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Modeling of germination and growth of ochratoxigenic isolates of *Aspergillus ochraceus* as affected by water activity and temperature on a barley-based medium

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Abstract

Aspergillus ochraceus is an ochratoxin producing fungus that can be found on stored cereal grains such as barley. The objective of this study was to determine the effects of water activity (a_w, 0.75–0.99) and temperature (10–30°C) on germination and growth on barley extract agar medium (BMEA) of three isolates of *Aspergillus ochraceus*. The three isolates showed an optimal a_w for germination and growth of 0.99–0.95 at 20–30°C, with a marked increase of the lag phases and decrease of germination and growth rates at the marginal levels of a_w and temperature assayed. Minimum level of a_w for germination was 0.80 and 0.85 for growth. Data were then modeled by an MLR regression and response surface models were obtained. These models may allow a rough prediction of germination/growth as a function of the storage temperature and moisture content of barley grains.

Keywords: Predictive microbiology; Growth model; *Aspergillus ochraceus*

1. Introduction

Ochratoxins are fungal secondary metabolites, which are potent nephrotoxin, teratogen, and carcinogen agents (Krogh, 1987), and also, their inhalation can lead to renal failure (Dipaulo et al., 1994). Ochratoxin A was first isolated in 1965 from cereals and other products inoculated with *Aspergillus ochraceus* (van der Merwe et al., 1965). Latter, it has been shown to be produced by several species in the *A. ochraceus* group, and by *A. alliaceus*, *A. albertensis*, *A. niger*, *A. carbonarius*, *Eurotium herbariorum* and *Penicillium verrucosum*. Ochratoxins contaminate grains, legumes, coffee, dried fruits, beer and wine, and meats (Frisvad, 1995; Pitt, 2000). Some studies have shown the presence of OTA-producing isolates of *A. ochraceus* in stored barley grains contaminated by OTA (Cvetnin and Pepeljnjak, 1990). The natural incidence of *A. ochraceus* on barley has been reported (Hill and Lacey, 1983; Sala, 1993). Barley samples naturally contaminated with OTA-producing *A. alliaceus* and *P. cyclopium* isolates were contaminated with high amounts of OTA (Czerwiecki et al., 2002).

Preventing germination and growth of *A. ochraceus* will eventually prevent the production of OTA by this species. This is the reason why predicting and preventing growth becomes important. Moreover, it has been shown that the range of water activity and temperature conditions for germination and fungal growth is wider than that for OTA production (Northolt, 1979). Therefore, if growth is restricted OTA will not be present.

Few reports deal with ecophysiology of ochratoxin producers in terms of germination and growth. Effects of environmental factors, such as temperature, pH and inoculum size on *A. ochraceus* growth have been studied for ochratoxin-producing strains (Paster and Chet, 1979; Haggblom, 1982; Damoglou et al., 1984; Delas et al., 1995). The effect of abiotic factors on OTA accumulation has been studied by some of these and other researchers (Ciegler, 1972; Trenk et al., 1971; Northolt et al., 1979; Milanez and Leitao, 1994). Most of these works, however, were carried out by testing just one single factor, such as temperature, and then the interaction between different environmental factors was

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not given. The combined effects of a_w and temperature on germination and growth of *A. ochraceus* have been studied on a maize meal extract agar (Marín et al., 1998), and on growth on a barley meal extract agar (Ramos et al., 1998).

Magan and Lacey (1988) suggested that lag times for growth, rates of germination and germ tube extension, were good criteria for comparing the capabilities of different fungal spores for colonizing grain surfaces under different a_w conditions. This parameter, water activity, is defined as the ratio of the vapor pressure of water over a substrate to that over pure water at the same temperature and pressure. Given a range of ecological factors, lag phases for germination, germination rates and growth rates can be used in the development of technology approaches for predicting spoilage of cereals (Leistner, 1994, 1995).

The objective of this study was (a) to determine the effects of a_w, temperature and their interactions on the spore germination and growth of OTA producing isolates of *A. ochraceus* on a barley meal extract agar, and (b) to obtain suitable models for prediction of germination and growth as a function of a_w and temperature which may be useful to predict safe storage life of cereals.

2. Materials and methods

2.1. Fungal isolates

Three ochratoxigenic isolates of *A. ochraceus* Wilhem (=*Aspergillus alutaceus* Berk & Curt) (3.113, 3.94 (NRRL 3174), and 3.38) were used. All of them were isolated from cereals and are deposited in the Food Technology Department Collection of the University of Lleida, Spain.

2.2. Medium

The basic medium used was a 3% (w/v) barley meal extract agar (BMEA) that was made by boiling 30 g of dry ground barley in 1 l of water for 30 min. The resulting mixture was filtered through a double layer of muslin and the volume was made up to 1 l. To determine the influence of a_w on mycelial germination and growth on this medium, experiments were carried out on media in which glycerol had been added to achieve levels of 0.75, 0.80, 0.85, 0.90, 0.95 and 0.99 a_w, (606.0, 553.6, 469.0, 352.1, 202.9 and 60.4 g glycerol l^{-1} of medium, respectively). Finally, a_w values were checked with a water activity meter (AquaLab, Pullman, WA, USA). Autoclaved media were poured into 9 cm diameter sterile plastic Petri dishes (20 ml/plate).

2.3. Inoculation, incubation and measurement

2.3.1. Inoculum preparation

Fungi were grown on BMEA for 7 days at 25°C to obtain heavily sporulating cultures. Spores were then suspended in sterile distilled water containing 0.005% of a wetting agent (tween 80). From this stock spore suspension, 1 ml was added to 2 ml of sterile water previously modified with glycerol to the required water availability treatment. The final water activities of the spore suspensions were 0.75, 0.80, 0.85, 0.90, 0.95 and 0.99a_w, and the final concentration of spores was in the range $1–5 \times 10^7$ spores ml^{-1} for the germination study and $1–5 \times 10^6$ spores ml^{-1} for the growth study.

2.3.2. Inoculation and incubation

2.3.2.1. Germination study. A 0.1 ml aliquot of the spore suspensions was pipetted onto BMEA plates of the same a_w and spread on the surface of the agar medium with a sterile bent glass rod as quickly and carefully as possible. Petri dishes with the same a_w were enclosed in polyethylene bags and incubated at 10°C, 20°C and 30°C. The experiment was repeated twice.

2.3.2.2. Growth study. Petri plates were needle-inoculated in the center with a 1.5×10^6 (spores ml^{-1}) fungal spore suspension. Plates with the same a_w conditions were enclosed in polyethylene bags and incubated. The assayed conditions were four a_w levels (0.85, 0.90, 0.95 and 0.99), and three different temperatures (10°C, 20°C and 30°C). All treatments were repeated twice.

2.3.3. Measurement

2.3.3.1. Germination study. Periodically three agar discs (5 mm diameter) were aseptically removed from each replicate plate using a cork borer, placed on a slide, and 50 single spores per disc (150 per replicate Petri plate; 450 per treatment) were examined microscopically. Spores were considered to be germinated when the germ-tube was equal to or greater than the diameter of the spore. The experiment was carried out for a maximum of 60 days.

2.3.3.2. Growth study. The Petri plates were examined daily or as necessary, and two diameters at right angles were measured of each colony. The experiment lasted 40 days.

2.4. Statistical treatment of the results and primary models obtention

In this study two variables were measured; (a) the percentage of germination versus time (in the germination study) and (b) colony diameters versus time (in the growth experiment).

(a) the percentage of germination at each a_w/temperature condition was plotted against time, and a nonlinear regression was used to estimate the maximum germination rate (h^{-1}) and lag phase (h) at the 95% confidence level. The Gompertz (1825) model was used as the fitting equation (Zwietering et al., 1990).

$$\%\ \text{germination} = A\ exp(-\exp(((\mu_m e/A)(\delta - t)) + 1)),$$

where A is the value where the % of germination becomes constant (100% in most cases); μ_m the maximum specific germination rate (h^{-1}); and δ the lag phase (h). Nonlinear regressions were made by using StatGraphics Plus version 2.1.

On the other hand, the percentages of germination were transformed $\sqrt{(x+0.5)}$ where x is the % of germination, in order to homogenize variance, and analysed by general linear model regression (GLM) using SAS version 8.2 (SAS Institute, Inc., Cary, NC, USA) for determination of effects of a_w, temperature, isolate, and two- and three-way interactions.

(b) Linear regression of colony radius against time was used to obtain growth rates (mm d^{-1}) under each set of conditions. Microsoft Excel version 97 was used for this purpose. Analysis of covariance for the different sets of results were carried out using the SAS version 8.2 (SAS Institute, Inc., Cary, NC, USA).

2.5. Obtention of secondary models by RSM

A polynomial multiple linear regression (MLR) and the resulting response surface models (RSM) were obtained with the Unscrambler version 7.6 (CAMO ASA), including the significant factors, interactions and quadratic terms. Models were obtained for prediction of lag phases prior to germination and visible growth.

3. Results

3.1. Water activity and temperature effects on lag phases prior to germination and germination rates

Table 1 shows minimum water activity levels for germination at different temperature levels. These values were 0.80a_w at 20–30°C and 0.90a_w at 10°C.

Statistical analyses showed that there were significant differences due to a_w, temperature, and two-way interactions (a_w × temperature and isolate × temperature) (Table 2). Under the driest conditions, 0.75a_w, germination was not observed; at 0.80a_w only occurred at 30°C for all three isolates and at 20°C for isolates 3.113 and 3.94. For these two isolates the decrease in 10°C in temperature at 0.80a_w increased the lag phases about 80–100 h (Fig. 1).

Table 1
Minimum water activity for germination/growth, at different temperatures, for the three isolates of *Aspergillus ochraceus* assayed on a barley meal extract agar after 60/40 days incubation

Isolates	Temperature (°C)		
	10	20	30
3.113	0.90/0.95	0.80/0.85	0.80/0.85
3.94	0.90/0.90	0.80/0.85	0.80/0.85
3.38	0.90/0.90	0.85/0.85	0.80/0.85

Table 2
Analysis of covariance of the effect of different isolates (I), temperature (T), water activity (a_w) and their interactions on the percentage of germination and growth of three OTA producing isolates of *Aspergillus ochraceus* on barley meal extract agar

Source of variation	Germination study			Growth study		
	DF	MS	F	DF	MS	F
Time	1	1101.750705	147.64**	1	588824.2881	1536.76***
I	2	4.344463	0.58^{ns}	2	2950.6241	77.08***
T	2	912.688464	122.31**	2	72938.3518	1905.48***
a_w	4	778.861863	104.37**	3	38805.3740	1013.77***
$I \times a_w$	8	5.939694	0.90^{ns}	6	434.1270	11.34***
$a_w \times T$	8	153.930104	20.63**	6	8537.1887	223.03***
$T \times I$	4	21.077455	2.82*	4	1166.1026	30.46***
$I \times a_w \times T$	16	10.221011	1.37^{ns}	12	89.4156	2.34*

DF, degree of freedom; MS, mean square.
^{ns}not significant.
***significant $P<0.0001$.
**significant $P<0.01$.
*significant $P<0.05$.

The shortest lag phases, less than 15 h, were found at 0.99–0.95a_w at 20–30°C. At marginal conditions of a_w and temperature the lag phases increased, more markedly at 0.80a_w at levels of 30°C and 20°C, and for all a_w levels at 10°C.

The effect of a_w on the lag phases increased with a reduction in temperature levels. A decrease in temperature from 30°C to 10°C, at 0.99a_w, resulted in an increase of the lag phases of 70–75 h, while at 0.90a_w the same change in temperature led to an increase of the lag phases about 90–200 h.

The highest germination rates for all isolates occurred at 0.99a_w regardless of temperature (Fig. 2), being at 20–30°C the final % of germination of spores about 100%. At 0.80a_w at 20°C and 30°C the germination rates were lower than 0.5 spores d^{-1} and the final % of germination was not higher than 50% for the three isolates. The isolate 3.113 at 0.90a_w and 10°C showed a long lag phase (205 h) and a low germination rate (0.5 spores d^{-1}); despite of this, it reached a 100% of germination of spores after 60 days. At 10°C and

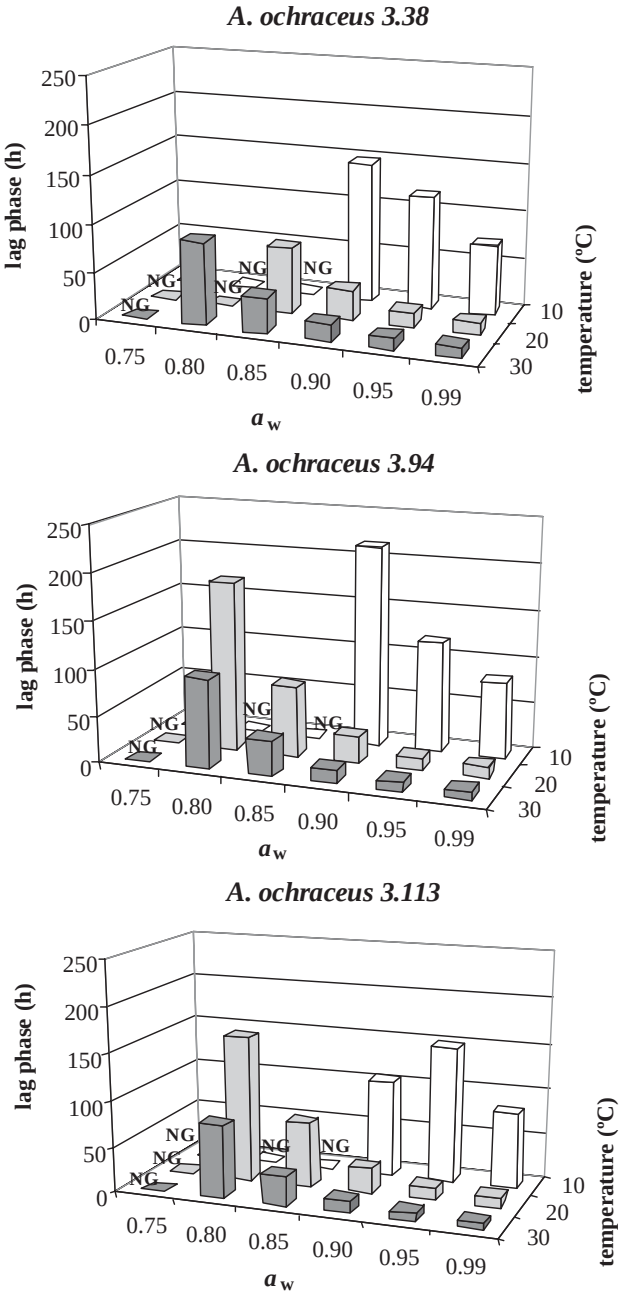

Fig. 1. Effect of water activity and temperature on lag phases prior to germination of three OTA producing isolates of *Aspergillus ochraceus* on BMEA (NG: no germination).

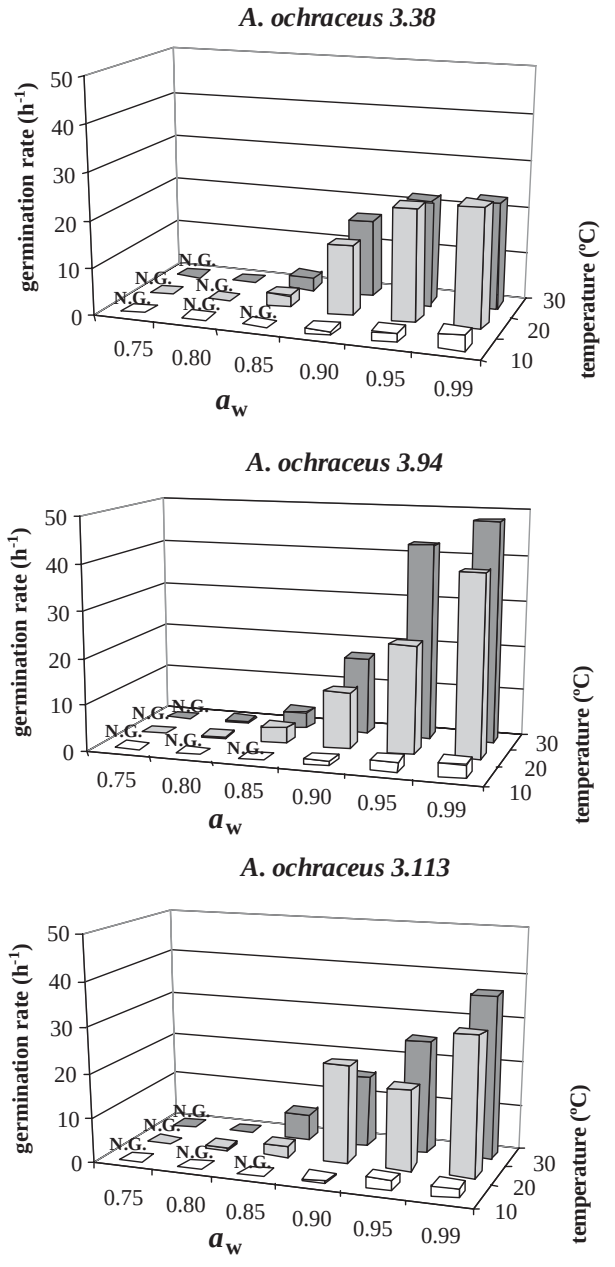

Fig. 2. Effect of water activity and temperature on germination rates of three OTA producing isolates of *Aspergillus ochraceus* on BMEA (NG: no germination).

0.95a_w it showed a lag phase higher than at 0.99 a_w, but a similar germination rate.

3.2. Water activity × temperature effect on mycelial growth

Statistical analyses of variance showed that there were significant differences due to a_w, temperature, isolate, two-and three-way interactions (Table 2).

Optimum level of a_w for growth of the three isolates was at 0.95–0.99. The maximum growth rates found were 3.97, 5.13 and 2.09 mm d^{-1} for isolates 3.38, 3.94 and 3.113, respectively (Fig. 3).

No growth occurred at 0.85a_w and 10°C for any of the isolates, neither at 0.90°C and 10°C for isolate 3.113. In general *A. ochraceus* 3.113 showed slower growth than the others. Analysis of covariance showed optimum growth at 30°C for isolates 3.113 and 3.38 while the isolate 3.94 showed similar growth at 20°C and 30°C. At 10°C the three isolates showed significant differences on growth. In general, there was a similar

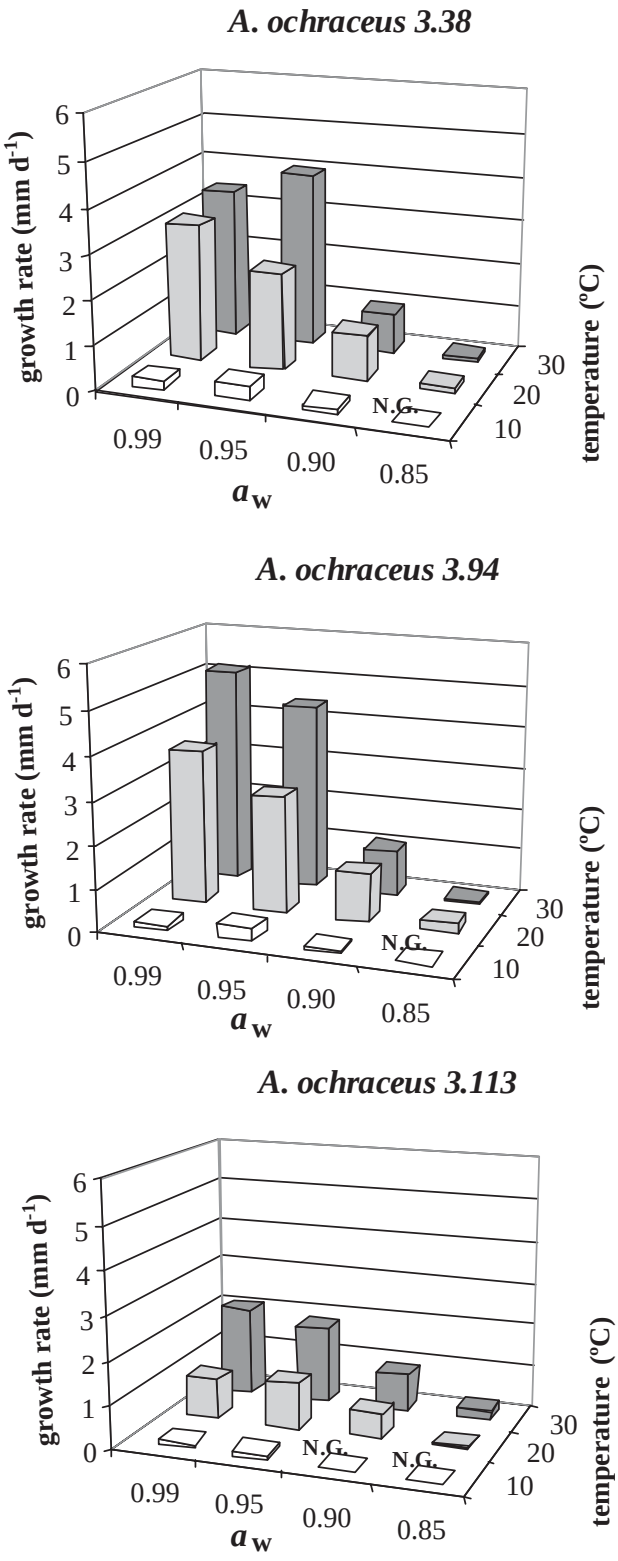

Fig. 3. Effect of water activity and temperature on the growth rate of three isolates of *A. ochraceus*, after 40 days of incubation on BMEA (NG: no growth).

trend for growth and germination, thus isolate 3.94 showed the highest growth and germination rates and the lower lag phases.

3.3. Predictive modeling of spore germination and fungal growth on barley-based agar medium

Lag phases prior germination and visible fungal growth were described by a polynomial model equation with six coefficients (b_0, b_1, …, b_{22}) (Table 3): lag phase $= b_0 + b_1T + b_2a_w + b12Ta_w + b_{11}T^2 + b_{22}\,a_w^2$. Coefficients of the single factors (a_w and temperature) were significant ($P<0.05$) in all models. The interaction effects ($a_w \times$ temperature) were significant ($P<0.01$) only in the germination models, while the square effects (a_w^2 and temperature^2) were significant ($P<0.01$) in the growth models.

When no germination or no growth occurred, the lag phases entered were the lasting of the experiments: 1440 h for germination and 40 days for growth. The inclusion of these data in the data matrix had advantages and drawbacks: the advantage was that the models gave better estimations of the lag phases at marginal conditions of a_w/temperature, however, the goodness of fit of the model was worse and the predicted values for short lag phases were not accurate at all and sometimes they were negative values which had to be interpreted as 0 values (Fig. 4). We decided to keep this approach, however, because the models are developed with the aim of predicting safe storage, then from a practical point of view, prediction at marginal conditions is more useful than around the optimum ones. Moreover, the model may make an underestimation of lag phases because the maximum entered (lasting of experiments), may be probably higher or infinite.

4. Discussion

This study has confirmed the impact of the close interaction between a_w and temperature on germination and growth of *A. ochraceus*. Moreover, it has been shown that different isolates may have a slightly different response to environmental factors regarding, mainly, their growth.

In general there was a parallelism between the increment of lag phases and the reduction of germination rates when a_w decreased for all temperature levels and isolates. The range of a_w conditions for germination was wider than that for mycelial growth regardless of temperature. Spores germination is the first stage in the fungal colonization of substrates, so if the conditions of a_w and temperature that prevent germination are established, this will be the starting point to prevent growth and OTA production. Germination of *A. ochraceus* had only been studied before on a maize extract agar (Marín et al., 1998); it happened at a minimum water activity of 0.85 at 10°C, while at 30°C the minimum water activity for germination was 0.80. In our study the results at 30°C were similar, while at 10°C

Table 3
Polynomial model equations obtained by MLR for effects of a_w and temperature (T) on lag phases prior germination (days) and visible fungal growth (days) on BMEA

Isolate	R^2	Equation
Germination study[a]		
3.113	0.832	Lag phase = $3427 - 31.8T - 3108.0a_w + 240.0Ta_w + 206.7T^2 + 58.6a_w^2$
3.94	0.835	Lag phase = $3490 - 31.3T - 3184.0a_w + 239.0Ta_w + 202.7T^2 + 62.1a_w^2$
3.38	0.818	Lag phase = $126.2 - 30.6T - 4792.0a_w + 242.9Ta_w + 17.1T^2 + 200.8a_w^2$
Growth study[b]		
3.113	0.858	Lag phase = $162.4 - 1.1T - 148.9a_w - 1.8Ta_w + 5.9T^2 + 5.7a_w^2$
3.94	0.895	Lag phase = $176.9 - 0.6T - 176.4a_w - 1.1Ta_w + 5.5T^2 + 5.5a_w^2$
3.38	0.907	Lag phase = $179.3 - 0.6T - 179.6a_w - 0.6Ta_w + 4.5T^2 + 5.9a_w^2$

R^2 = percentage of variation explained by the model.
[a] Values of interaction and square effects: $T^*a_w = 0.1183(T - 20) \times 14.2195(a_w - 0.898)$; $(T)^2 = ((0.1183(T - 20))^2$; $(a_w)^2 = ((14.2195(a_w - 0.898))^2$.
[b] Values of interaction and square effects: $T^*a_w = 0.1211(T - 20) \times 14.554(a_w - 0.898)$; $(T)^2 = ((0.1211(T - 20))^2$; $(a_w)^2 = ((14.554(a_w - 0.898))^2$.

the minimum water activity for germination was 0.90 for all isolates. Optimum conditions for germination were 20–30°C at 0.95–0.99a_w.

A. ochraceus has been described as a fungus which grows between 8°C and 37°C, with the optimum at 24–31°C (ICMSF, 1996). The minimum a_w for growth being 0.77°C at 25°C (Pitt and Christian, 1968), with the optimum 0.95–0.99 (ICMSF, 1996). Ramos et al. (1998) studied growth of *A. ochraceus* on BMEA; optimal growth was at 0.96–0.98a_w and 25–30°C were obtained for growth of three isolates assayed. A coincident optimum a_w range, 0.95–0.99, was found in the present study, as they found increasing growth rates with a_w, with a sharp decrease from 0.98 to 0.99a_w, then they found higher growth rates at 0.95 than at 0.99a_w, with the maximum in between. Inoculation systems were different in both studies: Ramos et al. (1998) inoculated with actively growing mycelium, while conidial suspensions were used in this study. Higher growth rates were found, however, in the later; spores suspensions were prepared from cultures grown on BMEA, while mycelium plugs were taken from cultures grown on PDA, then the need to adapt to the different medium composition may be the reason of the slower growth.

Minimum a_w for growth at 30°C was similar to that in maize extract agar (Marín et al., 1998), while at 10°C growth took place at 0.87a_w in maize extract agar and only at $\geqslant$0.90–0.95a_w in BMEA. In general, however, optimum growth conditions were similar both in barley and maize extract agar. It seems that maize composition may be more convenient for both germination and growth of *A. ochraceus* at 10°C. Similarly, Damoglou et al. (1984) observed growth of *A. ochraceus* in autoclaved barley grains at 0.85a_w at 10°C. This suggests that the nutrient source can affect the minimum water activity for germination, growth and growth rates (Wearing and Burgess, 1979), but it does not affect the optimum conditions (Marín et al., 1999). Growth of *A. ochraceus* in terms of fungal biomass was maximum at 25–30°C, in a study in maize kernels (38% moisture content) at 15–30°C (Delas et al., 1995).

This study has shown that these ochratoxigenic isolates are adapted to low water activity conditions, and they may easily colonize stored grain. Data and models obtained cannot be directly extrapolable to actually stored barley, but they may be a rough approximation. Polynomial secondary models were developed for prediction of lag phases prior to germination and visible growth. Secondary models have been developed for moulds by adjusting data to existing equations such as the 'square-root' model (Ratkowsky et al., 1982) or Arrhenius equation (Schoolfield et al., 1981). Regression analysis and the subsequent formation of a polynomial model is the main alternative, however, to the above approach, and a wide range of computer programs is available to perform such analyses (Gibson and Hocking, 1997). The multifactorial analysis allows to development of a model with a good quality of fit and taking account of possible interaction effects between the environmental factors. Prediction given by these models is valid only within the particular experimental domain (Delignette-Muller, 1997).

This work is an example of how different environmental conditions can affect the development of spoilage fungi of feeds and foods, using direct measurements of germination and growth, and estimated parameters as rate of germination, lag phases and rate of growth. Predictive mycology studies the behavior of moulds under different physico-chemical conditions. It can help in the identification of critical points in the production, storage and distribution process of foods and feeds. More research is needed to

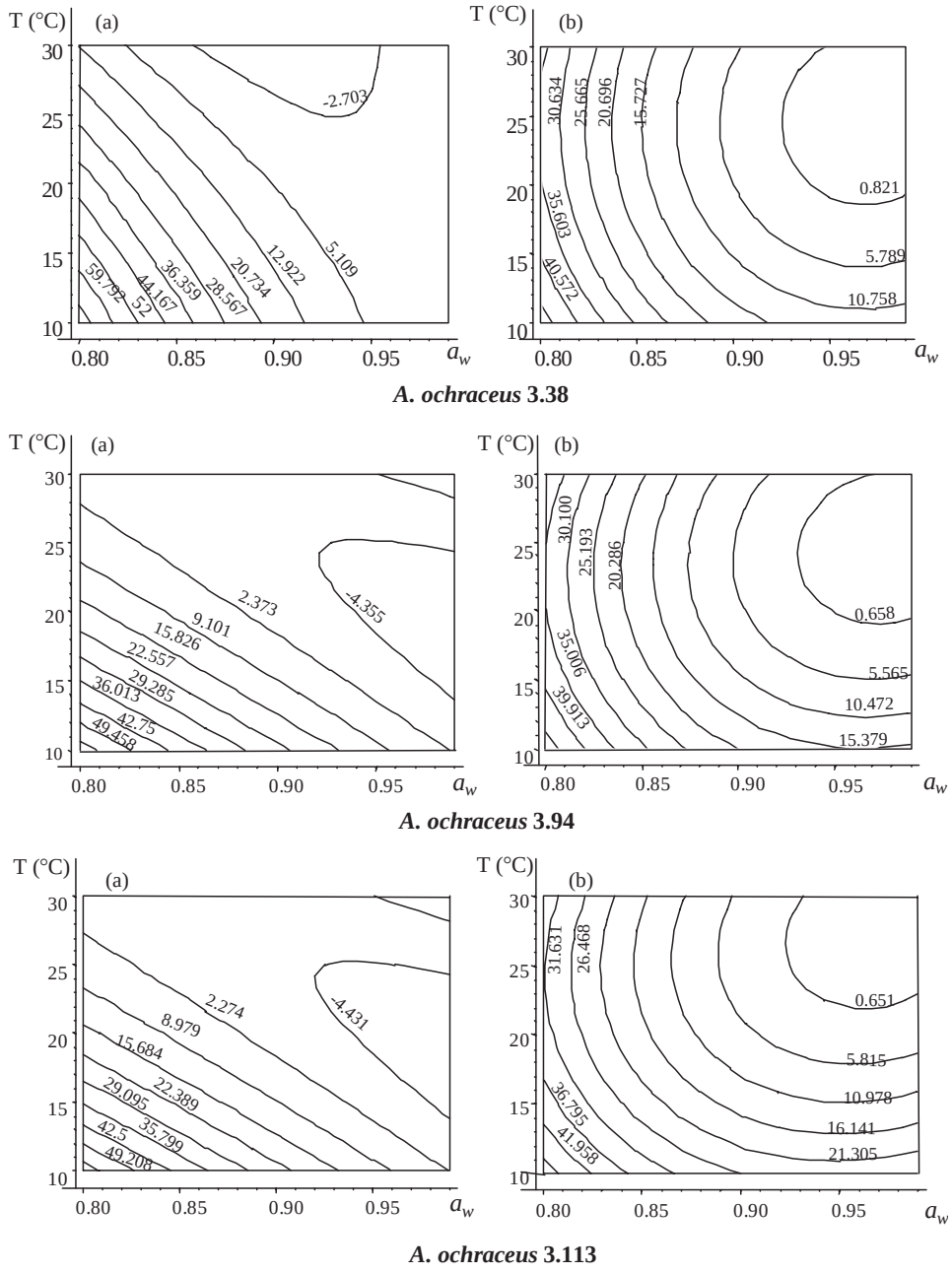

Fig. 4. Response surface contour plots showing the effect of a_w and temperature on (a) lag phases prior germination and (b) visible fungal growth of three ochratoxigenic isolates of *A. ochraceus* on BMEA.

improve the microbiological safety and shelf life of food products.

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