed that the initial time was the time at which spores were suspended in the solution. In fact, spores can swell as soon as they were suspended in an aqueous solution. As stated in the next section, spore suspensions are usually standardised to a certain concentration. Therefore, it was recommended that dilution of the spore suspensions, if necessary, be done quickly as possible. This definition applies without any problem to fresh spores, but may cause some concern with frozen or freeze-dried spores.

Some participants are using sterile bent glass rods for opening the sporangium of Zygomycetes, but the technique consisting of gently brushing the surface of the mycelium with an inoculating loop or other similar device is also suitable for this purpose.

2.2. Inoculation protocols

Inoculating mature spores as a needlepoint is probably the closest technique to what was happening naturally. Due to a possible alteration of the water activity by pouring a solution, this technique is used for studying xerophilic fungi (Pitt and Hocking, 1977). This technique is suitable for studying mould growth, but with some fungi, especially *Penicillium* and *Aspergillus*, it is important to minimise colonies from stray spores (Pitt and Hocking, 1999). However this technique cannot be standardised easily. Most of the recent germination studies were using standardised inoculum. Two groups can be defined depending on whether spore suspensions were spread or poured.

2.2.1. Spreading technique

Petri dishes are swirled so that the inoculum covered the entire surface of the agar. Generally, a high number of spores (greater than $1.0\ 10^4$) and a large volume of suspension are utilised (see Table 1). When spore suspensions are spread over the surface of the agar medium, some patches of this medium are aseptically removed at regular intervals for microscopic observations. Therefore, this technique is invasive, time-consuming and the risk of post-inoculation contamination is increased. In addition, the same spore cannot be studied throughout germination.

2.2.2. Pouring technique

Using this technique a small volume of suspension (less than 100 μl) is inoculated at the same spot (see Table 1). Therefore, fewer spores than those spread with the other technique are

Table 1
Comparison between the spreading and the pouring inoculation techniques cited in the literature

Inoculation technique	Volume	Number of spores	Moulds	References
Spreading	10 µl	$1.5\ 10^4$	*Aspergillus niger*	Plascencia-Jatomea et al. (2003)
	50 µl	$5.0\ 10^4$	*Microsphaeropsis* spp.	Carisse and Bernier (2002)
	0.1 ml	$1.0\ 10^6$–$5.0\ 10^6$	*Aspergillus ochraceus*	Pardo et al. (2005)
	0.1 ml	$1.0\ 10^5$	*Penicillium digitatum*, *P. italicum*, *Geotrichum candidum*	Plaza et al. (2003)
	0.1 ml	$1.0\ 10^5$–$5.0\ 10^5$	*Fusarium moniliforme*, *F. proliferatum*	Marín et al. (1996)
	0.5 ml	0.5–$1.0\ 10^6$	*Coniothyrium minitans*	McQuilken et al. (1997)
Pouring	1 µl	225	*Mucor racemosus*	Dantigny et al. (2002)
	5 µl	$1.7\ 10^4$–$2.0\ 10^4$	*Wallemia sebi*	Patriarca et al. (2001)
	10 µl	$1.0\ 10^4$	*Penicillium chrysogenum*	Sautour et al. (2001b)
	60 µl	300	*Leptosphaeria maculans*	Huang et al. (2001)

inoculated. In contrast to previous technique, spores can be examined microscopically without opening the dishes.

Although we do not recommend any particular technique as they are both widely used. The final objective was to monitor from 10 to 20 spores per microscope field at 100×–400×.

2.3. Experimental device for monitoring germination

An experimental device developed for an easy assessment of germination was shown in Fig. 1.

The device aimed at observing microscopically the spores through the lid, without opening the dishes. In each dish, three patches of germination medium were applied to the lid, and different control solutions can be poured into the bottom. To apply the medium to the lid, a sterile dish was opened in a laminar flow cabinet. Three small glass cylinders were placed on the internal side of the lid and filled with sterile germination medium to a thickness of about 1 mm. After the medium had solidified, the glass cylinders were removed and each surface was ready for inoculation. A droplet of spore suspension was poured at the centre of each patch. After inoculation, Petri dishes were hermetically closed. Providing Petri dishes were marked, the germination of the same spore can be monitored throughout the experiments (Dantigny et al., 2005b).

3. Definitions

3.1. When is a spore germinated?

The definition of when a spore is considered germinated is based on a comparison between the length of the germ tube and the diameter of the spore that is assumed round shaped. Depending on whether spores were considered germinated, (when the length of the germ tube was equal to (i) one half or (ii) twice the spore diameter), the germination time of *Mucor racemosus* varied by about 30% and 25% at 25 and 15 °C, respectively.

It was shown that the definition had an important effect on the germination time. However, none of these definitions was used. All participants agreed to consider a spore germinated when the length of the longest germ tube was greater to equal the greatest dimension of the swollen spore. This definition that applied also to a non-round shaped spore and to a spore with more than one germ tube was also in accordance with the literature.

The development of image analysis for monitoring spore germination (Paul et al., 1993; Oh et al., 1996) was mentioned. However, few laboratories have developed this technique. It would have been unrealistic to provide a definition of a technique that is not routinely used.

3.2. Germination time

Germinated spores eventually form mycelium. Based on this observation, the germination time was defined by some authors as the time at which mycelium was observed (Table 2). But, in most cases the germination time was based on microscopic observations. A morphological definition was given for defining a germinated spore in the Section 3.1. When a population of spores is considered, the definition should be based on another criterion.

In fact, all spores were not germinated at the same time. Each spore was characterised by an individual germination time. For a population, the percentage of germinated spores was calculated as: $P\ (\%) = (N_{\text{germinated spores}} / N_{\text{total spores}}) \times 100$.

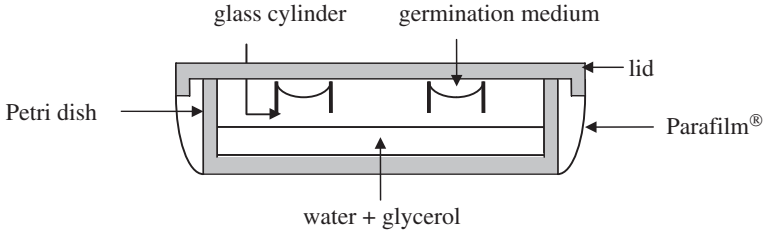

Fig. 1. Example of experimental device developed for observing mould spores microscopically without opening the dish (Sautour et al., 2001a).

Table 2
Definitions of the germination time reported in the literature

Definition	Percentage criterion	Reference
Change in the appearance of the inoculum	na	Ayerst (1969)
Visible mycelium on inoculated points	1/50th	El Halouat and Debevere (1997)
Significant number of germ tubes	na	Pitt and Hocking (1977)
ns	ns	Hocking and Pitt (1979)
ns	ns	Wheeler et al. (1988)
Percentage of germinated spores	10% of total spores	Magan and Lacey (1984)
Percentage of germinated spores	50% of total spores	Hocking and Miscamble (1995) Patriarca et al. (2001)
Percentage of germinated spores	90% of total spores	Sautour et al. (2001b)
Percentage of germinated spores	50% of viable spores	Huang et al. (2001)

na: not applicable, ns : not stated.

From an initial value, P_0=0%, the percentage of germinated spores increased with time up to a maximum percentage of germination, P_{max}. The definition of the germination time varied greatly, depending on the source (Table 2). It appeared during the workshop that people concerned with food spoilage moulds were setting the germination time definition at a low percentage (say 10%). In contrast, people concerned with producing starters or fungal metabolites were using a greater percentage (say 90%).

The germination time, t_G, is dependent on the percentage set for its definition, especially under non-optimal conditions. For example, the germination time of *Fusarium moniliforme* at 0.90 a_w was equal to 36 and to 80 h, at P=10% and P=90%, respectively (calculated from the data of Marín et al., 1996). The need for having a precise definition of the germination time was illustrated in Table 2. Chronologically the definitions given by Hocking vary from a significant number of the germ tubes (Pitt and Hocking, 1977) to a percentage of germinated spores equal to 50% (Hocking and Miscamble, 1995).

It was decided during the workshop that 50% represented a good trade-off between 10% and 90%. In addition, this value has been widely used in the literature as seen in Table 2. However, under harsh environmental conditions some spores were unable to initiate a germ tube, thus leading to a maximum percentage of germination less than 100%. For this reason, the definition based on a percentage of germinated spores equal to 50% of the viable spores (Huang et al., 2001) should be preferred.

4. Models

Biological growth parameters (e.g., radial growth rate, lag time, germination time…) are very often calculated from model equations. The probability that a time at which spores are examined coincided with germination time is close to zero. Therefore, spores are examined at regular intervals, thus enabling a germination curve: P (%)=$f(t)$ to be drawn. Two models for fitting the germination data can be used (Dantigny et al., 2003):

the Gompertz equation:

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

where A (%) was the asymptotic P value at $t\rightarrow+\infty$, μ_m (h^{-1}) was the slope term of the tangent line through the inflection point (t_G) as defined further, λ_{ge} (h) was the t-axis intercept of the tangent through the inflection point and t was the time (h).

the logistic function: $$P = \frac{P_{max}}{1+\exp[k(\tau-t)]} \quad (2)$$

where P_{max} (%) was the asymptotic P value at $t\rightarrow+\infty$, k (h^{-1}) was the slope term of the tangent line through the inflection point, τ (h) was the inflection point where P equals half of the P_{max} and t was the time (h).

By using any of these equations, it is possible to estimate accurately the time necessary to reach a certain percentage of germination. For example, the time at which 50% of the maximum percentage of germination is defined as:

$t_G = \lambda_{ge} + A/(\mu_m e(1))$ and
$t_G = \tau$ for equations (1) and (2), respectively.

Preliminary results on the germination of *Penicillium chrysogenum* had demonstrated that the choice of the model had no significant effect on the estimation of the germination time. However, from a theoretical point of view, the models are different. Both the models exhibited an inflection point at t_G. But the logistic function is symmetrical with respect to that point, not the Gompertz equation. By choosing a model, assumptions on the physiology of the germination are made implicitly.

5. Physiology of the germination

Assuming the definition of the germination time as the time at which 50% of the viable spores had germinated, two groups can be distinguished within the population. Individual spores characterised by individual germination times less than t_G (first group), and greater than t_G (second group). If the growth of the germ tube of the whole population is constant, and if the individual germination times amongst each group are equally, normally distributed, then the germination curve should be symmetrical as compared to the inflection point. Preliminary results on the germination of *P. chrysogenum* suggested that experimental data were fit more accurately with the logistic function than with the Gompertz equation.

At present, the available studies showed a linear increase of the length of the germ tubes during the early germination phase. Unfortunately, in these studies, the rate of elongation was determined from an average of the measured length of 20 (Snow, 1949) and 5 to 6 (Trinci, 1971) germ tubes. In order to verify this assumption, experiments allowing the measurement of the

length of individual spores throughout germination should be carried out.

6. Alternative methods to microscopic observation

The development of alternative methods to microscopic observation would appear a significant breakthrough in the studies of germination. Firstly, microscopic observations are tedious and time-consuming. Secondly, the opacity of products has made model validation very difficult. To overcome these disadvantages, alternative methods aimed at substituting microscopic observations for macroscopic ones. For example, an arbitrary parameter "rejection time" (i.e., the time required to form a 2 mm diameter colony) was introduced by Horner and Anagnostopoulos (1973) to express the shelf life of jam. The rejection time was affected by the lag for growth and the radial growth rate, μ (mm d^{-1}) of the colony. An arithmetic plot of increase in colony radius, r, against time was, after the lag phase, λ (d), initially linear, as found for fungi by Trinci (1969). A simple linear model with breakpoint was used for representing the plot:

$$r = \mu(t - \lambda) \quad (3)$$

To calculate the lag, this linear section of the graph is extrapolated to a zero increase in diameter. The intercept on the time axis is defined as the lag. In order to substitute microscopic observations for macroscopic ones, the relationship between the lag and the germination time was assessed for *M. racemosus* (Dantigny et al., 2002). In these studies, it was shown that, for a number of inoculated spores arbitrarily set to 225, the lag coincided with the completion of the germination process (say $P \geq 95\%$). It was shown for *P. chrysogenum* that the lag was dependent on the initial number of spores. This was explained by the ability of a large number of spores to form more readily a visible colony than a fewer number of spores (Sautour et al., 2003).

The effect of the number of spores inoculated, N, on the lag was described by the following relationship (González et al., 1987):

$$\lambda = \lambda_1 - k \cdot \log(N) \quad (4)$$

where λ_1 was the theoretical maximum lag obtained for one spore.

In order that the lag for growth equals the germination time as defined in Section 3.2., a greater number of spores than 225 should be inoculated. Eq. (4) can be used to calculate the number of spores that should be inoculated for having the lag equals to the germination time. This alternative approach for determining macroscopically the germination time should now be calibrated for various moulds.

7. Conclusions and recommendations

The number of participants to this workshop proved that standardising techniques for studying germination was a great concern. This workshop allowed these techniques to be listed. Fortunately, the use of different techniques has no influence on the acquisition of accurate raw data. For example, the nature of the diluting solution has no significant effect on germination kinetics.

Germination studies were often based on microscopic observations. For this purpose, two types of techniques for the inoculum preparation were described. The first one consisted of spreading the suspension of spores whereas the second one involved applying a droplet onto the surface of the agar medium. The second technique allows microscopic observations without opening the dishes. More spores are spread than those that are poured. Hence the number of spores per microscopic field is independent from the technique chosen.

In contrast, it was demonstrated that biological parameters such as the germination time were greatly dependent from the definitions. It was clear that people involved in food spoilage preferred definitions of the germination time based on a very low level of germinated spores. In contrast, people whose interest was producing starters or fungal metabolites advocated germination of all the spores. Other definitions were reported in the literature. This observation was helpful in reaching a conclusion that the accepted definition of the germination time should not be purpose dependent. It was also acknowledged that a definition for a biological parameter was often related to a model.

The Gompertz equation and the logistic function are available for describing germination kinetics. The time for which the curve exhibited an inflection point can be easily calculated from the model equations. In addition that time has a biological meaning. It represents the time at which 50% of the viable spores had germinated (Huang et al., 2001). Accordingly, that definition was recommended for the germination time.

A model is characterised by two aspects: (i) a predictive one and (ii) a descriptive one. The predictive aspect of a model allowed results to be obtained without carrying out additional experiments. For this purpose, a model should be validated on a food product by means of challenge tests. Due to the opacity of products, alternative methods for assessing germination should be developed. These methods are based on macroscopic observations. The plot of the colony radius versus time can be extrapolated to calculate a lag. It was shown that the germination time can be substituted for the lag providing the inoculum size was carefully controlled. This approach appeared effective but should be calibrated for the moulds of concern.

Two models (i.e., the Gompertz model and the logistic function) can be used for calculating the germination time. By using a model it was underlined that assumptions on the physiology of the germination were implicitly assumed. A model enables the codification of the knowledge and assumption of the physiology. The distribution of the rate of elongation of the germ tubes amongst spores remains unknown, thus preventing from favouring a model.

Predictive mycology aiming at predicting fungal growth and production of metabolites is not restricted to food spoilage. This field can extend its use to biotechnology, phytopathology, as suggested by the large audience of the workshop.

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