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Impact of Environmental Conditions on the Germination Time of *Penicillium chrysogenum*

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Abstract In predictive mycology, most of the studies have been concerned with the influence of some environmental factors on fungal growth and production of mycotoxins at steady-state. However, germination should be the main step to be focused on, because a product is spoiled shortly after fungal conidia had germinated. In most of the studies dedicated to fungi, germination experiments were carried out immediately after spores were produced and harvested. Very few studies were concerned with the effect of storage conditions on the germination of fungal spores. The aim of this study is to show the impact of relative humidity, time of storage and temperature on germination time for the mould free shelf-life of food products determination. The effects of relative humidity, RH, time of storage, t and temperature, T on τ were assessed according to a Doehlert design in the range, 20-100%, 2-28 days and 5-25°C, respectively. The paramount influence of the relative humidity during storage on the germination time of the conidia of *Penicillium chrysogenum* was highlighted. An increase of the germination time was shown at reduced RH's, for increased periods of time and at lower temperatures. We can also observe a germination time, more important for conidia stored compared to fresh conidia obtained in the laboratory. The key factor was relative humidity, but time may be also of paramount importance for storage periods that exceed many weeks.

Keywords: *germination fungi, mould, Penicillium chrysogenum, storage, humidity, temperature*

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1. Introduction

In laboratory studies, freshly harvested conidia are used to study the effect of environmental factors on germination time. In contrast, conidia that are disseminated into the environment can spend a period of time under unfavourable conditions prior to germination. The standardized protocol for producing spore suspensions can be summarized as follows. Fresh spores are obtained after mycelium has been grown on various semi-synthetic media (0.99 aw) such as Potato Dextrose Agar (PDA) or Malt Extrat Agar (MEA) [1]. However, media characterised by lower aw are required for growing xerophilic fungi such as Wallemia sebi. Then, spore suspensions are obtained by flooding the mycelium with an aqueous solution usually set at aw of the subsequent experiments. It has been shown that harvesting spores in isotonic conditions resulted in better viability due to the compatible solutes, such as sugar polyols, remain in the spores [2]. Spore suspensions are also usually filtered so that the large mycelium fragments are removed and standardized to a desired concentration. In order to produce spores within the minimum period of time, the environmental conditions are set at the optimum for growth in terms of water activity and temperature. Additional objectives can be to increase the number of produced spores [3] or to avoid erratic [4]. In laboratory, spores are produced under optimum conditions, but such conditions are very unlikely in the environment [5]. It is also important to be in same conditions than contamination by fungi for improve free shelf-life of food products determination. Therefore, the objective of this study was to assess the effects of RH, time of storage and temperature on the germination time of *Penicillium chrysogenum* by means of a Doehlert design, in the range 20-100%, 2-28 days and 5-25°C, respectively.

2. Materials and Methods

2.1. Mould

Penicillium chrysogenum was isolated from a spoiled pastry product and identified according to the descriptions of Samson *et al*. (1995) [6].

2.2. Production of Dry Harvested Conidia

Potato Dextrose Agar (PDA) medium, 0.99 a_w was central point inoculated and incubated at 25°C for 7 days. Conidia were collected by turning the plates upside-down then gently tapping the bottom of the dishes. Dry-

harvested conidia were collected on the lid of the dishes, the bottom parts were substituted for sterile ones. Conidia were stored into closed boxes that contained glycerol solutions to control RH and placed into incubators at various temperatures for different periods of time according to the experimental design.

2.3. Germination Assessment

The stored conidia were re-suspended into sterile saline solution (NaCl, 9g/l of water) containing Tween 80, 0.05% (vol/vol). After counting the conidia on a Malassez cell, the suspensions were standardized to $1x10^6$ conidia ml^{-1}. 10µl of the suspension was poured at the surface of a thin layer of PDA medium to allow automatic monitoring of the germination without opening the devices [7]. At least 100 spores (20-25 per microscopic field) were examined through the Petri dish lid every hour. Experiments were carried out in triplicate. The length of the germ tubes was measured by means of a Leica DMLB (x200) (Leica, Rueil-Malmaison, France) connected to a IXC 800 (I2S, Pessac, France) camera. Pictures were analyzed using Matrox Inspector 2.2 (Matrox Electronics Systems Ltd, Dorval, Canada). Spores were considered germinated when the length of the germ tubes was equal to the greatest dimension of the swollen spore [8].

2.4. Germination Model

The asymmetric model [9]:

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

was used to determine the percentage of viable spores, P_{max}, the asymptotic P value at $t \to +\infty$ and the germination time τ(h), the point where P equals half of the P_{max}.

2.5. Experimental Design

An experimental domain was defined over 20-100%, 2-28 days and 5-25°C. The Doehlert design allows the description of a region around an optimal response and contains k^2+k+1 points for k variables. For 3 variables, a set of 13 experiments was required. In this study, the germination time τ (h) was the experimental response. The influence of three environmental factors (*i.e.* variables): RH (X1), time (X2), and temperature (X3) on τ was assessed. The experimental values of these three factors are listed in Table 1. The coded values in the range (0-1) are used for the determination of the model coefficients.

2.6. Analysis and Interpretation of the Results

Multiple regression analysis based on the least square method was performed by using Nemrod software (LPRAI, Marseille, France). The analysis concerned the linear and quadratic effects of the three factors and their interactions. Thus, the equation giving T_{90} was a second-order polynomial model with 10 coefficients (b_0, b_1, b_{12}...b_{23}):

$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3$ where X_1, X_2 and X_3 = coded factors studied.

The significance of the coefficients was evaluated by multiple regression analysis based upon the F-test with unequal variance.

3. Results and Discussion

All replicates were characterized by the same values or values that did not differ from more than 0.1h. Therefore the statistical analysis was performed on the mean germination time, otherwise all parameters coefficients of the model would be significant. The mean germination times, τ (h) were reported in Table 1.

Table 1. Experimental values obtained by applying the Doehlert matrix to assess the effect of relative humidity, time and temperature (factors) on the germination time of *Penicillium chrysogenum* (response) and Parameter estimates obtained by fitting the germination time of *P. chrysogenum* to a second-order polynomial model

Experiment	Experimental values			Germination time (h)
	RH (%)	Time (day)	Temperature (°C)	
1	100	15	15	17.5
2	20	15	15	21.4
3	80	28	15	19.8
4	40	2	15	19.0
5	80	2	15	17.7
6	40	28	15	21.0
7	80	19.3	25	19.0
8	40	10.7	5	20.1
9	80	10.7	5	18.0
10	60	23.7	5	19.6
11	40	19.3	25	20.2
12	60	6.3	25	17.5
13	60	15	15	19.6

Coefficient	b_0	b_1	b_2	b_3	b_{11}	b_{22}	b_{33}	b_{12}	b_{13}	b_{23}
Factor	Mean	RH	t	T	RH^2	t^2	T^2	RH.t	RH.T	t.T
Value	19.6	-1.71	1.27	-0.20	-0.15	-0.25	-0.72	0.06	0.053	1.04
p-value	<0.01	<0.01	<0.01	0.206	31.8	10.1	<0.01	68.2	0.194	<0.01

Depending on the storage conditions, τ varied in the range 17.5-21.4 h. The average germination time for the central point of the experimental design, RH 60%, 15 days, 15°C, was equal to 19.6h (experiment 13). This value is in accordance with the response means, b_0, estimated by the polynomial model (Table 1).

The suitability of the polynomial model to fit the experimental data is strengthened by a value of the determination coefficient, $r^2 = 0.979$.

All the coefficients for the main effects, b_1, b_2 and b_3 were significant with p-values less than 0.01. The relative effect of the factors can be ranked according to the absolute value of these coefficients. By a decreasing order of significance, the most important factors were RH, time and temperature. As compared to the response means and regardless of the other factors, the germination time decreased for positive values of X_1 because b_1 negative lead to $b_1.X_1$ also negative. The decrease of the germination time with increasing RH was shown on Figure 1. At 15°C, τ was equal to about 20h after a storage of 15 days at 20% RH, as compared to about 17h at 100% RH. This result can be explained by an initiation of the germination process (i.e., swelling) during storage at high relative humidity, thus decreasing τ.

In contrast, b_2 was positive, thus an increase of time, X_2, delayed germination. At 15°C, the germination time of the conidia of *P. chrysogenum* was equal to about 18h and 20.5h after being stored for 0 and 30 days respectively, Figure 1.

The effect of temperature on the germination time was less clear than that of RH and time because the coefficient of the major effect b_3 was not as significant as b_1 and b_2. The quadratic effect of temperature, b_{33} should be also be taken into account as highly significant, in addition to the interactive effect between time and temperature, b_{23}. Assuming, $b_2.X_2$ positive (for $X_2>0$) and $b_3.X_3$ also positive (for $X_3<0$), the interactive effect is negative ($b_{23}.X_2.X_3<0$) thus antagonistic. The contour plot (Figure 1, right) showed that for a short period of storage, the germination time decreased at increased temperatures. Conversely, for long period of storage, the germination time increased at increased temperatures.

The physiological state is an important factor for explaining biological responses, such as fungal spore germination and viability [5]. The physiological state is affected by environmental conditions during the production of spores and also during storage. In general, temperature, conidial moisture content, and the humidity of the storage atmosphere are the major factors that influence spore viability [10]. For most fungi, their ability to germinate decreases as temperature, conidial moisture content, or relative humidity (RH) increase [11]. At a fixed RH, increasing temperature (but below that which kills spores) generally decreases the viability of fungal spores, whereas lower temperatures (above freezing) increase viability. The relationship between RH and viability of fungal spores does not appear to be as simple; most persist longer at lower humidity, conversely, some species die more rapidly at lower humidity. An isolate of *Aspergillus flavus* was reported to lose viability rapidly at 75% RH while persisting much longer at lower or higher RH [12]. The effect of the period of time after discharge of ascospores of *Gibberella zeae* from perithecia on germination was also studied [13]. It was shown that freshly discharged ascospores germinated within 4h at 20°C and 100% RH, but the rate of germination and the percentage of viable ascospores decreased over time. Humidity during storage was a key factor in germination of *G. zeae*. By incubating ascospores at 53% RH, the percentage of viable spores decreased from 93 to 6% within 10 min. Up to our knowledge, there are no studies that focused on the effect of the storage conditions on the germination time of fungal spores.

4. Conclusions

In the present study the effects of 3 factors, RH, time and temperature were assessed at 5, 7 and 3 levels, respectively. For this kind of study, full factorial designs are usually applied. In such a case, all the experiments are carried out, thus leading to 5x7x3=105 experiments as compared to only 13 with the Doehlert design. Concerning the present study, the paramount influence of the relative humidity during storage on the germination time of the conidia of *Penicillium chrysogenum* was highlighted. Obviously, longer period of storage should be examined as this could affect the germination time but also the viability of the conidia. Finally, it is very difficult to draw conclusions based on one mould only, because a great variability on the experimental responses was shown depending on the mould species. The present study should be extended to other fungi.

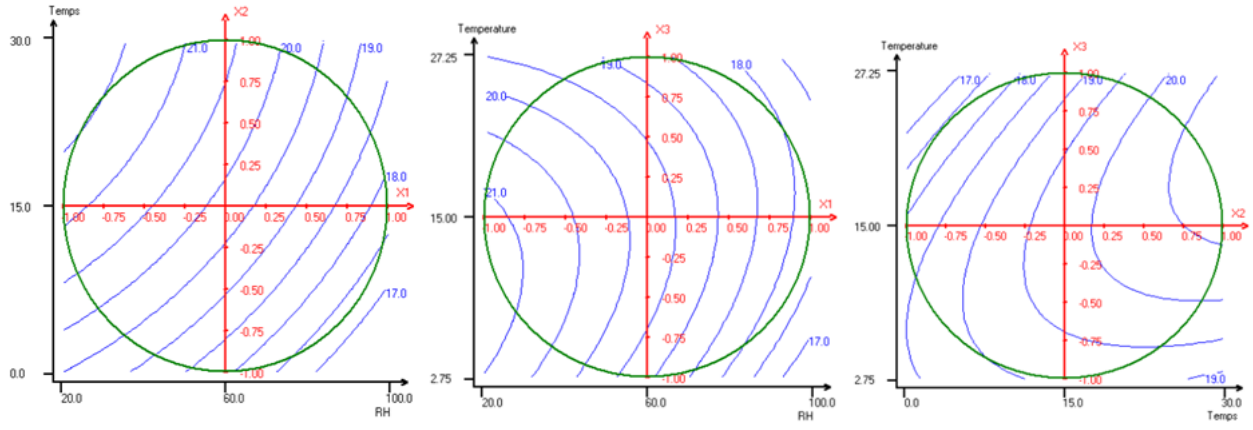

Figure 1. Contour plots of the influence of time and RH at 15°C (left), T and RH at 15 days (centre), T and time at 60% RH (right) on the germination time of *Penicillium chrysogenum* on Potato Dextrose Agar

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