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Effect of temperature and water activity on growth and ochratoxin A production boundaries of two *Aspergillus carbonarius* isolates on a simulated grape juice medium

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Running title: Effect of temperature and a_w on growth and OTA boundaries of *A. carbonarius*

Abstract

Aims: To develop and validate a logistic regression model to predict the growth and ochratoxin A (OTA) production boundaries of two *A. carbonarius* isolates on a synthetic grape juice medium as a function of temperature and a_w.

Methods and results: A full factorial design was followed between the factors considered. The a_w levels assayed were 0.85, 0.88, 0.90, 0.92, 0.94, 0.96, 0.98 and the incubation temperatures were 10, 15, 20, 25, 30, 35, and 40°C. Growth and OTA production responses were evaluated for a period of 25 days. For growth boundaries, the degree of agreement between predictions and observations was > 99% concordant for both isolates. The erroneously predicted growth cases were 3.4-4.1% false positive and 0.7-1.4% false negative. No growth was observed at 10°C and 40°C for all a_w levels assayed, with the exception of 0.98 a_w/40°C where weak growth was observed. Similarly, OTA production was correctly predicted with a concordance rate > 0.98% for the two isolates with 0.7-1.4% accounting for false positives and 2.0-2.7% false negatives. No OTA production was detected at 10°C or 40°C regardless of a_w, and at 0.85 a_w at all incubation temperatures. With respect to time, the OTA production boundary shifted to lower temperatures (15-20°C) compared to the growth boundary which shifted to higher temperature levels (25-30°C). Using two literature datasets for growth and OTA production of *A. carbonarius* on the same growth medium, the logistic model gave 1 false positive and 3 false negative predictions out of 68 growth cases and 13 false positive predictions out of 45 OTA production cases.

Conclusions: The results of this study suggest that the logistic regression model can be successfully used to predict growth and OTA production interfaces for *A. carbonarius* in relation to temperature and a_w.

Significance and Impact of the Study: The proposed modelling approach helps the understanding of fungal-food ecosystem relations and it could be employed in HACCP implementation plans to predict the risk of contamination of grapes and grape products by *A. carbonarius.*

Keywords: *Aspergillus carbonarius*, fungal growth, ochratoxin A, predictive mycology, probabilistic models, wine grapes

Introduction

Mycotoxins are natural secondary metabolites produced by fungi on agricultural commodities in the field and during storage over a wide range of climatic conditions. Ochratoxin A (OTA) is a major mycotoxin produced by several fungal species belonging to the genera *Penicillium* section *Circumdati* and *Aspergillus* section *Nigri* that has nephrotoxic, immunosuppressive, teratogenic and carcinogenic effects on animals and humans (O'Brien and Dietrich 2005; Murphy *et al.* 2006; Richard 2007). *Aspergillus carbonarius* has a central role in OTA contamination of grapes and wines (Abarca *et al.* 2001; Esteban *et al.* 2004; Battilani *et al.*, 2006; Bellí *et al.* 2007; Pateraki *et al.* 2007; Visconti *et al.* 2008), as several strains of the fungus have been isolated and identified in wine producing countries around the Mediterranean basin (Serra *et al.* 2003; Battilani *et al.* 2004; Bellí *et al.* 2004a; Mitchell *et al.* 2004; Tjamos *et al.* 2004, 2006) and other parts of the world (Chulze *et al.* 2006; Leong *et al.* 2006; Romero *et al.* 2007). The presence of OTA in foods is a major concern in food safety and the European Union has established maximum OTA levels at 2 μg kg^{-1} for wine, grape juice, grape nectar and grape must intended for direct human consumption, and at 10 μg kg^{-1} for dried vine fruits (currants, raisins and sultanas) (European Commission 2005).

Predictive microbiology aims at the determination of the responses of a given microorganism combining mathematical models with experimental data under certain environmental conditions. In general, predictive models can be divided into two main categories, namely kinetic and probabilistic models. The former determine microbial responses in relation to time and provide estimates for parameters such as lag phase duration and growth rate. The latter determine the probability of microbial growth or toxin production, i.e. whether growth or toxin production might occur or not, under a specific range of environmental factors (Whiting 1995). So far, several modelling approaches have been developed for *A. carbonarius* to quantify the effect of temperature and a_w on fungal growth and OTA production (Bellí *et al.* 2004b, 2005; Magan and Aldred 2005; Pardo *et al.* 2005; Marín *et al.* 2006; Tassou *et al.* 2007; Romero *et al.* 2007). However, these studies have been focused almost exclusively on the development of kinetic models providing information in the form of growth rate and lag phase duration or produce response surface and contour plots, whereas there is little information available on the development of a probabilistic approach to quantify fungal growth and OTA production boundaries for a specific range of environmental conditions. Recently, Marín *et al.* (2008) has developed a probabilistic model to determine the growth/no growth boundaries and OTA production by *A. carbonarius* in pistachio nuts. To the best of our knowledge, no similar probabilistic model has been developed so far for *A. carbonarius* on grapes and grape products. The objectives of the present work are (i) to develop probabilistic models to predict the growth/no growth and OTA production boundaries of two ochratoxigenic isolates of *A. carbonarius* from Greek wine grapes on a synthetic grape juice medium, and (ii) to validate the performance of the developed models with independent data from the literature.

Materials and methods

Fungal isolates and growth medium

This work was carried out on two ochratoxigenic isolates of *Aspergillus carbonarius* (ATHUM 5659 and 5660) isolated previously from wine grapes in the Peloponnesus region of southern Greece (Tassou *et al.* 2007). Both isolates were found to produce OTA when tested in Czapek yeast extract agar (CYA) in amounts exceeding 8 μg g^{-1} of substrate, determined by the method of Bragulat *et al.* (2001) after incubation at 25°C for 7 days. Studies were carried out *in vitro* using a

synthetic nutrient medium (SNM) with composition similar to grapes between véraison and ripeness. The medium had the following composition: D(+) glucose, 70 g; D(-) fructose, 30 g; L(-) tartaric acid, 7 g; L(-) malic acid, 10 g; $(NH_4)_2HPO_4$, 0.67 g; KH_2PO_4, 0.67 g; $MgSO_4 \cdot 7H_2O$, 1.5 g; NaCl, 0.15 g; $CuCl_2$, 0.0015 g; $FeSO_4 \cdot 7H_2O$, 0.021 g; $ZnSO_4 \cdot 7H_2O$, 0.0075 g; (+) Catechin hydrate, 0.05 g; agar, 25 g; distilled water, *ca* 1000 ml. The pH of the medium was adjusted to 3.5 with KOH (2 M). The a_w of this basal medium was 0.98, measured by a Novasina Thermoconstander RTD 33 (Novasina AG, Zürich, Switzerland) water activity meter at 20°C. SNM was modified to 0.85, 0.88, 0.90, 0.92, 0.94, and 0.96 a_w by adding different amounts of glycerol (Mitchell *et al.* 2004).

Inoculation and incubation conditions

Both fungal isolates were grown on SNM medium for a period of 10 days at 25°C to obtain sporulating cultures. Spore suspension was obtained adding 15 ml of sterile phosphate buffer solution (pH 7.0) containing 0.1% of a wetting agent (Tween 80; Merck, Darmstadt, Germany) and softly scraping the surface of the medium with a sterile glass rod to suspend the spores in the liquid phase. The resulting suspension was filtered through sterile medical tissue to remove any mycelial fragments. The final concentration of spores was assessed by a Neubauer counting chamber (Brand, Wertheim, Germany) and adjusted to 10^6 spores ml^{-1} with the same buffer medium. SNM agar plates containing *ca* 20 ml of solidified growth medium were needle inoculated centrally. Plates with the same a_w level were sealed with parafilm and wrapped in polyethylene to minimise desiccation and finally incubated at the required temperatures (10, 15, 20, 25, 30, 35, and 40°C). The effect of temperature and a_w on fungal growth and OTA production was investigated by means of a full factorial design. Four replicated plates for each treatment were used and the whole experiment was repeated twice (n = 8).

Fungal growth assessment and OTA determination

Fungal growth was observed on a daily basis for an overall period of 25 days and the first day where visible growth was evident in each a_w/temperature treatment was recorded. OTA production was determined after 5, 10, 15, 20, and 25 days incubation following a previously described HPLC screening method (Bragulat *et al.* 2001). An agar plug of 3 mm radius was taken from the central area of each colony, weighed, vortexed with 0.5 ml of methanol and kept in the solvent for 1 hr. The extracts were filtered (Millex® Syringe Driven Filter Unit, Millipore Co. Bedford, MA, USA) and stored at 4°C until HPLC analysis. Analysis was performed with a HPLC system (Hewlett Packard Series 1100), equipped with an Agilent 1100 fluorescence detector (330 nm excitation wavelength; 460 nm emission wavelength). Chromatographic separations were performed with a C18 Waters Spherisorb ODS2 column (5 μm, 250×4.6 mm). The flow rate of the mobile phase used (acetonitrile: water: acetic acid; 51:47:2) was 1 ml min^{-1}. The detection limit of the method was 0.01 μg OTA g^{-1} of SNM.

Modelling of the growth/no growth interface

For each replicate response of the two fungal isolates, visible growth or no growth were scored as values of 1 or 0, respectively. Data were fitted to a logistic regression model based on the approach of Ratkowsky and Ross (1995) in order to determine the growth/no growth boundaries of the fungi under the assayed environmental factors. The model employed was a second-order logistic regression model in the form shown in the following equation:

$$\log it(P) = \ln\left[\frac{P}{1-P}\right] = a_0 + a_1 t + a_2 T + a_3 a_w + a_4 t^2 + a_5 T^2 + a_6 a_w^2 + a_7 tT + a_8 t a_w + a_9 T a_w \quad (1)$$

where, P is the probability of growth (in the range of 0-1), a_i are coefficients to be estimated, a_w is the water activity of the medium, t (days) is incubation time, and T (°C) is temperature. The equation was fitted using Minitab® version 14.1 (Minitab Inc., State College, PA, USA) logistic regression procedure. The automatic variable selection option with a stepwise 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 using Microsoft Excel Solver. The following statistical indices were calculated to measure the goodness-of-fit of the developed models: the Hosmer-Lemeshow goodness-of-fit statistic, the maximum rescaled R^2 and the concordance rate (McKellar and Lu 2001; Koutsoumanis and Sofos 2005; Skandamis *et al.* 2007).

Modelling of OTA production

For the same set of a_w/temperature conditions a separate logistic regression model was developed to quantify the effect of both factors on OTA production. For this reason, OTA analysis results were assigned values of either 1 when OTA concentration was above the limit of detection (>l.d.), or 0 when OTA concentration was below the limit of detection (<l.d.). Logistic regression was used to calculate the probability of OTA production given a certain combination of storage conditions (a_w and temperature) and incubation time. A similar second order logistic regression model was developed as follows:

$$\log it(P) = \ln\left[\frac{P}{1-P}\right] = b_0 + b_1 t + b_2 T + b_3 a_w + b_4 t^2 + b_5 T^2 + b_6 a_w^2 + b_7 tT + b_8 t a_w + b_9 T a_w \quad (2)$$

where, P is the probability of OTA production (in the range of 0-1), and b_i are coefficients to be estimated. The equation was fitted using Minitab® version 14.1 (Minitab Inc., State College, PA, USA) logistic regression procedure. The automatic variable selection option with a stepwise selection method was used to choose the significant effects (P<0.05). The predicted OTA interfaces for P=0.1, 0.5, and 0.9 were calculated using Microsoft Excel Solver. The goodness-of-fit of the developed model was assessed by the same statistical indices as mentioned above.

Comparison of the developed models with independent data

The predictions at 50% probability level of models for the two isolates of *A. carbonarius* were compared with two literature data sets in which conditions for growth were similar to those used for the development of the logistic models. Specifically, the first validation data set was that of Bellí *et al.* (2005) in which the growth and OTA production of eight isolates of *A. carbonarius* were monitored on the same synthetic grape juice medium in relation to temperature (15-37°C) and water activity (0.90-0.99 a_w). The second data set was again of Bellí *et al.* (2004b) who studied the effect of water activity (0.90-0.995 a_w) and temperature (10-37°C) on the growth rate of ten isolates of *Aspergillus* section *Nigri* from which four were *A. carbonarius*, on the same synthetic grape juice medium. In both data sets, the value of 0.99 a_w was not included in the validation approach as it was outside the initial a_w range used in our work for the development of the probabilistic model. It has to be noted that in both publications the reported growth data are provided in the form of kinetic parameters (growth rates, mm day^{-1}) and not as incidence (probability) of growth/no growth. For the purpose of validation, values of 0 or 1 have been assigned by the authors of the present work to validation data for a selected time period of 20 days, where, according to our experience, growth or no growth should have been occurred.

Results

The parameter estimates and statistics with the significant effects ($P < 0.05$) of the logistic regression model for the growth of the two fungal isolates are shown in Tables 1 and 2. The degree of agreement between predictions and observations was 99.4% concordant and 0.6% discordant for *A. carbonarius* ATHUM 5659 (Table 1) indicating successful data fitting. Overall, 5.5% of observed data fell on the "wrong" side of the predicted boundary at a probability level of 0.5, from which 4.1% were false positives (i.e. growth predicted when no growth was observed) and 1.5% false negatives (i.e. no growth predicted but growth observed). The goodness-of-fit was also evaluated by the Hosmer-Lemeshow statistic (χ^2 =11.98, df = 8, P = 0.152) and the maximum rescaled R^2 (0.845) which proved the good adjustment of the model to the observations. A similar pattern was observed for the other isolate of *A. carbonarius* ATHUM 5660 (Table 2). In this case, the concordance of the model was 99.5%, whereas 3.4% and 0.7% of the predictions were false positive and false negatives, respectively. The Hosmer-Lemeshow statistic and the maximum rescaled R^2 showed high agreement of predicted with observed probability of growth and hence adequate fit of the data.

Plots of probability of growth for a_w and temperature at 5, 15 and 25 days of incubation are presented in Figures 1 and 2. It is characteristic that the probability plot shifted to lower temperatures for the same a_w level, especially between 5 and 15 days for both fungal isolates. In addition, the probability of growth for *A. carbonarius* ATHUM 5660 was higher at the lowest a_w assayed (0.85) at 15 and 25 days (Fig. 2) indicating that this fungal isolate could be more xerophilic compared with *A. carbonarius* ATHUM 5659. The predicted growth interfaces with respect to time at probabilities of 0.1, 0.5 and 0.9, together with the observed growth/ no growth data from which the predictions were derived are depicted in Figures 3 and 4 for each isolate. These graphs are also representative of the low percentage of model disagreement with the experimental data. No growth was observed at 10°C and 40°C, regardless of a_w level. As time increased, the predicted growth interface shifted to lower water activity values for both isolates. The advancement of the interface was clearer between 5 and 15 days, but from this time onwards little change was evident, with the exception of 40°C and 0.98 a_w where slow fungal growth was observed at 25 days.

A probabilistic approach was also employed for OTA production using a full second order logistic regression model for each fungal isolate. The developed models showed high agreement of prediction with observed probability for OTA production, as was evident from the high concordance rate (98.9-99.1) and the R^2 statistic values (0.832-0.856) (Tables 3 and 4). Increasing probabilities for OTA were predicted at 15 days, compared to those after 5 days, particularly at the lower a_w levels assayed (0.88-0.94) (Figs. 5 and 6). The lowest probability for OTA production (P = 0.03) was observed at a_w 0.85 and 19-20°C even after 25 days of storage. Probability profiles at 15 and 25 days presented similar patterns with the exception of a_w 0.88 where increased values were estimated for both fungal isolates with respect to time. The predicted OTA interface at probabilities of 0.1, 0.5 and 0.9 is shown in Figures 7 and 8. No OTA was detected either at 10°C or at 40°C regardless of a_w. Similarly, no OTA was detected at a_w 0.85 at the different temperature levels. At 5 days, predictions with P = 0.5 enclosed all OTA production cases for *A. carbonarius* ATHUM 5960 (Fig. 8). The same was not observed for the other isolate as three a_w/temperature conditions were left below the interface line (Fig. 7). With regard to time, the interface shifted to lower a_w values and at 25 days the P = 0.5 interface line enclosed all the OTA production cases for both isolates.

Validation was carried out with literature data from two independent data sets. In the first literature study (Bellí *et al.* 2005) the logistic model predicted growth in all cases (100%), while growth was not actually observed at 0.90 a_w and 15°C for the eight strains of *A. carbonarius* assayed (Table 5). A similar situation was observed with the second literature study (Bellí *et al.* 2004) where the model gave three false negative (i.e. no growth predicted when growth was observed) predictions at 10°C and 0.95, 0.98 a_w (Table 6) for four strains of the fungus examined. Predicted OTA responses from the first literature study are shown in Table 7. There was an overall discordance for 13 a_w/temperature conditions for which the logistic model predicted OTA production above the detection limit, while the observed OTA concentration was below the detection limit. However, all the erroneously predicted cases were on the safe side (fail positive).

Discussion

The present study describes the applicability of a probabilistic modelling approach for the influence of a_w and temperature on growth and OTA production of two ochratoxigenic isolates of *A. carbonarius* from Greek wine grapes. Models to predict the likelihood of growth of microorganisms as a function of intrinsic and extrinsic factors were first explored in the 1970s (Genigeorgis 1981; Gibson *et al.* 1987), known as "probability" models. Later on it became necessary to manage the risk to consumers from foodborne pathogens and ensure presence/absence of a certain microorganism in a food commodity, thus leading to the development of "growth/no growth boundary" or "interface" modelling (Ratkowsky and Ross 1995). In recent years the need for modelling microbial growth limits has been increasingly recognised (McMeekin *et al.* 2002). Such models can be useful in the development of processes that allow production of safer food products and could also be important for deciding food safety regulations (Schaffnet and Labuza 1997). So far predictive mycology has not received the same level of attention compared to food-borne pathogenic bacteria and only recently the concepts for modelling fungal development have been reviewed (Dantigny *et al.* 2005). Probability models, although not extensively used in predictive mycology, can provide useful information and define the response of the fungus in boundary conditions of growth and toxin production.

The present logistic model was fitted successfully to the experimental data as the agreement between observed and predicted probabilities was > 99% concordant for fungal growth (Tables 1-2) and > 98% for OTA production (Tables 3-4) for both isolates. It proved difficult to find appropriate literature data to compare our logistic model as no similar approach has been employed so far for *A. carbonarius* in grapes. However, these values are comparable with those reported by Marín *et al.* (2008) for *A. carbonarius* growth and OTA production in pistachio nuts, where the relevant concordant rates were 95.6% (for fungal growth) and 94.6% (for OTA production). The maximum rescaled R^2 of 0.845/0.869 (for growth) and 0.832/0.856 (for OTA) obtained in the present study was higher that that reported in the above mentioned work, i.e. 0.786 and 0.715 for growth and OTA production, respectively. The higher values of R^2 reported in this work could be explained by the fact that our experiment was carried out on a synthetic and well-defined laboratory medium, whereas in the other work the fungus was inoculated directly on pistachio nuts.

With respect to time, the growth boundary shifted to higher temperature levels (25-30°C) (Figs. 3-4) whereas the OTA production boundary shifted to lower temperatures (15-25°C) (Figs. 7-8) indicating that OTA production does not occur at its best under the same conditions for growth. The extension of the growth boundary with time was similar for the two isolates, with the lowest a_w for growth at 0.85-0.88 depending on incubation temperature. Growth at these a_w

values, although not consistent with literature data (Mitchell *et al.* 2004; Bellí *et al.* 2005; Leong *et al.* 2006), could be attributed to adaptation to regional climatic conditions, thus making these isolates more tolerant to xerophilic conditions. As observed in the probability plots (Figs. 1-2), probabilities of growth over 0.8 were predicted in synthetic grape medium with 0.90-0.98 a_w incubated at 15-35°C in 5 days time. As the a_w in grapes during ripening is 0.95-0.98 and the prevailing temperatures at harvest many vary between 30 and 35°C, or lower depending on regional conditions, there is increased probability of fungal growth and subsequent OTA contamination. The same probability level ($P > 0.8$) for OTA production for the same temperature range and time was attained in growth media with 0.94-0.98 a_w for *A. carbonarius* ATHUM 5659 (Fig. 7) and 0.96-0.98 a_w for *A. carbonarius* ATHUM 5660 (Fig. 8), indicating that the range of a_w for OTA production is narrower than that for growth. However, fungal growth and OTA production is much more complicated under realistic conditions as reported by Marín *et al.* (2006). Environmental fluxes, especially day temperatures may not be appropriate for OTA production but may support hyphal extension increasing the potential for OTA production under lower temperatures at night.

Validation with independent literature data showed that the developed logistic model could adequately predict the growth/no growth cases of other *A. carbonarius* strains at a probability level of 0.5. Some disagreement was only observed with 1 false positive (Table 5) and 3 false negative (Table 6) predictions out of 68 total growth cases. The false negative cases were not predicted successfully as they were located at the boundaries of the domain of the model. Finally, the model predicted OTA responses reasonably well as the agreement with literature data for OTA absence was 27 out of 40 (67.5%) cases and 5 out of 5 (100%) cases for OTA presence. The misclassified cases of the model could be attributed to the great variability of different *A. carbonarius* stains in OTA production between countries and also within the same country even under the same environmental conditions (Mitchell *et al.* 2004).

In conclusion, the results of this study indicate that logistic regression models can be successfully employed to predict the boundaries for growth and OTA production of *A. carbonarius* on a synthetic grape juice medium and also for other mycotoxin producing species. However, due to the variability of *A. carbonarius* strains in growth potential and OTA production, further research is needed to develop and validate more extensively such models with additional regional experimental data from grapes and grape products.

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Figure 1 The effect of temperature and water activity on the predicted probability of *Aspergillus carbonarius* ATHUM 5659 growth on a synthetic grape juice medium for 5, 15 and 25 days.

Figure 2 The effect of temperature and water activity on the predicted probability of *Aspergillus carbonarius* ATHUM 5660 growth on a synthetic grape juice medium for 5, 15 and 25 days.

Figure 3 Growth/no growth boundaries of *Aspergillus carbonarius* ATHUM 5659 after 5, 15 and 25 days incubation on a synthetic grape juice medium. Solid symbol: growth, open symbol: no growth; solid line $P = 0.9$; dotted line $P = 0.5$; dashed line $P = 0.1$.

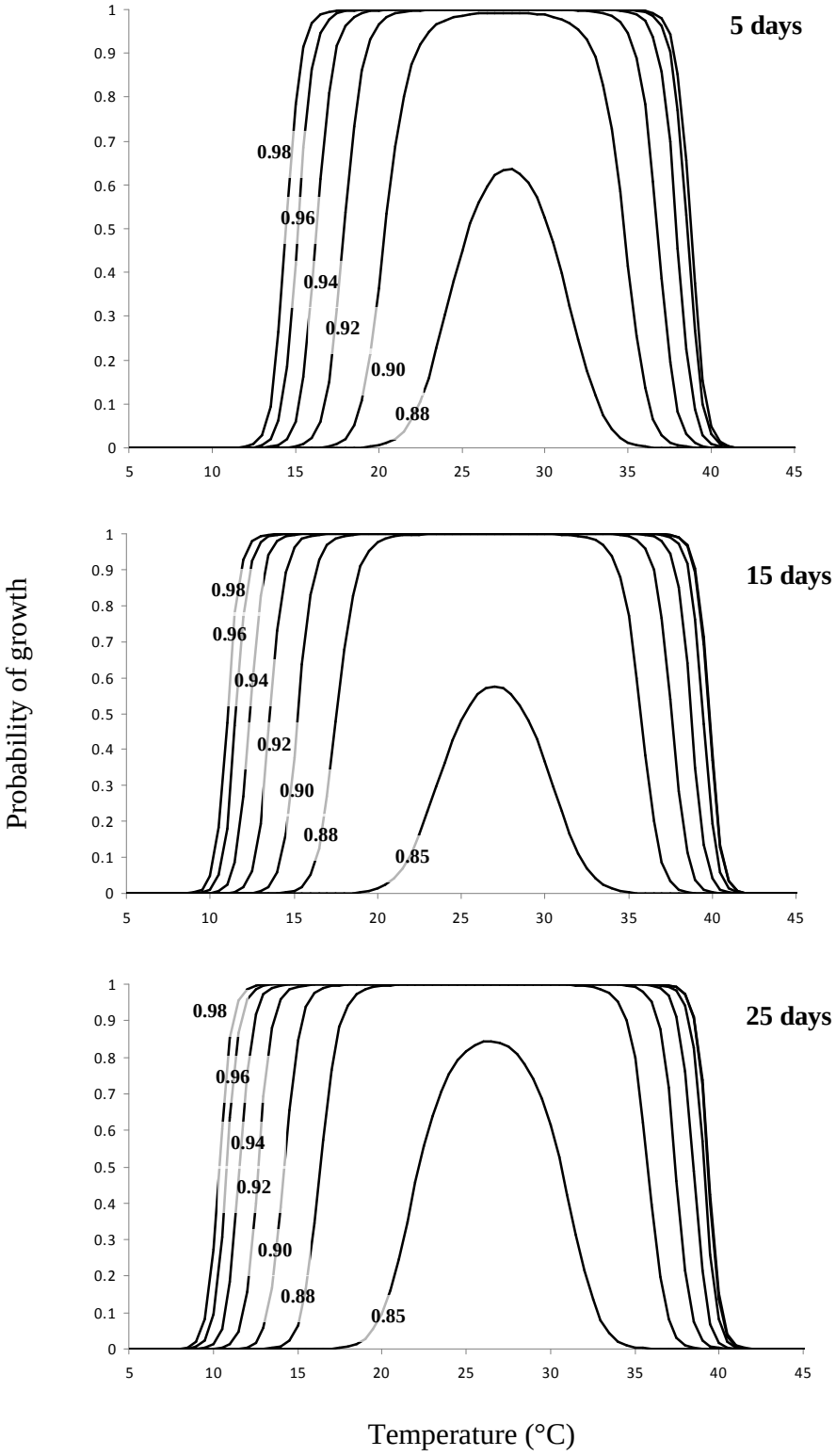
Figure 4 Growth/no growth boundaries of *Aspergillus carbonarius* ATHUM 5660 after 5, 15 and 25 days incubation on a synthetic grape juice medium. Solid symbol: growth, open symbol: no growth; solid line $P = 0.9$; dotted line $P = 0.5$; dashed line $P = 0.1$.

Figure 5 The effect of temperature and water activity on the predicted probability of *Aspergillus carbonarius* ATHUM 5659 OTA presence on a synthetic grape juice medium for 5, 15 and 25 days.

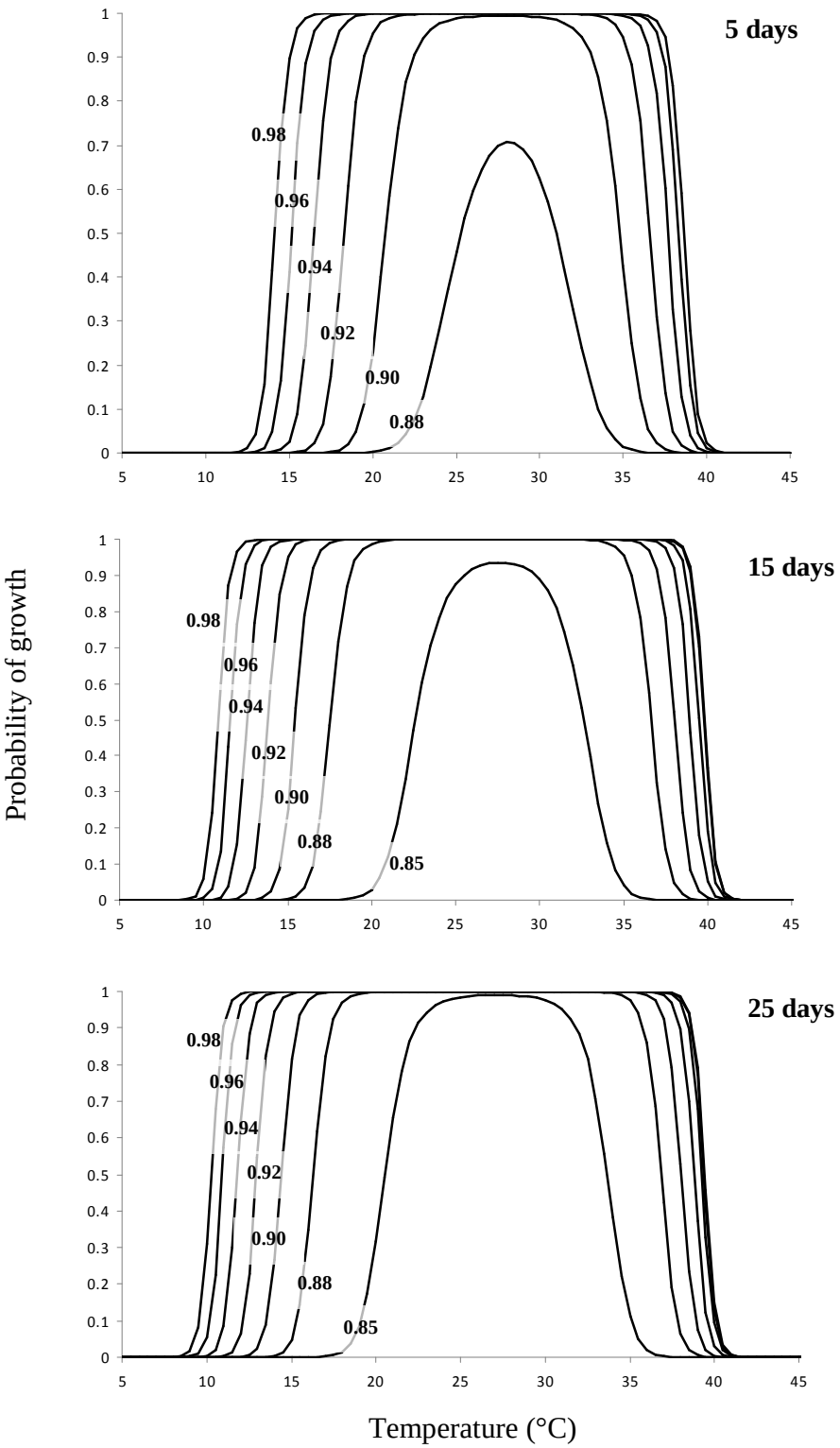
Figure 6 The effect of temperature and water activity on the predicted probability of *Aspergillus carbonarius* ATHUM 5660 OTA presence on a synthetic grape juice medium for 5, 15 and 25 days.

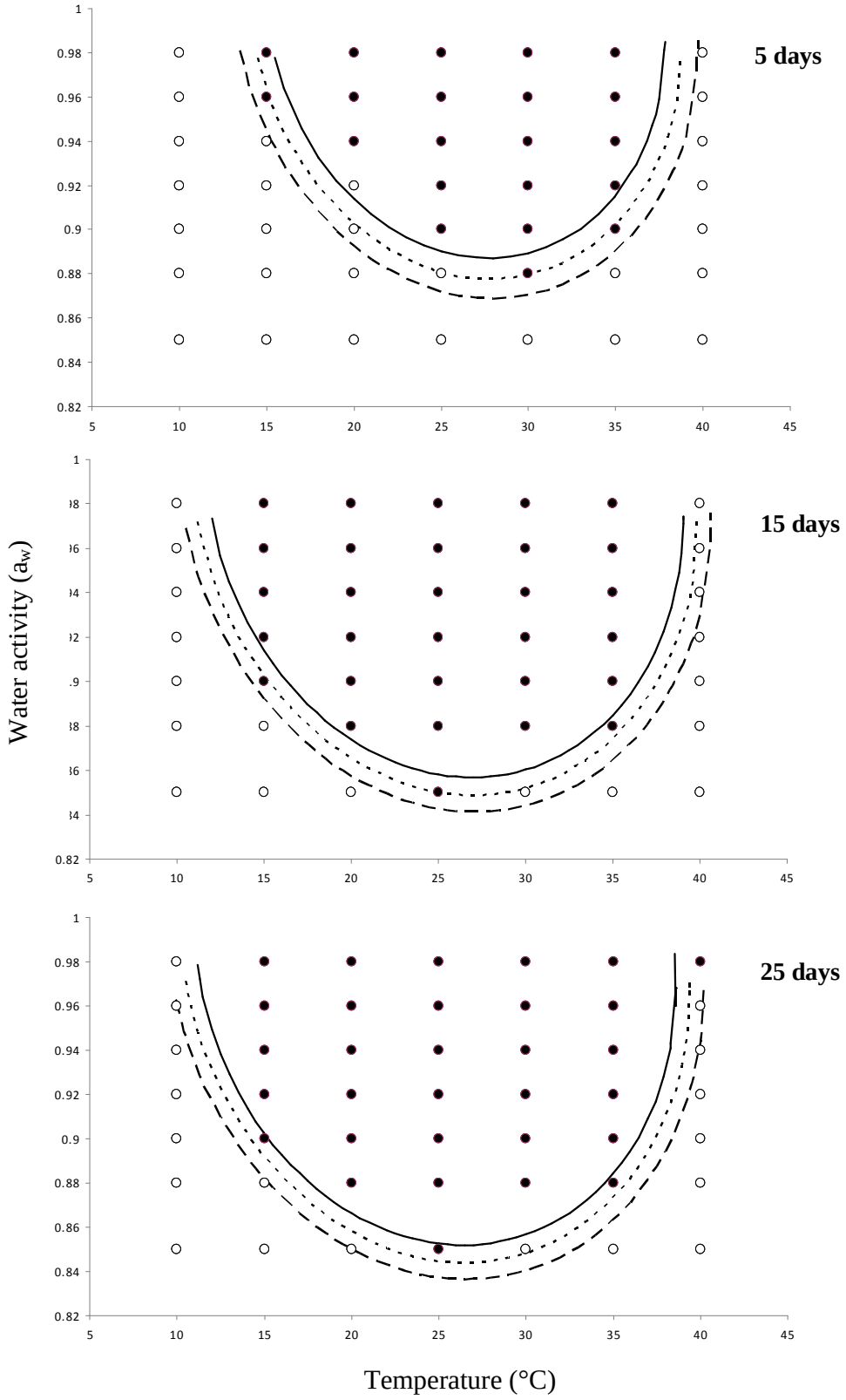
Figure 7 OTA production boundaries of *Aspergillus carbonarius* ATHUM 5659 after 5, 15 and 25 days incubation on a synthetic grape juice medium. Solid symbol: OTA presence (>l.d.), open symbol: OTA absence (<l.d.); solid line $P = 0.9$; dotted line $P = 0.5$; dashed line $P = 0.1$.

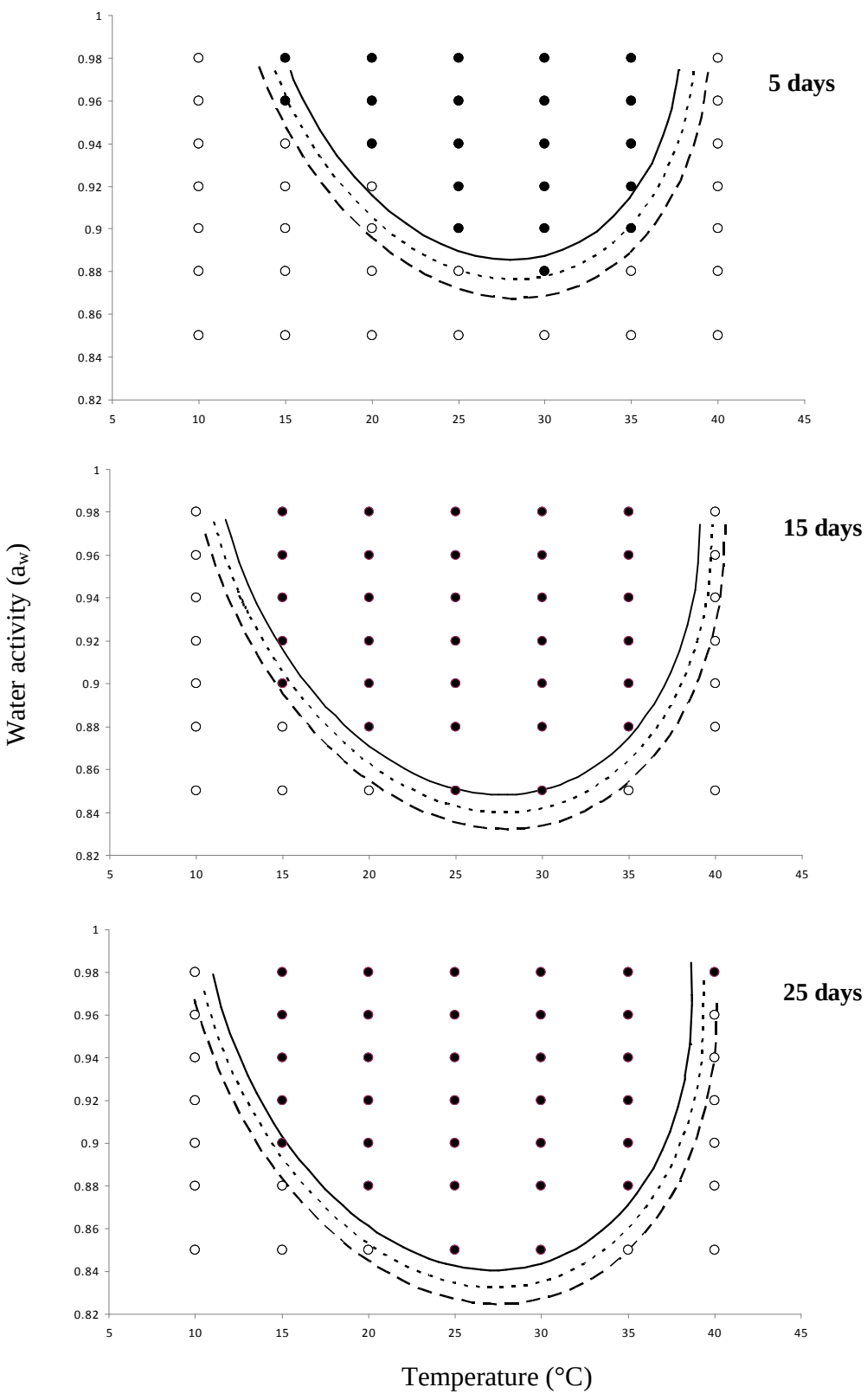
Figure 8 OTA production boundaries of *Aspergillus carbonarius* ATHUM 5660 after 5, 15 and 25 days incubation on a synthetic grape juice medium. Solid symbol: OTA presence (>l.d.), open symbol: OTA absence (<l.d.); solid line $P = 0.9$; dotted line $P = 0.5$; dashed line $P = 0.1$.


5 days
15 days
25 days
0.98
0.96
0.94
0.92
0.90
0.88
0.85
Probability of growth
Temperature (°C)

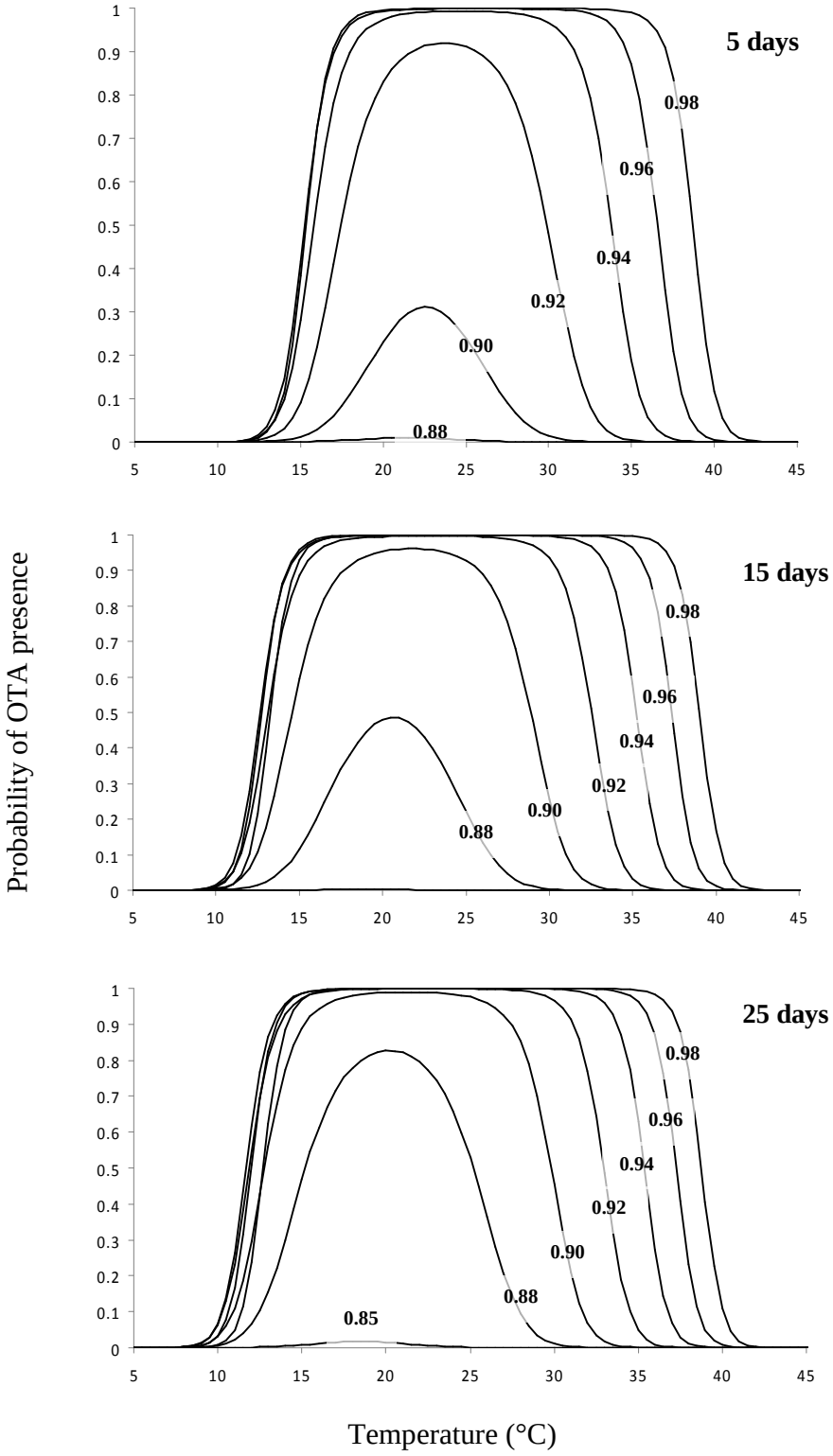
1


5 days
0.98
0.96
0.94
0.92
0.90
0.88
15 days
0.98
0.96
0.94
0.92
0.90
0.88
0.85
25 days
0.98
0.96
0.94
0.92
0.90
0.88
0.85
Probability of growth
Temperature (°C)

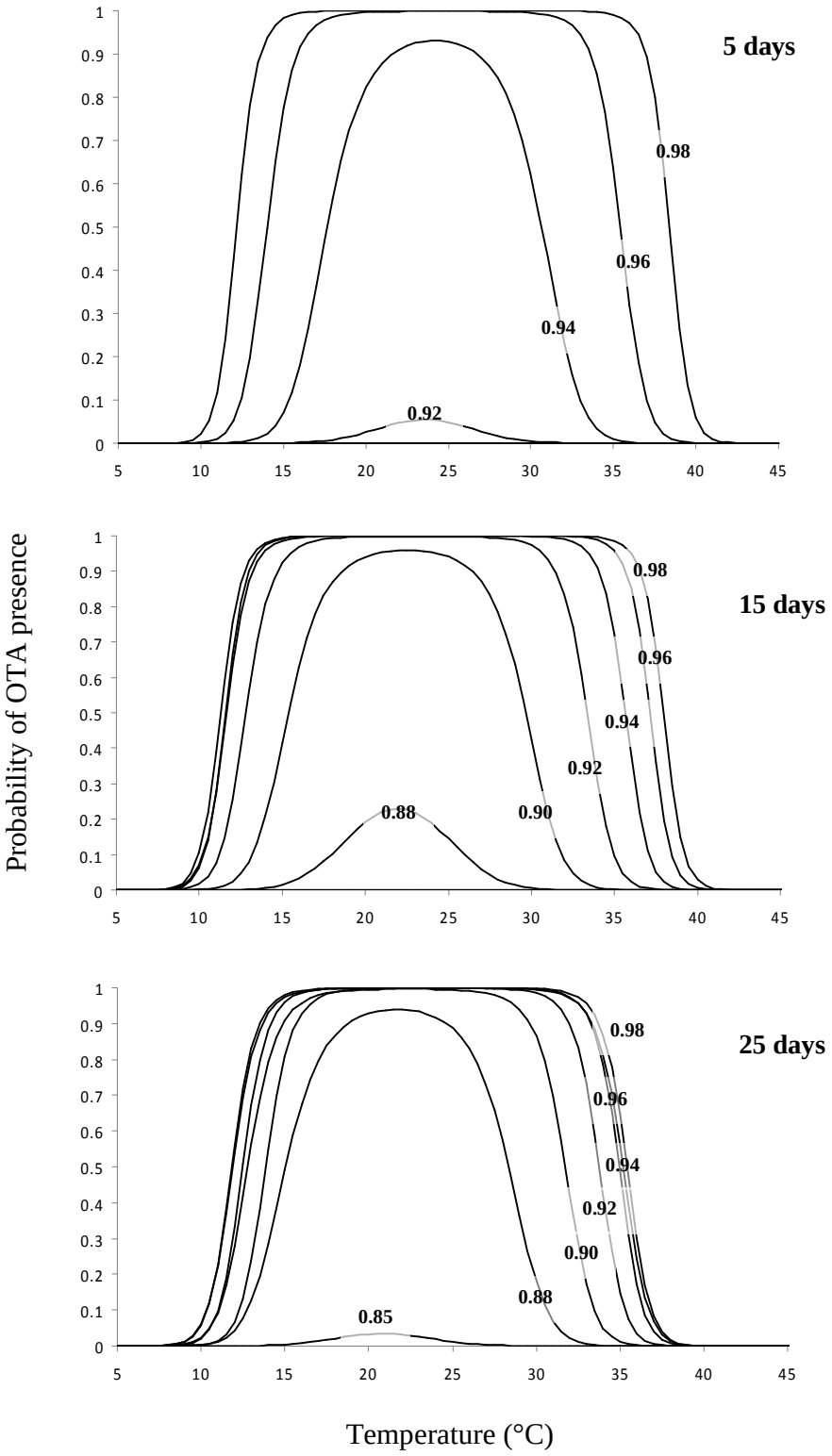

5 days
15 days
25 days
Water activity (a_w)
Temperature (°C)


5 days
15 days
25 days
Water activity (a_w)
Temperature (°C)
1
0.98
0.96
0.94
0.92
0.9
0.88
0.86
0.84
0.82
5
10
15
20
25
30
35
40
45

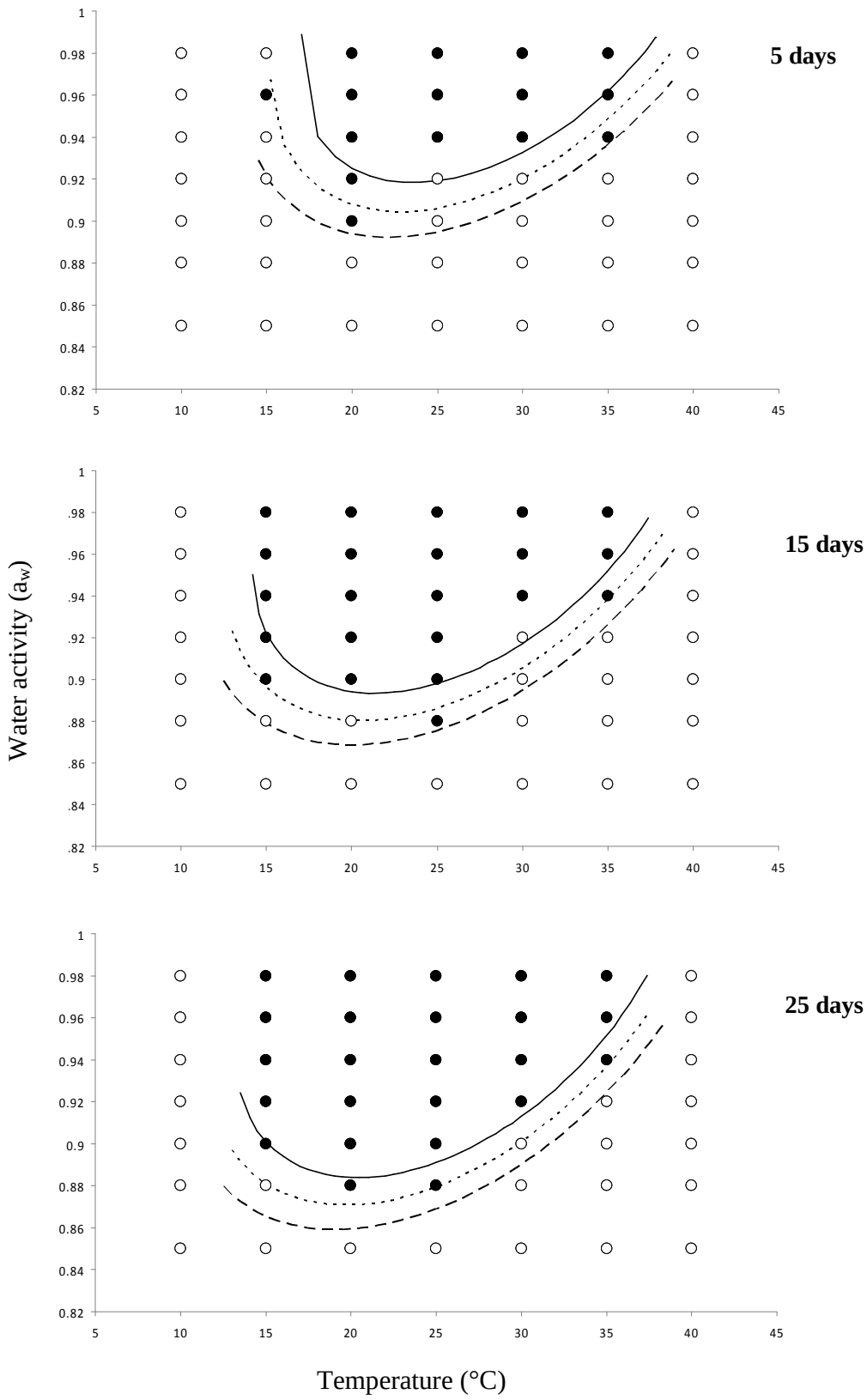
1


5 days
0.98
0.96
0.94
0.92
0.90
0.88
15 days
0.98
0.96
0.94
0.92
0.90
0.88
25 days
0.98
0.96
0.94
0.92
0.90
0.88
0.85
Probability of OTA presence
Temperature (°C)

1


5 days
0.98
0.96
0.94
0.92
15 days
0.98
0.96
0.94
0.92
0.90
0.88
25 days
0.98
0.96
0.94
0.92
0.90
0.88
0.85
Probability of OTA presence
Temperature (°C)

1



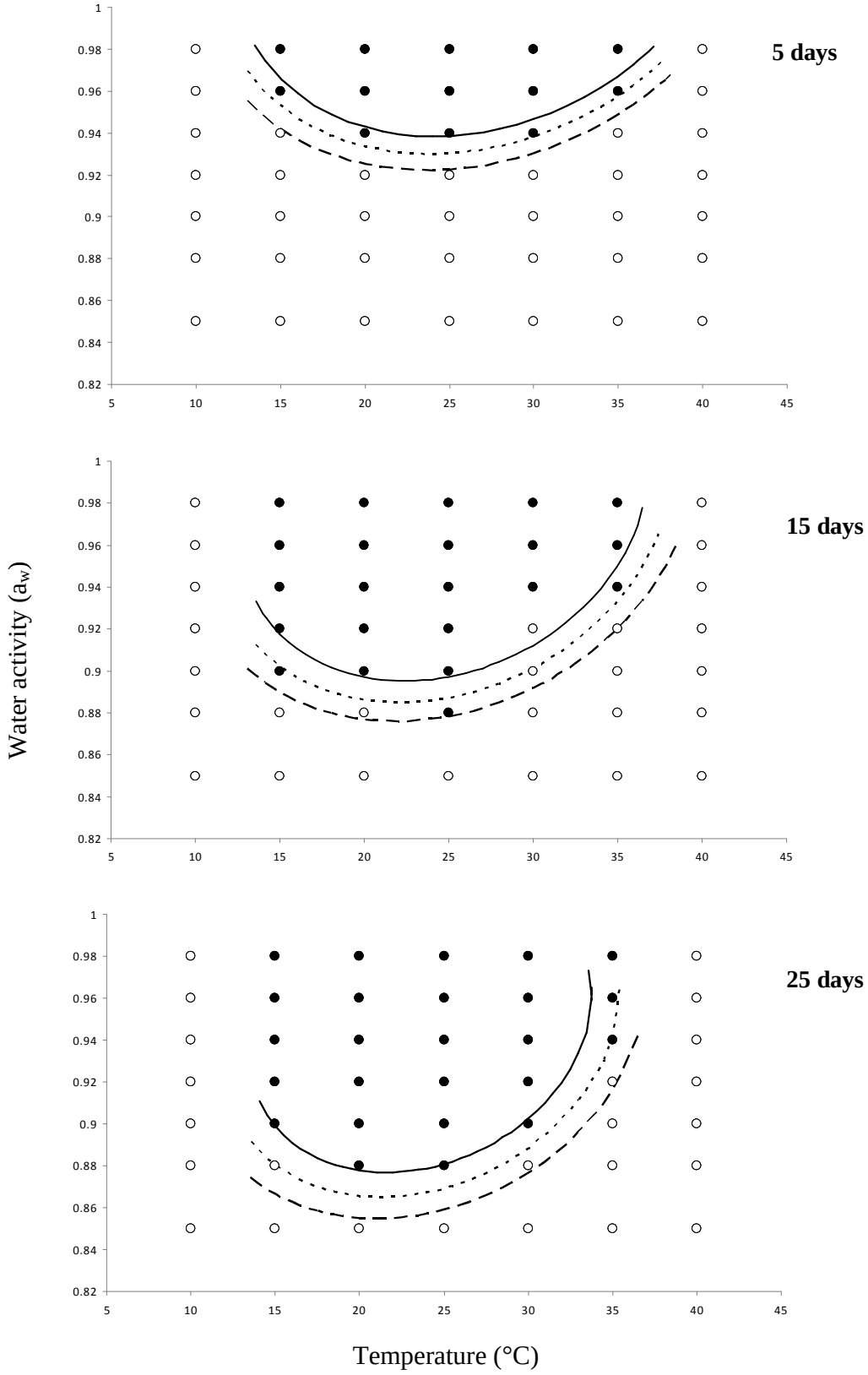

5 days
15 days
25 days
Water activity (a_w)
Temperature (°C)

Table 1 Estimated parameters and statistical indices of the logistic regression model for the growth/no growth interface of *Aspergillus carbonarius* ATHUM 5659

Parameter	Estimated value	Standard Error	*P*
Intercept	-1163.41	243.25	0.000
t	3.76	1.22	0.002
T	7.45	1.40	0.000
a_w	2144.02	475.82	0.000
t^2	-0.035	0.013	0.008
T^2	-0.095	0.017	0.000
a_w^2	-1040.71	242.24	0.000
$t \cdot T$	-0.022	0.006	0.001
$t \cdot a_w$	-1.95	1.14	n.s.
$T \cdot a_w$	-2.30	0.78	0.003
Hosmer-Lemeshow			11.98 (df = 8, P = *0.152*)
Maximum rescaled R^2			0.845
Concordant rate (%)			99.4
Discordant rate (%)			0.6
False positive [a]			4.1%
False negative [b]			1.4%

[a] Growth was not observed when the model predicted growth at probability $P > 0.5$

[b] Growth was observed when the model predicted no growth at probability $P < 0.5$

n.s.: Not significant ($P > 0.05$)

Table 2 Estimated parameters and statistical indices of the logistic regression model for the growth/no growth interface of *Aspergillus carbonarius* ATHUM 5660

Parameter	Estimated value	Standard Error	*P*
Intercept	-1083.51	260.13	0.000
t	4.85	1.42	0.001
T	9.39	1.74	0.000
a_w	1894.01	504.45	0.000
t^2	-0.039	0.014	0.006
T^2	-0.106	0.018	0.000
a_w^2	-874.51	254.41	0.001
$t \cdot T$	-0.022	0.006	0.001
$t \cdot a_w$	-2.91	1.33	0.030
$T \cdot a_w$	-3.72	0.98	0.000
Hosmer-Lemeshow			0.82 (df = 8, P = 0.999)
Maximum rescaled R^2			0.869
Concordant rate (%)			99.5
Discordant rate (%)			0.5
False positive [a]			3.4%
False negative [b]			0.7%

[a] Growth was not observed when the model predicted growth at probability $P > 0.5$

[b] Growth was observed when the model predicted no growth at probability $P < 0.5$

Table 3 Estimated parameters and statistical indices of the logistic regression model for OTA presence of *Aspergillus carbonarius* ATHUM 5659

Parameter	Estimated value	Standard Error	*P*
Intercept	-1619.59	247.17	0.001
t	2.73	1.46	0.042
T	-3.36	1.10	0.002
a_w	1709.83	528.52	0.001
t^2	-0.009	0.011	n.s.
T^2	-0.062	0.008	0.000
a_w^2	-933.25	284.14	0.001
$t \cdot T$	-0.011	0.004	0.033
$t \cdot a_w$	-2.12	1.50	n.s.
$T \cdot a_w$	6.94	1.42	0.000
Hosmer-Lemeshow			4.05 (df = 8, P = 0.852)
Maximum rescaled R^2			0.856
Concordant rate (%)			98.9
Discordant rate (%)			1.1
False positive [a]			1.4%
False negative [b]			2.7%

[a] OTA absence was observed when the model predicted presence at probability $P > 0.5$

[b] OTA presence was observed when the model predicted absence at probability $P < 0.5$

n.s.: Not significant ($P > 0.05$)

Table 4 Estimated parameters and statistical indices of the logistic regression model for OTA presence of *Aspergillus carbonarius* ATHUM 5660

Parameter	Estimated value	Standard Error	*P*
Intercept	-1395.49	285.59	0.000
t	16.07	3.11	0.000
T	-0.247	0.098	0.008
a_w	2640.74	580.71	0.000
t^2	-0.051	0.011	0.001
T^2	-0.061	0.008	0.000
a_w^2	-1276.59	300.49	0.000
$t \cdot T$	-0.006	0.005	n.s.
$t \cdot a_w$	-15.18	3.01	0.000
$T \cdot a_w$	3.47	1.09	0.002
Hosmer-Lemeshow			12.14 (df = 8, P = 0.145)
Maximum rescaled R^2			0.832
Concordant rate (%)			99.1
Discordant rate (%)			0.9
False positive [a]			0.7%
False negative [b]			2.0%

[a] OTA absence was observed when the model predicted presence at probability $P > 0.5$

[b] OTA presence was observed when the model predicted absence at probability $P < 0.5$

n.s.: Not significant ($P > 0.05$)

Table 5 Validation of growth/no growth logistic model using independent data of Bellí *et al.* 2005 [a]

a_w	Temperature (°C)	Observed [b] growth	Logistic model [c]
0.90	15	0	1
0.93	15	1	1
0.95	15	1	1
0.90	20	1	1
0.93	20	1	1
0.95	20	1	1
0.90	30	1	1
0.93	30	1	1
0.95	30	1	1
0.90	35	1	1
0.93	35	1	1
0.95	35	1	1
0.90	37	1	1
0.93	37	1	1
0.95	37	1	1

[a] Data of eight strains of *A. carbonarius* (W9, W37, W38, W89, W104, W120, W128, W198) for which there was no observed growth at 15°C and 0.90 a_w

[b] After 20 days of incubation, growth = 1; no growth = 0

[c] Growth prediction by logistic model using $P > 0.5$ denotes growth = 1; no growth = 0

Table 6 Validation of growth/no growth logistic model using data of Bellí *et al.* 2004

Strain	a_w	Temperature (°C)	Observed growth [a]	Logistic model [b]
A. carbonarius (36br4) *A. carbonarius* (A0933) *A. carbonarius* (Mu644)	0.90	10	0	0
	0.93	10	0	0
	0.95	10	1	0
	0.98	10	1	0
	0.90	15	1	1
	0.93	15	1	1
	0.95	15	1	1
	0.98	15	1	1
	0.90	20	1	1
	0.93	20	1	1
	0.95	20	1	1
	0.98	20	1	1
	0.90	25	1	1
	0.93	25	1	1
	0.95	25	1	1
	0.98	25	1	1
	0.90	30	1	1
	0.93	30	1	1
	0.95	30	1	1
	0.98	30	1	1
	0.90	37	1	1
	0.93	37	1	1
	0.95	37	1	1
	0.98	37	1	1
A. carbonarius (01UAs294)	0.90	10	0	0
	0.93	10	0	0
	0.95	10	0	0
	0.98	10	1	0
	0.90	15	1	1
	0.93	15	1	1
	0.95	15	1	1
	0.98	15	1	1
	0.90	20	1	1
	0.93	20	1	1
	0.95	20	1	1
	0.98	20	1	1
	0.90	25	1	1
	0.93	25	1	1
	0.95	25	1	1
	0.98	25	1	1
	0.90	30	1	1
	0.93	30	1	1
	0.95	30	1	1
	0.98	30	1	1
	0.90	37	1	1
	0.93	37	1	1
	0.95	37	1	1
	0.98	37	1	1

[a] After 20 days of incubation, growth = 1; no growth = 0

[b] Growth prediction by logistic model using $P > 0.5$ denotes growth = 1; no growth = 0

Table 7 Validation of logistic model for OTA presence/absence using data of Bellí *et al.* 2005

Strain	a_w	Temperature (°C)	Observed [a]	Logistic model [b]
	0.90	15	0	0
	0.93	15	0	0
	0.95	15	0	1
	0.90	20	0	0
	0.93	20	0	1
	0.95	20	1	1
A. carbonarius (W9)	0.90	30	0	0
A. carbonarius (W38)	0.93	30	0	1
	0.95	30	1	1
	0.90	35	0	0
	0.93	35	0	0
	0.95	55	0	1
	0.90	37	0	0
	0.93	37	0	0
	0.95	37	0	0
	0.90	15	0	0
	0.93	15	0	0
	0.95	15	0	1
	0.90	20	0	0
	0.93	20	0	1
A. carbonarius (W89)	0.95	20	1	1
A. carbonarius (W128)	0.90	30	0	0
A. carbonarius (W198)	0.93	30	0	1
	0.95	30	0	1
	0.90	35	0	0
	0.93	35	0	0
	0.95	35	0	1
	0.90	37	0	0
	0.93	37	0	0
	0.95	37	0	0
	0.90	15	0	0
	0.93	15	0	0
	0.95	15	0	1
	0.90	20	0	0
	0.93	20	1	1
	0.95	20	1	1
A. carbonarius (W37)	0.90	30	0	0
	0.93	30	0	1
	0.95	30	0	1
	0.90	35	0	0
	0.93	35	0	0
	0.95	35	0	1
	0.90	37	0	0
	0.93	37	0	0
	0.95	37	0	0

[a] After 7 days of incubation, presence (>l.d.) = 1; absence (<l.d.) = 0

[b] Predicted OTA by logistic model using $P > 0.5$ denotes presence = 1; absence = 0