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Modelling the effect of temperature and water activity in the growth boundaries of *Aspergillus ochraceus* and *Aspergillus parasiticus*

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ABSTRACT

The aim of this work was to model the growth of *Aspergillus parasiticus* and *Aspergillus ochraceus*, both mycotoxin producers, near to the growth/no growth boundaries and validate those models in sterile maize grain, peanuts and coffee beans. Malt extract agar was adjusted to six different water activities: 0.93, 0.91, 0.89, 0.87, 0.85 and 0.80. Plates were incubated at 10, 15, 20, 25, 30, 37 and 42 °C. For each of the 42 conditions, 10 Petri dishes were inoculated. Both kinetic and probability models were applied to colony growth data. The results of the present study indicate that the developed probability modelling approach could be satisfactorily employed to quantify the combined effect of temperature and water activity on the growth responses of *A. ochraceus* and *A. parasiticus*. However, validation of kinetic results led to poor goodness of prediction. In this study, the validation samples were placed near to the expected boundaries of the models in order to test them under the worst situation. Probability of growth prediction under extreme growth conditions was somewhat compromised, but it can be considered acceptable.

1. Introduction

Mycotoxins are a chemical risk in food products of increasing concern due to the wide range of food commodities where they can be found. Consumption of mycotoxin contaminated food has been associated with several cases of human poisoning or mycotoxicoses, sometimes resulting in death (Molina and Giannuzzi, 1999). *Aspergillus* species have the ability to grow in various climates and, in food products, are among the most important moulds causing both spoilage and mycotoxin production. *Aspergillus ochraceus* and *Aspergillus parasiticus* are responsible for synthesis of ochratoxin A (OTA) and aflatoxins (B1, B2, G1 and G2), respectively. *A. ochraceus* was the first OTA-producing species described (Van der Merwe et al., 1965) and is capable of producing OTA when grows on foods during storage (Leistner, 1984; Gareis and Scheuer, 2000; Spotti et al., 2001, 2002; Castella et al., 2002; Lund and Frisvad, 2003; Comi et al., 2004; Matrella et al., 2006; Pietri et al., 2006; Cantoni et al., 1982a, b, 2007). *A. parasiticus* grows rapidly on a wide variety of natural substrates under favourable conditions of temperature and humidity (Bagheri-Gavkosh et al., 2009). This mould can produce aflatoxins in food and feeds and can pose serious health hazards to human and animal.

Mycotoxins are associated to the presence of fungal inoculum on susceptible substrates. Despite the absence of direct correlation between the extent of mould growth and mycotoxin production, prevention of fungal growth effectively conduces to prevention of mycotoxin accumulation (Garcia et al., 2009). In addition, growth is a variable which presents less intraspecific variability, and the kinetics of growth are more known, thus a good alternative to prevent mycotoxin accumulation might be prediction and prevention of growth (Marín et al., 2008a). The growth of moulds in foods and feeds depends on the effects of multiple variables like pH, water activity (a_w), solute concentrations, temperature, atmosphere composition, time, etc. But generally a_w and temperature are regarded as the principal controlling factors determining the potential for growth (Panagou et al., 2003; Plaza et al., 2003; Dantigny et al., 2005).

Mathematical modelling can be a tool to predict and, consequently, to prevent the growth of mycotoxigenic moulds. Secondary kinetic models describe microbial response in relation to environmental factors providing estimates for parameters of growth: lag phase (λ) and maximum growth rate (μ_{max}). Probabilistic models study the probability that mould growth or mycotoxin production occur. An integrated description of the microbial response could be given by first establishing the likelihood of growth through a probability model and then predicting the growth parameters, such as specific growth rate and lag time, provided that growth is expected

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Nomenclature section

a_w	water activity
$a_{w\ min}$	minimum a_w for growth
$a_{w\ max}$	maximum a_w for growth
$a_{w\ opt}$	a_w at which μ_{max} is optimal
D	colony diameter (mm)
D_{max}	final diameter of mycelium attained in Petri dishes
μ_{max}	maximum growth rate (mm/d)
μ_{opt}	maximum growth rate at optimal conditions (mm/d, μm/h)
λ	time needed for visible growth of the fungus (lag time, days)
P	probability of growth (in the range of 0–1)
T	temperature (°C)
T_{min}	minimum temperature for growth (°C)
T_{max}	maximum temperature for growth (°C)
T_{opt}	T at which μ_{max} is optimal (°C)

(Masana and Baranyi, 2000). Logistic regression is a useful approach to model the growth boundaries (growth/no growth interface) of microorganisms in different areas of food science, such as product development, formulation and food processing (Sosa-Morales et al., 2009). Most published models take into account several factors (water activity, temperature, incubation time) in most cases close to their optimal levels, nevertheless foods and feeds are stored at marginal environmental conditions for mould growth. These researches have been frequently made on solid media adjusted to different a_w and incubated to different temperatures. However, results obtained on culture media cannot necessarily be extrapolated to natural ecosystems where there are other factors that influence on the mould growth. For this, validation is an essential step after modelling, enabling researchers to understand the applicable range of models and also the limits of their performance (Jagannath and Tsuchido, 2003).

The objectives of the present work were (i) to develop kinetic models for *A. ochraceus* and *A. parasiticus* on a synthetic growth medium as a function of temperature and water activity (reducing fungal growth may also reduce mycotoxin production); (ii) to develop growth/no growth interface models (with the objective of preventing from mycotoxin production), and (iii) to validate the developed models with data from independent experiments on selected food. Few previous studies have reported on validated models for moulds. As far as we know this is the first study specifically dedicated to model and validate mould growth at the limits of growth. The objective was to test whether the performance of the predictive models may fail at the boundaries.

2. Materials and methods

2.1. Fungal isolates and preparation of inoculum

Two mycotoxigenic isolates were included in this research, one of *A. parasiticus* (UdL-TA 3.18) isolated from peanuts and one of *A. ochraceus* (Udl-TA 3.53) isolated from coffee. The references in brackets are the codes of cultures held in the Food Technology Department Culture Collection of University of Lleida, Spain. The isolates were sub-cultured on malt extract agar (MEA) plates and incubated at 25 °C for 7 days to obtain heavily sporulating cultures. After incubation, a sterile inoculation loop was used to remove the conidia of each mould from MEA plates and they were suspended in 5 ml of H_2O/glycerol solutions with different water activity levels: 0.93, 0.91, 0.89, 0.87, 0.85 and 0.80.

The suspensions were then filtered through glass wool into sterile 10 ml tubes to remove mycelial fragments. Then the spore suspension was centrifuged at 4000 rpm, at 4 °C for 15 min. The pellet was resuspended with the required H_2O/glycerol solution. The number of spores per ml was then determined using a Thoma counting chamber and the final concentration was adjusted to 10^5 spores/ml.

2.2. Medium

The basic medium used in this study was malt extract agar (MEA) with six different water activities. The a_w of the medium was modified to 0.93, 0.91, 0.89, 0.87, 0.85 and 0.80 by the addition of 304.3 g/l, 376 g/l, 483.4 g/l, 565 g/l and 675 g/l of glycerol respectively. The medium was autoclaved and poured into 9 cm sterile Petri dishes. The pH of the media was in the range of 6.0–6.2. The a_w of each medium was checked with an AquaLab Series 3 (Decagon Devices, Inc., WA, USA) with an accuracy ±0.003.

2.3. Inoculation and incubation

MEA plates were inoculated centrally with a needlepoint load. Previous repeated experiments showed that the number of spores inoculated through this technique was 10–100 spores. In particular, 26.0 CFU ± 15.8 have been obtained through this protocol. Inoculation load has been shown to affect lag phase duration, although not growth rates (Sautour et al., 2003). Increased lag phases (up to 23%) were in general observed for *Aspergillus carbonarius* and *Penicillium expansum* when inoculum increased from 0–1 to 10–100 spores (Garcia et al., 2010).

Petri dishes with the same a_w level were enclosed in polyethylene bags in order to maintain a constant water activity and incubated at 10, 15, 20, 25, 30, 37 and 42 °C. Bags do not affect growth by diminishing oxygen availability neither increasing CO_2 concentration as confirmed before comparing growth with plates incubated without bags. The effect of temperature and a_w on the growth response of both fungi was investigated by means of a full factorial design. For each treatment 10 Petri dishes were inoculated (n = 10).

2.4. Growth assessment

Fungal growth was observed on a daily basis for an overall period of 90 days by diameter measurements at right angles with the aid of a ruler and a binocular magnifier. The diameter of the colonies was plotted against time.

2.5. Validation

Validation was carried out directly in sterile green coffee beans, peanuts and maize with modified a_w. Validation of models was

Table 1
Validation set of conditions for *A. ochraceus* and *A. parasiticus* models.

A. ochraceus		*A. parasiticus*	
Water activity	Temperature	Water activity	Temperature
0.85	25	0.85	25
0.85	30	0.85	30
0.87	20	0.87	20
0.87	37	0.87	30
0.89	15	0.87	37
0.89	20	0.89	15
0.89	37	0.89	20
0.91	15	0.89	37
0.93	10	0.91	15
0.93	25	0.93	25

carried out in peanuts and maize for *A. parasiticus* and in coffee and maize for *A. ochraceus*. Table 1 shows incubation conditions chosen for validation of each mould after inoculation. Except for a suitable condition, the combinations were chosen near the growth/no growth boundaries in order to validate the models under those conditions in which prediction may be a key point in food safety. Resolution and accuracy of the measurements in the validation experiments might be compromised by the heterogeneity of the matrices.

Sterile nuts, beans and grains were adjusted to the required a_w levels and poured in Petri plates forming a single layer. Inoculation was carried out as in agar plates. Plates with the same a_w were enclosed in sealed containers along with beakers containing water glycerol solution of the same a_w as the plates which were renewed periodically in order to maintain constant a_w (Dallyn, 1978). For each condition 10 Petri dishes were inoculated. Growth assessment was carried out as for MEA experiments.

2.5.1. Water activity adjustment of the substrates

Seeds were sterilized by autoclave at 120 °C for 20 min. Water activity was adjusted by aseptically adding amounts of sterile distilled water to the substrates in sterile bottles. The bottles were cooled down to 4 °C for 48 h with periodic hand-shaking during this time. The amount of water necessary to reach the different water activity levels was determined by calibration curves (water activity-ml water to be added/g substrate) previously made (Table 2). Final a_w values of each substrate were confirmed with an AquaLab Series 3 (Decagon Devices, Inc., WA, USA) with an accuracy ±0.003. The pH values of maize, peanuts and coffee beans were 6.0, 6.0 and 5.7 ± 0.1, respectively.

2.6. Model development

2.6.1. Kinetic model

A typical two-step modelling approach, including primary and secondary modelling, was employed to quantify the effect of temperature and a_w on the kinetic parameters of *A. ochraceus* and *A. parasiticus*. Initially, estimates of the growth rates of the fungi were obtained by plotting colony diameter changes against time. For each treatment, a non-linear regression was applied to estimate the maximum growth rate (μ_{max}, mm d^{-1}), lag phase before growth (λ, days), and maximum colony diameter, if applicable, by fitting the experimental data to the primary model of Baranyi and Roberts (1994) (1) by using Statgraphics Plus 5.1 with the non-linear regression option.

$$D = \mu_{max}A - \ln\left[1 + \frac{\exp(\mu_{max}A) - 1}{\exp(D_{max})}\right] \quad (1)$$

$$A = t + \left(\frac{1}{\mu_{max}}\right)\ln\left[\exp(-\mu_{max}t) + \exp(-\mu_{max}\lambda) - \exp(-\mu_{max}t - \mu_{max}\lambda)\right] \quad (2)$$

The average estimates of μ_{max} and λ were further fitted to secondary models to describe the effect of temperature and a_w on fungal growth rate. The models are described by the following equations:

a) The combined effects of temperature and water activity were determined according to the gamma concept (Zwietering et al., 1992), based on previous cardinal models (Rosso et al., 1993; Rosso and Robinson, (2001).

$$\sqrt{\mu_{max}}(T, a_w) = \mathrm{CTPM}_2(T, a_w) = \sqrt{\mu_{opt}\cdot\tau(T)\cdot\rho(a_w)} \quad (3)$$

where

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

and

$$\rho(a_w) = \left(\frac{(a_w - a_{w\,min})^2\cdot(a_w - 1)}{(a_{w\,opt} - a_{w\,min})\cdot\left[(a_{w\,opt} - a_{w\,min})(a_w - a_{w\,opt}) - (a_{w\,opt} - 1)(a_{w\,opt} + a_{w\,min} - 2a_w)\right]}\right) \quad (5)$$

where $a_{w\,max} = 1$ according to Sautour et al. (2001).

b) Extended Davey model (Davey, 1989)

$$\sqrt{\mu_{max}}(\text{or } \ln\lambda) = a_0 + a_1a_w + a_2a_w^2 + a_3/T + a_4/T^2 \quad (6)$$

Where T is absolute temperature (°K), and $a_0 \ldots a_4$ are coefficients to be estimated.

c) General polynomial model

$$\sqrt{\mu_{max}}(\text{or } \ln\lambda) = a_0 + a_1a_w + a_2a_w^2 + a_3T + a_4T^2 + a_5Ta_w \quad (7)$$

Where T is temperature (°C) and $a_0 \ldots a_5$ are coefficients to be estimated.

Similar results should be expected from b) and c) quadratic models, both of them polynomial however, it was decided to include both of them as did Samapundo et al. (2005, 2007) in their studies.

The goodness of fit of the modelling approach was evaluated by the root mean square error (RMSE), which measures the average deviation between observed and predicted values. The smaller the value of this index the better the fit of the model to the experimental data:

Table 2
Amount of water to be added to each substrate for different a_w levels.

a_w	Water (ml/100 g)		
	Maize	Peanut	Green coffee
0.85	2.0	4.5	10.5
0.87	4.0	5.0	12.3
0.89	5.0	6.0	15.5
0.91	7.5	9.5	21.5
0.93	11.0	12.0	24.5

$$\text{RMSE} = \sqrt{\frac{\sum(\text{predicted} - \text{observed})^2}{n-p}} \quad (8)$$

Where n is the number of observations and p is the number of parameters to be estimated.

For validation, the indices proposed by Ross (1996) were used:

2.6.1.1. Bias factor (BF). The BF answers the question whether, on average, the observed values lie above or below the line of equivalence and, if so, by how much.

$$\text{bias factor} = 10^{\left(\sum \log(\mu_{\text{predicted}}/\mu_{\text{observed}})/n\right)} \quad (9)$$

μ is maximum growth rate or other modelled variable
n is the number of observations used in the calculation

Perfect agreement between predictions and observations will lead to a bias factor of 1.

2.6.1.2. Accuracy factor (AF). The AF averages the minimum 'distance' between each point and the line of equivalence as a measure of how close, on average, predictions are to observations. The AF is, thus, a measure of average deviation and may be used as a simple measure of the level of confidence one may have in the model's predictions.

$$\text{accuracy factor} = 10^{\left(\sum|\log(\mu_{\text{predicted}}/\mu_{\text{observed}})|/n\right)} \quad (10)$$

The larger the value, the less accurate is the average estimate.

2.6.2. Modelling of the growth/no growth interface

For each treatment of the two fungal species, growth data were converted into probabilities of growth by assigning the value of 1 in the case where visible fungal growth was evident, and 0 in the case of absence of growth during the overall period of the experiment. The resulting data were fitted to a logistic regression model as described previously (Ratkowsky and Ross, 1995) to determine the growth/no growth boundaries of the fungi under the different a_w and temperature levels assayed. The model employed was a full second-order logistic regression model (Battey et al., 2002) that includes also the linear term for time:

$$\text{Logit}\,P = \ln\left(\frac{P}{1-P}\right) = b_0 + b_1 a_w + b_2 T + b_{11} a_w^2 + b_{22} T^2 + b_{12} a_w T + \text{time} \quad (11)$$

Where b_i are the coefficients to be estimated. The equation was fitted by using Statgraphics® Plus version 5.1 (Manugistics, Inc, Maryland, USA) linear logistic regression procedure. The automatic variable selection option with a backward stepwise factor selection method was used to choose the significant effects ($P < 0.05$). The predicted growth/no growth interfaces for $P = 0.1$, 0.5 and 0.9 were calculated and plotted using Microsoft Excel 2003 Solver.

3. Results

3.1. Kinetic primary model

Growth of *A. ochraceus* in solid medium followed, in general, biphasic Baranyi's function (with no upper asymptote) except at 37 °C/0.85–0.89a_w and at 10 °C with 0.91–0.93a_w, where growth followed a sigmoidal curve. For *A. parasiticus* the growth also followed biphasic Baranyi's function; only at 0.85a_w/20 °C a sigmoidal growth curve was observed.

Maximum growth rate (μ_{max}) and lag phase (λ) were estimated through Baranyi's primary model (Table 3). For both moulds under marginal conditions, μ_{max} decreased and λ increased. No growth was observed under extreme conditions (eg. >37 °C, <10 °C, <0.85a_w for *A. ochraceus* and >42 °C, <15 °C, <0.85a_w for *A. parasiticus*). In the case of *A. ochraceus*, the higher μ_{max} was observed at 0.93a_w/30 °C with the lowest λ. For *A. parasiticus* the higher μ_{max} was found at 0.93a_w/37 °C but the minor λ was at 0.93a_w/30 °C.

3.2. Secondary models for the effects of a_w and temperature on the growth rate and lag phase

Response of the moulds to the environmental conditions studied was examined with several existing models. Based on analysis of residues, the square root transformation was introduced to stabilize the variance of the fitted values for the growth rate ($\sqrt{\mu}$), confirming the suitability of this transformation as suggested by Dantigny and Bensoussan (2008).

The cardinal values of environmental factors (minimum, maximum and optimum value) of the secondary cardinal model are shown in Table 4, whereas the fitted models are presented in Fig. 1. In particular, optimum a_w values were poorly estimated as they were outside the experimental domain, and models should not be applied outside this domain. Taking into account that the experimental design included only suboptimal water activity levels, the model exhibited reasonably good fit to experimental data in terms of calculated RMSE. *A. parasiticus* was rather fast growing under the best conditions of temperature and a_w tested, based on the estimates of μ_{opt} values of 1.8 mm d^{-1} compared to 1.0 mm d^{-1} for *A. ochraceus*. While $a_{w\,min}$ estimates for growth were similar for both fungi, the optimum estimated temperatures for growth varied from 27.4 °C for *A. ochraceus* to 31.1 °C for *A. parasiticus*, explaining the better adaptation of the later to higher temperatures. Oppositely to what expected, the estimated T_{min}, however, was lower for *A. parasiticus* than for *A. ochraceus*.

For subsequent models, based on analysis of residues, natural logarithm transformation was used ($\ln \lambda$) for lag phases. In particular, the general polynomial model showed better performance than extended Davey model for both growth rate (RMSE = 0.125–0.189) and lag time (RMSE = 0.493–0.696) (Table 5). Most coefficients resulted significant. As far as statistical evaluation is concerned, the models presented better performance for *A. ochraceus* than for *A. parasiticus*. For *A. ochraceus*, lag phases were predicted under 7 days in the range 20–34 °C at 0.87a_w, and in the range 16–35 °C at 0.93a_w, whereas lag phases over 30 days were predicted at 0.80 regardless of temperature level, outside the range 18–38 °C at 0.85a_w, and outside the range 12–40 °C at 0.89a_w (Fig. 2). For *A. parasiticus*, lag phases were predicted under 7 days in the range 22–32 °C at 0.87a_w, and in the range 18–38 °C at 0.93a_w, whereas lag phases over 30 days were predicted at 0.80 regardless of temperature level, outside the range 18–36 °C at 0.85a_w, and outside the range 15–40 °C at 0.89a_w.

3.3. Validation of kinetic models on food matrices

Plotting observed versus predicted growth rate values for the four food matrices (figures not shown), it could be visually concluded that the best predictions were done for *A. parasiticus* in maize at 0.93a_w/25 °C, and that in general there was a poor accordance between predictions and observed values. Moreover, small differences were observed in the predictions by the three models. Bias and accuracy factors (Bf and Af) were calculated for both species and models (Table 6).

Validation of *A. ochraceus* growth model in coffee beans led to acceptable Bf and Af at 0.93a_w/25 °C (0.63–0.67 and 1.19–1.28, respectively) suggesting that the model was conservative as higher values were predicted than the observed ones. Worse indices were

Table 3
Estimated maximum growth rates (μ_{max}) and lag times (λ, starting from 10 to 100 inoculated spores) for the growth of *Aspergillus ochraceus* and *Aspergillus parasiticus* on malt extract agar at various temperatures and water activity levels.

Temperature (°C)	Water activity	*Aspergillus ochraceus*		*Aspergillus parasiticus*	
		μ_{max} (mm d^{-1}) ± SE	λ (days) ± SE	μ_{max} (mm d^{-1}) ± SE	λ (days) ± SE
10	0.80	–	–	–	–
	0.85	–	–	–	–
	0.87	–	–	–	–
	0.89	–	–	–	–
	0.91	0.14 ± 0.02	48.3 ± 5.0	–	–
	0.93	0.06 ± 0.01	35.3 ± 9.2	–	–
15	0.80	–	–	–	–
	0.85	–	–	–	–
	0.87	–	–	–	–
	0.89	0.18 ± 0.01	14.8 ± 2.4	0.10 ± 0.02	39.3 ± 9.7
	0.91	0.30 ± 0.03	15.5 ± 4.4	0.24 ± 0.02	23.7 ± 3.7
	0.93	0.30 ± 0.03	11.0 ± 3.2	0.28 ± 0.02	18.0 ± 3.3
20	0.80	–	–	–	–
	0.85	0.22 ± 0.01	10.8 ± 0.7	0.18 ± 0.08	30.6 ± 3.6
	0.87	0.34 ± 0.01	10.0 ± 0.6	0.22 ± 0.00	12.2 ± 1.5
	0.89	0.39 ± 0.03	5.3 ± 0.5	0.28 ± 0.03	9.2 ± 0.9
	0.91	0.56 ± 0.01	4.6 ± 0.4	0.40 ± 0.01	6.2 ± 0.7
	0.93	0.68 ± 0.01	3.9 ± 0.2	0.49 ± 0.02	4.9 ± 0.2
25	0.80	–	–	–	–
	0.85	0.30 ± 0.03	4.2 ± 1.0	0.38 ± 0.03	5.9 ± 0.4
	0.87	0.43 ± 0.03	3.4 ± 0.8	0.61 ± 0.03	3.6 ± 0.4
	0.89	0.58 ± 0.04	2.9 ± 0.8	0.78 ± 0.05	2.9 ± 0.3
	0.91	0.94 ± 0.03	2.9 ± 0.4	1.06 ± 0.02	2.5 ± 0.3
	0.93	1.04 ± 0.06	2.4 ± 0.3	1.08 ± 0.05	2.1 ± 0.2
30	0.80	–	–	–	–
	0.85	0.32 ± 0.01	4.6 ± 0.6	0.41 ± 0.03	4.2 ± 0.3
	0.87	0.45 ± 0.02	3.2 ± 0.6	0.65 ± 0.04	3.2 ± 0.3
	0.89	0.63 ± 0.03	3.5 ± 1.0	0.47 ± 0.05	1.1 ± 0.6
	0.91	0.94 ± 0.03	2.6 ± 0.5	1.17 ± 0.03	1.9 ± 0.1
	0.93	1.15 ± 0.08	2.3 ± 0.2	1.40 ± 0.10	1.5 ± 0.2
37	0.80	–	–	–	–
	0.85	0.10 ± 0.20	22.5 ± 1.3	–	–
	0.87	0.29 ± 0.10	15.1 ± 1.1	0.57 ± 0.05	6.2 ± 1.0
	0.89	0.20 ± 0.03	12.0 ± 1.5	0.88 ± 0.06	4.6 ± 0.2
	0.91	0.23 ± 0.02	9.3 ± 1.7	1.18 ± 0.05	2.3 ± 0.2
	0.93	0.27 ± 0.02	7.5 ± 1.5	1.50 ± 0.07	1.7 ± 0.3
42	0.80	–	–	–	–
	0.85	–	–	–	–
	0.87	–	–	–	–
	0.89	–	–	–	–
	0.91	–	–	–	–
	0.93	–	–	0.48 ± 0.10	4.8 ± 1.7

SE, standard error, $n = 10$.
–, no growth observed for 90 days.

Table 4
Estimated values and statistics of the coefficients of the cardinal model for *Aspergillus ochraceus* and *Aspergillus parasiticus* at different conditions of temperature and a_w.

Species	Parameter	Estimated value ± SE	RMSE
Aspergillus ochraceus	μ_{opt} (square root)	1.00 ± 0.02	0.128
	T_{min}	6.48 ± 0.71	
	T_{max}	41.83 ± 0.15	
	T_{opt}	27.36 ± 0.27	
	$a_{w\ min}$	0.78 ± 0.01	
	$a_{w\ opt}$	0.93 ± 0.00	
Aspergillus parasiticus	μ_{opt} (square root)	1.33 ± 0.06	0.175
	T_{min}	5.66 ± 0.89	
	T_{max}	42.47 ± 0.16	
	T_{opt}	31.15 ± 0.31	
	$a_{w\ min}$	0.77 ± 0.01	
	$a_{w\ opt}$	0.94 ± 0.01	

SE, standard error derived from non-linear regression.

obtained for the 9 extreme growth conditions assayed. Bf were unacceptably low for validation in maize grain due to the absence of growth in maize under many conditions; acceptable Af were obtained at 0.93a_w/25 °C, but they revealed a poor predictive power under marginal conditions. No differences were observed among the applied models. Similarly, better results were observed for the validation in coffee beans for lag phases predictions compared to results in maize.

Good validation results were obtained in maize for *A. parasiticus* at 0.93a_w/25 °C (Bf = 0.98–1.10; Af = 1.03–1.08); however the models performed poorly in maize under the remaining conditions included in the validation set. The same trend was observed in peanuts at 0.93a_w/25 °C (Bf = 0.90–1.02; Af = 1.06–1.20), while bias factors under extreme conditions revealed too fast predicted growth compared to the observed one. No differences were observed among the applied models. Similarly, better results were observed for the validation in peanuts of lag phases predictions compared to results in maize.

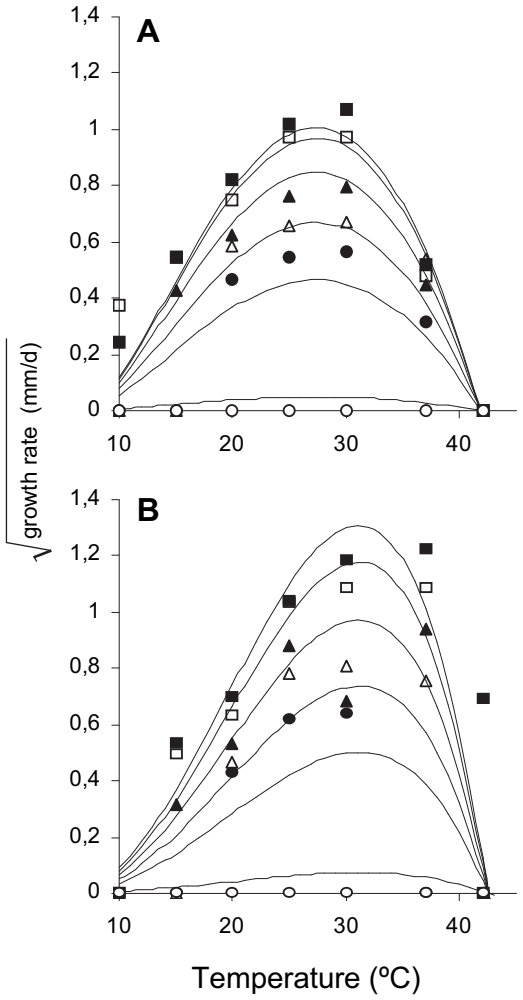

Fig. 1. Square root of growth rates (mm d^{-1}) of A) *A. ochraceus* and B) *A. parasiticus* as a function of temperature at different a_w levels (■, 0.93a_w; □, 0.91a_w; ▲, 0.89a_w; △, 0.87a_w; ●, 0.85a_w; ○, 0.80a_w). Regression lines are fitted to cardinal model.

3.4. Probability models

The mould growth responses for each of the 10-fold repeated experiments are shown in Table 7. *A. ochraceus* grew under 25 of the assayed conditions, and *A. parasiticus* under 21. The replicated samples did not always show the same response, more markedly when conditions were far from the optimum ones (e.g. at 15 and 37 °C). No growth was observed at 0.80a_w, regardless of the temperature level.

A full second-order logistic regression model including all the linear, quadratic and interaction terms along with the time term was generated. Predicted probabilities with a value of >0.50 were considered positive responses (growth). With this criterion, the degree of agreement between observations and predictions was 93.0% concordant and 7.0% discordant for *A. ochraceus* and 94% concordant and 6% discordant for *A. parasiticus*. Maximum rescaled R^2 were of 0.684 and 0.738, respectively. Backward stepwise regression did not eliminate any of the linear or quadratic terms of the logistic model, as all of them were statistically significant ($P < 0.05$), thus the models consisted of 7 terms:

For *A. ochraceus*:

$$\text{Logit}(P) = -704.005 + 1373.68a_w + 5.3746T + 0.0329\text{time} - 681.468a_w^2 - 3.7892a_wT - 0.0384T^2$$

For *A. parasiticus*:

$$\text{Logit}(P) = -607.505 + 1220.57a_w + 2.8928T + 0.0387\text{time} - 642.99a_w^2 - 0.3006\,a_wT - 0.0481T^2$$

where P is the probability of growth and T is the incubation temperature in °C.

The interaction of a_w with temperature was statistically significant. Plots of probability of growth for temperature and a_w at 7 and 30 days of incubation are presented in Fig. 3. It is graphically depicted that the probability plot shifted to lower water activities for the same temperature level as time advanced, for both fungal isolates. Probability data observed after 1 month were almost equal to those observed after 3 months. In addition, the probability of growth for *A. parasiticus* was lower at the lowest temperatures assayed (10–20 °C) indicating delay in fungal growth of this species. For instance, after 7 days at 0.91a_w/15 °C no growth was observed for *A. parasiticus* as the estimated probability was <0.50, whereas for *A. ochraceus* growth was evident ($P > 0.90$) at the same conditions.

As observed in the figures, probabilities of growth for *A. ochraceus* over 0.80 were predicted in the range 0.87–0.93a_w at 19–35 °C. Thus for safe storage of food products, a $a_w < 0.85$ should

Table 5
Model coefficients for the modelled μ_{max} and λ of *A. ochraceus* and *A. parasiticus*.

	Coef.	μ_{max} (mm d^{-1})		λ (days)	
		A. ochraceus	*A. parasiticus*	*A. ochraceus*	*A. parasiticus*
		Estimated value ± SE	Estimated value ± SE	Estimated value ± SE	Estimated value ± SE
Davey	a_0	−264.6 ± 9.4	−352.5 ± 15.8	1.21 10^3 ± 37.3	1.42 10^4 ± 58.6
	a_1	4.7 ± 10.6 ns	68.8 ± 16.8	−180.6 ± 42.1	−319.7 ± 62.4
	a_2	−35.5 ± 4.2	−21.8 ± 6.5	210.4 ± 16.8	146.8 ± 24.3
	a_3	1.56 10^5 ± 4.16 10^3	1.92 10^5 ± 6.95 10^3	−6.72 10^5 ± 1.65 10^4	−7.62 10^5 ± 2.58 10^4
	a_4	−2.59 10^7 ± 6.11 10^5	−2.78 10^7 ± 9.71 10^5	1.09 10^8 ± 2.42 10^6	1.13 10^8 ± 3.61 10^6
	RMSE	0.128	0.198	0.509	0.736
Polynomial	a_0	−38.7 ± 3.1	−23.9 ± 4.8	206.6 ± 12.4	146.7 ± 17.5
	a_1	73.7 ± 7.2	45.3 ± 10.9	−408.8 ± 28.3	−285.8 ± 40.0
	a_2	−35.7 ± 4.1	−22.9 ± 6.2	211.9 ± 16.3	151.q ± 23.0
	a_3	0.3 ± 0.0	0.1 ± 0.0	−1.3 ± 0.1	−0.6 ± 0.1
	a_4	−0.0 ± 0.0	−0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	a_5	−0.2 ± 0.0	0.1 ± 0.0	0.6 ± 0.1	−0.2 ± 0.1 ns
	RMSE	0.125	0.189	0.493	0.696

SE, standard error derived from non-linear regression.
ns, not significant.

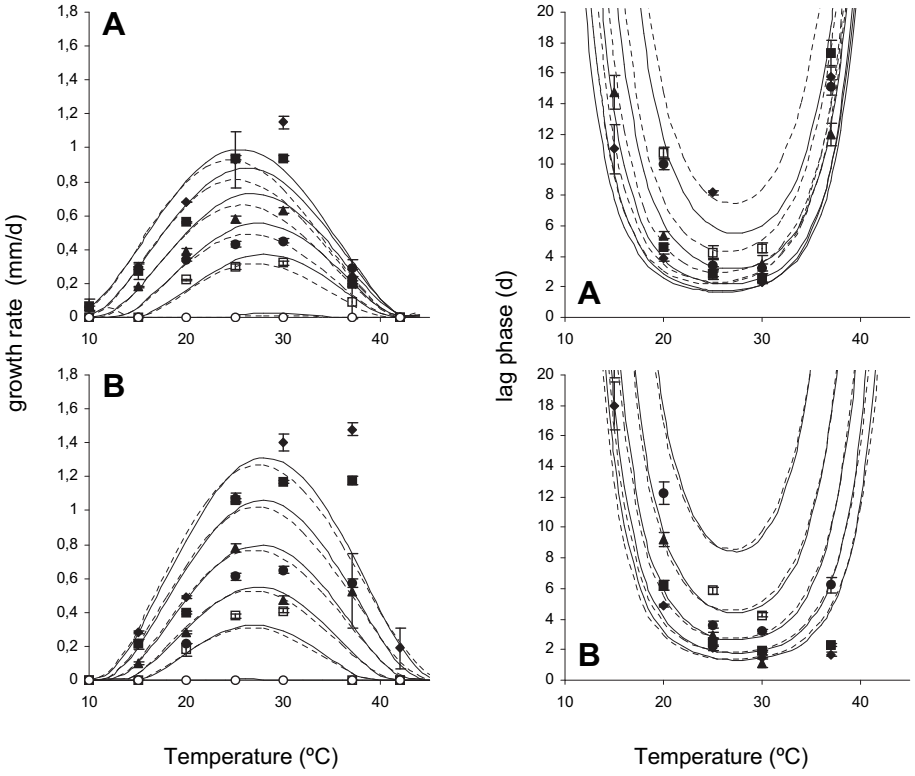

Fig. 2. Growth rates (mm d^{-1}) and lag phases (d, starting from 10 to 100 inoculated spores) of A) *A. ochraceus* and B) *A. parasiticus* as a function of temperature at different a_w levels (◆, 0.93a_w; ■, 0.91a_w; ▲, 0.89a_w; ●, 0.87a_w; □, 0.85a_w; ○, 0.80a_w). Regression lines are fitted to polynomial (—) and extended Davey (–) models. Error bars show standard deviation ($n = 10$).

be maintained and temperatures in the range 21–35 °C should be avoided ($P < 0.5$); alternatively, cool storage (<10 °C) could be applied. Probabilities of growth for *A. parasiticus* over 80% were predicted in the range 0.87–0.93a_w at 19–35 °C. Thus for safe storage of food products, a $a_w < 0.85$ should be maintained and temperatures in the range 20–34 °C should be avoided ($P < 0.5$); alternatively, cool storage (<14 °C) could be applied.

The predicted growth/no growth boundaries at probabilities of 0.1, 0.5 and 0.9, together with the observed growth/no growth cases from which the predictions were derived are presented in

Table 6
Bias and accuracy factors obtained for the validation set.

Species	Food matrix	Model	Validation set	Growth rate (mm d^{-1})		Lag phase (d)	
				Bias factor	Accuracy factor	Bias factor	Accuracy factor
A. ochraceus	Maize grain	cardinal	0.93a_w/25 °C	0.12	1.72	–	–
			Extreme (9)	0.11	26.86	–	–
		Davey	0.93a_w/25 °C	0.13	1.65	7.69	1.82
			Extreme (9)	0.13	29.93	5.18	15.30
		Polynomial	0.93a_w/25 °C	0.12	1.76	10.02	2.37
			Extreme (9)	0.11	33.60	6.82	20.16
	Coffe beans	cardinal	0.93a_w/25 °C	0.64	1.25	–	–
			Extreme (9)	0.48	1.91	–	–
		Davey	0.93a_w/25 °C	0.67	1.19	1.81	1.97
			Extreme (9)	0.56	2.13	1.51	1.68
		Polynomial	0.93a_w/25 °C	0.63	1.28	2.36	2.57
			Extreme (9)	0.48	2.39	1.99	2.21
A. parasiticus	Maize grain	cardinal	0.93a_w/25 °C	1.09	1.07	–	–
			Extreme (9)	0.09	24.06	–	–
		Davey	0.93a_w/25 °C	0.99	1.03	2.53	2.34
			Extreme (9)	0.10	30.13	5.23	13.40
		Polynomial	0.93a_w/25 °C	0.98	1.04	2.49	2.31
			Extreme (9)	0.10	30.97	5.11	13.33
	Peanuts	cardinal	0.93a_w/25 °C	1.01	1.07	–	–
			Extreme (9)	0.29	1.02	–	–
		Davey	0.93a_w/25 °C	0.92	1.18	0.52	1.07
			Extreme (9)	0.33	1.22	0.65	2.23
		Polynomial	0.93a_w/25 °C	0.90	1.20	0.53	1.08
			Extreme (9)	0.33	1.26	0.66	2.21

Extreme (9), the 9 validation experiments listed in Table 1, except condition 0.93a_w/25 °C.

Table 7
Experimental variables with logistic growth responses after 1 month for the growth of *A. ochraceus* and *A. parasiticus* in MEA.

a_w	Temperature (°C)	Observed outcome[a]	
		A. ochraceus	*A. parasiticus*
0.80	10	0	0
0.80	15	0	0
0.80	20	0	0
0.80	25	0	0
0.80	30	0	0
0.80	37	0	0
0.80	42	0	0
0.85	10	0	0
0.85	15	0	0
0.85	20	10	9
0.85	25	10	10
0.85	30	10	10
0.85	37	3	0
0.85	42	0	0
0.87	10	0	0
0.87	15	0	0
0.87	20	10	10
0.87	25	10	10
0.87	30	10	10
0.87	37	10	10
0.87	42	0	0
0.89	10	0	0
0.89	15	10	7
0.89	20	10	10
0.89	25	10	10
0.89	30	10	10
0.89	37	10	0
0.89	42	0	0
0.91	10	1	0
0.91	15	9	9
0.91	20	10	10
0.91	25	10	10
0.91	30	10	10
0.91	37	9	10
0.91	42	0	0
0.93	10	8	0
0.93	15	10	10
0.93	20	10	10
0.93	25	10	10
0.93	30	10	10
0.93	37	9	10
0.93	42	0	0

[a] Number of positive MEA plates at the given a_w out of the 10 inoculated MEA plates after a 90 days incubation at the given temperature.

Fig. 4 for both *Aspergillus* species. This figure confirms graphically the high percentage of logistic model agreement with experimental data.

3.5. Validation of probability models on food matrices

Predicted mould growth responses after one month were compared to those observed in the food matrices for the same period (Table 8). The models predicted growth reasonably successfully in peanuts and coffee (70 and 80% concordance, respectively) considering that the validation samples were located at the boundaries of the domain of the model. For maize, however, there was an agreement of 30 and 40%, for *A. ochraceus* and *A. parasiticus*, respectively, between predicted and observed probabilities. The only condition tested under suitable conditions for growth (0.93a_w/25 °C) led to predicted $P = 1$ for both *A. ochraceus* and *A. parasiticus*, which agreed with the observed probability of growth of *A. parasiticus* in maize and peanuts and *A. ochraceus* in coffee, while the observed probability for *A. ochraceus* in maize was 0.60.

4. Discussion

Most work in predictive microbiology, dealing with bacterial pathogens and spoilage bacteria, has been carried out under a wide range of temperatures, but mainly under high water availabilities which are common in fresh products prone to be colonized by bacteria. Moulds, however, colonize and spoil mainly those foods (e.g. stored cereals and nuts) in which the reduced water activity prevents bacterial growth. Thus in food mycology it is important the use of predictive models developed under suboptimal water activities, and in the whole range of storage temperatures.

Two main modelling approaches may be applied for prevention of mycotoxin accumulation in food commodities. The first one consists of predicting and preventing any growth of mycotoxigenic species; the second modelling approach involves direct mycotoxin analyses. In this work, the first approach has been considered; from our experience, although there is a correlation between growth and mycotoxin production, it is not possible to predict mycotoxin accumulation from kinetic growth data: i) Mycotoxin accumulation does not occur at its best under the same conditions as growth; ii) moreover, not all fungal growth results in mycotoxin formation (Marín et al., 2006). The second approach involving modelling of mycotoxin concentration, may encounter the problem that given the different abilities to synthesize mycotoxins by the different strains of a given species, extrapolation from the models obtained with one or several strains might not be representative for the majority of the strains (Marín et al., 2008a). In addition, growth is a variable which presents less intraspecific variability, and the kinetics of growth are more known, thus we considered prediction and prevention of growth a good alternative to prevent mycotoxin accumulation (Marín et al., 2008b).

The results of this work showed that the examined mycotoxigenic *Aspergillus* species were rather slow growing fungi, with maximum growth rates under the conditions assayed of 1.15 and 1.50 mm d^{-1}, for *A. ochraceus* and *A. parasiticus*, respectively. Previous published works dealing with radial growth rate of *A. ochraceus* showed maximum values of approx. 2–4 mm d^{-1} around 0.93a_w in different agar media and coffee beans (Marín et al., 1998; Ramos et al., 1998; Pardo et al., 2004a, 2005a,b; Suárez-Quiroz et al., 2004), except that of Pardo et al. (2004b) in barley grains, which showed maximum growth rates of 1 mm d^{-1} at such reduced a_w levels. Although many studies deal with growth of *Aspergillus* section *Flavi*, few give details on growth requirements specific for *A. parasiticus*.

Several secondary models have been developed to quantify the effect of these factors on fungal growth (Garcia et al., 2009). Rosso et al. (1993) proposed a model that included three cardinal parameters for temperature (T_{min}, T_{max}, T_{opt}) and the specific growth rate at optimum temperature. This model was later extended to include also water activity (Rosso and Robinson, 2001; Sautour et al., 2001). While estimated $a_{w\ min}$ was similar for both fungal species (0.77 and 0.78), the derived cardinal values for temperature (T_{min}, T_{max}, T_{opt}) for *A. ochraceus* were 6, 42 and 27, and 6, 42 and 31 for *A. parasiticus*. Minimum a_w for growth has been reported at 0.80 (Pardo et al., 2004b; Suárez-Quiroz et al., 2004), however, no a_w levels were tested within the range 0.75–0.80, thus our estimation of $a_{w\ min}$ is probably accurate. Regarding cardinal temperatures for *A. ochraceus*, minimum and maximum temperatures have been reported at 10 and 37 °C, respectively (Marín et al., 1998; Ramos et al., 1998; Suárez-Quiroz et al., 2004), while the optimum one was 25–30 °C (experiments done in 5 °C intervals), in agreement with the estimated values by cardinal model. A great advantage of this model is that all parameters have a physiological meaning which clearly facilitates initial parameter estimations and may also aid in future incorporation into the model of underlying

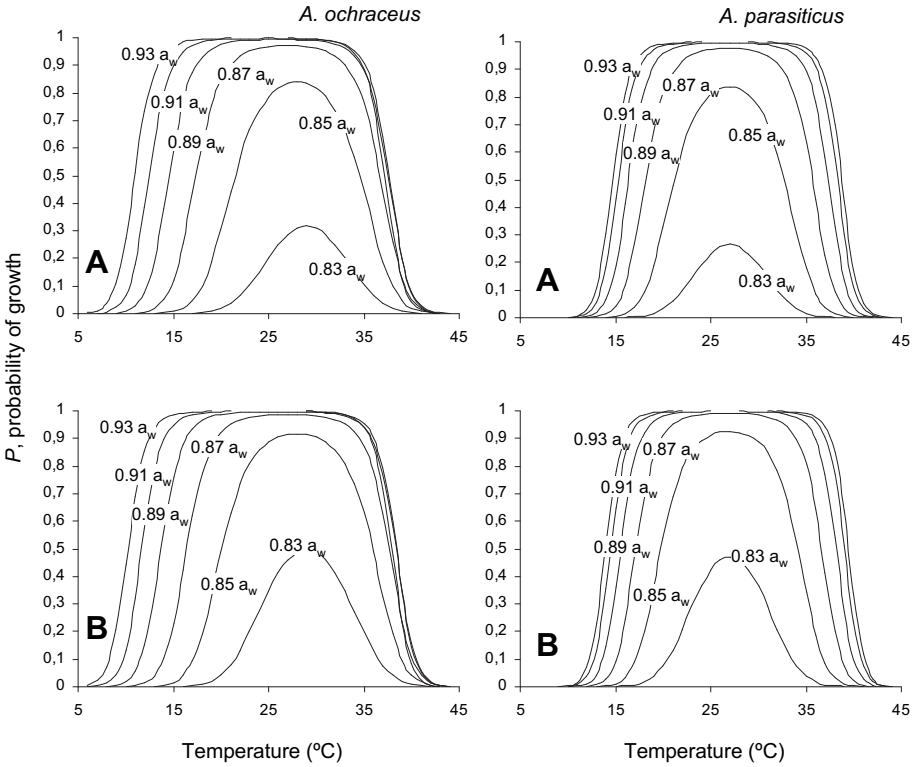

Fig. 3. The predicted effect of temperature and water activity on probability of *Aspergillus ochraceus* and *Aspergillus parasiticus* growth in MEA incubated for A) 7 and B) 30 days.

cell biological mechanisms (Brul and Klis, 1999). In contrast, the estimated coefficients of the polynomial models have no meaning. In addition, similar or slightly worse goodness of fit was observed for the polynomial models in terms of RMSE compared to those obtained for the cardinal model (RMSE = 0.128 for *A. ochraceus*; RMSE = 0.175 for *A. parasiticus*). Consequently, from our results cardinal model should be favoured over the polynomial ones.

Modelling of lag phase data is of interest because it allows direct prediction of the safe storage/transport periods for a given food commodity. Observed lag phases when growth occurred ranged from 2 to 48 days for *A. ochraceus*, and from 1 to 39 days for *A. parasiticus* (Table 3). Short lag times were observed for *A. ochraceus* at 25–30 °C, with a sharp increase at 37 °C, while they decreased gradually at lower temperatures. In the case of *A. parasiticus*, similar values were observed at 25–37 °C, while there was a sharp increase at 20–15 °C. However, modelling of lag phase data obtained under marginal growth conditions becomes difficult, because of no growth results. In our case, the general polynomial model performed better than that proposed by Davey (1989) for both species (RMSE = 0.493–0.696).

For practical purposes, like safe storage prediction, an alternative to lag time calculation may be the use of probability of growth estimation through linear logistic regression. While kinetic models allow for calculation of lag time ± 95% confidence interval, from probability models we can infer the time interval in which initiation of growth may be more probable (e.g. $0.50 < p < 0.90$). For example, at 20 °C and 0.87a_w, lag time confidence intervals were [8.7, 11.3] and [9.3, 15.2] for *A. ochraceus* and *A. parasiticus*, respectively; those intervals corresponded to 0.85–0.86 and 0.82–0.85 probability of growth, respectively. Probabilistic models using logistic regression have been extensively employed to define the growth boundaries of important foodborne pathogens such as *Listeria monocytogenes* (Koutsoumanis and Sofos, 2005; Gysemans et al., 2007; Vermeulen et al., 2007) and *Escherichia coli* (Presser et al., 1998; Skandamis et al., 2007), but their application is rather limited in fungi, where the majority of developed models are kinetic, providing information in terms of growth rate and lag phase or produce response surface and contour plots.

The developed logistic regression models were successfully fitted to the experimental data as the agreement between observed and predicted probabilities was >93% concordant for both fungal species and R^2 were >0.68. High values for both performance indices have been reported previously for the growth of *A. carbonarius* in pistachio nuts (Marín et al., 2008b) and in synthetic grape juice medium (Tassou et al., 2009). It must be noted that polynomial type logistic regression models are empirical in nature and do not contribute to the understanding of the mechanism involved in microbial growth inhibition. However, this type of models offer the possibility to include the no growth responses, which are particularly important when efforts are conducted to prevent an event, such as fungal growth, providing thus a significant degree of quality and safety from spoilage (López-Malo et al., 2000). Nevertheless, Ratkowsky and Ross (1995) modelled growth/no growth of *Shigella flexneri* through logistic regression with equations which contained biological meaningful terms (pH, temperature, a_w, and gas concentration).

Probability of growth of *A. ochraceus* higher than 0.9 was estimated after 8 days over 0.91a_w in the range 15–35 °C. However growth could be delayed by cooling to 10 °C; in such case *P* was <0.1 for 20 days at 0.91a_w, for 51 days at 0.90a_w and indefinitely under this a_w value. Similarly an extension of the unaltered period could be achieved through a_w control, for example *P* did not reach 0.1 in one month at 0.86/15 °C, and over 90 days at 0.82a_w outside the interval 25–34 °C or at 0.80a_w outside the interval 30–31 °C. Probability of growth of *A. parasiticus* higher than 0.9 was estimated after 3 weeks over 0.91a_w in the range 17–37 °C. However growth could be delayed by cooling to 15 °C; in such case *P* was <0.1 for 27 days at 0.87 or for >90 days under 0.86a_w. Growth was delayed for

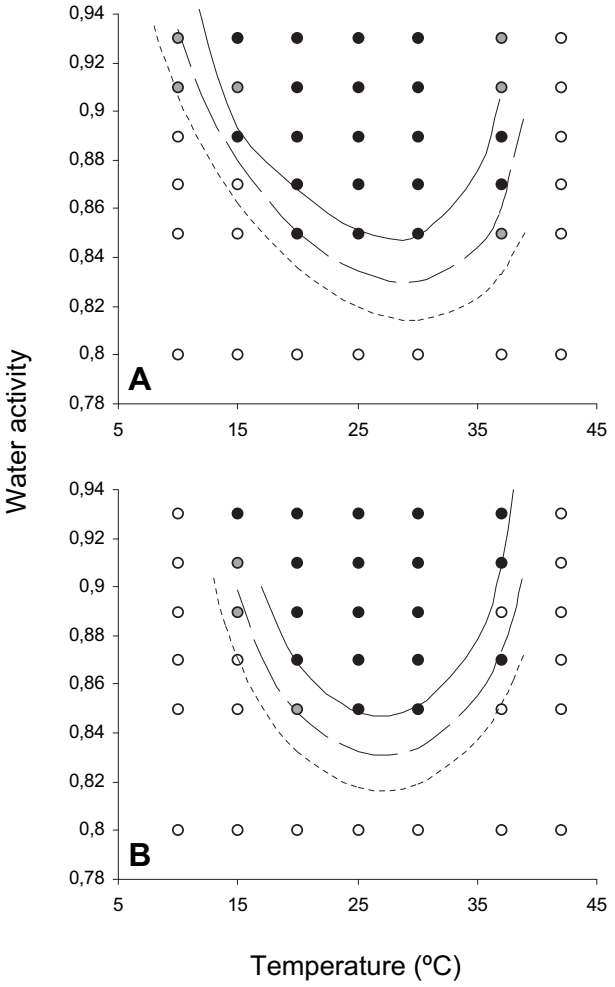

Fig. 4. The predicted growth/no growth boundaries for one month with respect to a_w and temperature at probabilities of 0.1, 0.5 and 0.9 for A) *Aspergillus ochraceus* and B) *Aspergillus parasiticus*. ●, 100% observed growth; ●, 10–90% observed growth; ○, no growth observation.

2 months at 0.90/12 °C and for >90 days under decreasing values of a_w and temperature. Similarly an extension of the unaltered period could be achieved through a_w control, for example, *P* did not reach 0.1 in 40 days at 0.85a_w/15 °C, over 90 days at 0.82a_w outside the interval 23–31 °C or at 0.80a_w. Taking into account the sorption curves of maize, peanuts (Chen, 2000) and coffee (Pittia et al., 2007) the safe moisture contents would be approx. 18, 11 and 18% (dry weight basis), respectively, as suggested by the models developed in this work. Thus in terms of its use in the implementation of HACCP plans the probabilistic approach shown in this study may be of more interest than kinetics modelling.

Very few studies in scientific literature have externally validated the models developed to describe fungal growth, and they rarely present accuracy and bias factors. For example, Battey et al. (2001) constructed a probability model for *A. niger* and *Penicillium spinulosum* on a beverage analogue and validated it in an external set of beverage analogues, too. Samapundo et al. (2007) modelled *A. flavus* and *A. parasiticus* growth on irradiated maize grain, and performed the numerical validation on an external independent set of experiments on irradiated maize grain, too. Baert et al. (2007) developed a series of models for *P. expansum* growth in apple puree agar medium, which were validated in apples. Marín et al. (2008b) validated the probability of growth and OTA models developed for *A. carbonarius* on pistachio nuts in an independent set of experiments prepared on pistachio nuts. However developing models on real food substrates requires much effort, and models developed in a food substrate cannot be extrapolated to other food products. A better approach would be to develop the models in general synthetic media and validate results on food substrates, as done in the present work, however the deviations shown confirm that models developed on synthetic media do not present a realistic picture of microbial responses on real food systems, as they do not take into account one of the most important parameters, i.e. that of food matrix.

Validation of kinetic models was carried out in peanuts and maize for *A. parasiticus* and in coffee and maize for *A. ochraceus*. The domain of the models included suboptimal growth conditions (always under 0.93a_w); within this framework, validation set was constructed including 9 extreme conditions plus a centered condition in the domain. The objective was to test whether the performance of the predictive models may fail at the boundaries. Validation of *A. ochraceus* growth models on coffee beans led to acceptable results under most conditions, while the models for *A. parasiticus* predicted too fast growth under the extreme conditions. Growth of the fungi in maize was in general much slower than predicted by the models. The optimum growth rate is very much dependant on the substrate thus probably explaining the bias and accuracy factors obtained for maize grains. No significant differences were found among the performance of the three models used. The location of grains, beans and nuts in a single layer in Petri plates, which was the method used for validation is

Table 8
Comparison of predicted and observed mould growth responses (one month) of *Aspergillus ochraceus* and *A. parasiticus* in raw food matrices.

a_w/ Temperature		*A. parasiticus*			*A. ochraceus*		
		Predicted probability of growth	Observed outcomes in maize[a]	Observed outcomes in peanut[a]	Predicted probability of growth	Observed outcomes in maize[a]	Observed outcomes in coffee[a]
0.85	25	0.91	0.0	0.4	0.87	0.0	1.0
0.85	30	0.89	0.0	1.0	0.90	0.0	1.0
0.87	20	0.91	0.0	0.0	0.92	0.2	1.0
0.87	30	0.98	0.6	1.0	–	–	–
0.87	37	0.41	0.9	0.8	0.63	0.0	0.1
0.89	15	0.36	0.1	0.0	0.76	0.0	1.0
0.89	20	0.98	0.3	1.0	0.99	0.0	1.0
0.89	37	0.77	0.5	1.0	0.77	0.8	1.0
0.91	15	0.65	0.4	1.0	0.95	1.0	0.9
0.93	10	–	–	–	0.44	0.6	1.0
0.93	25	1.00	1.0	1.0	1.00	0.6	1.0

[a] Number of positive maize, peanuts or coffee plates at the given a_w out of the 10 inoculated plates after a 90 days incubation at the given temperature.

not a realistic situation, because the free spaces among particles may compromise fungal growth, making it slower. Is it likely that the resolution and accuracy of measurements in validation studies is lower than on agar plates due to the lack of homogeneity in the matrices tested.

Validation with independent data showed that the developed logistic model could satisfactorily predict the responses of both fungal species at probability level of 0.5. Specifically, the agreement with growth data in food matrices was 70% in coffee, 80% in peanuts and 30–40% for maize.

In conclusion, the results of the present study indicate that the probability developed modelling approach could be satisfactorily employed to quantify the combined effect of temperature and water activity on the growth responses of *A. ochraceus* and *A. parasiticus*. However, validation of kinetic results led to poor goodness of predictions. Boundary models may be important in predicting the most suitable combinations of environmental factors to prevent fungal growth, providing thus a significant degree of quality and safety. In this study, the validation samples were placed near the expected boundaries of the model in order to test it under the worst situation. Probability of growth prediction under extreme growth conditions was somewhat compromised, but it can be considered acceptable. The risk in using the models presented here in real situations may be the differences in the initial inoculum size and the unrealistic constant conditions of temperature and moisture content.

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