

Assessment of intraspecies variability in fungal growth initiation of *Aspergillus flavus* and aflatoxin B₁ production under static and changing temperature levels using different initial conidial inoculum levels

Laila Aldars-García ^a, Sonia Marín ^{a*}, Vicente Sanchis ^a, Naresh Magan ^b, Angel Medina ^b

^a Food Technology Dept, XaRTA-UTPV, Agrotecnio Center, University of Lleida, Spain

^b Applied Mycology Group, Environment and AgriFood Theme, Cranfield University, Cranfield, Bedford MK43 0AL, UK

Corresponding author Sonia Marín: (smarin@tecal.udl.cat, Tel 34 973 702542, Fax 34 973 702596)

Laila Aldars-García: laldarsgarcia@tecal.udl.cat

Naresh Magan: n.magan@cranfield.ac.uk

Vicente Sanchis: vsanchis@tecal.udl.cat

Ángel Medina: a.medinavaya@cranfield.ac.uk

Abstract

Intraspecies variability in fungal growth and mycotoxin production has important implications for food safety. Using the Bioscreen C we have examined spectrophotometrically intraspecies variability of *A. flavus* using 10 isolates under different environments, including temperature shifts, in terms of growth and aflatoxin B₁ (AFB₁) production. Five high and five low AFB₁ producers were examined. The study was conducted at 5 isothermal conditions (from 15 to 37 °C) and 4 dynamic scenarios (between 15 and 30 °C). The experiments were carried out in a semisolid YES medium at 0.92 a_w and two inoculum levels, 10² and 10³ spores/mL. The Time to Detection (TTD) of growth initiation was determined and modelled as a function of temperature through a polynomial equation and the model was used to predict TTD under fluctuating conditions using a novel approach. The results obtained in this study have shown that a model can be developed to describe the effect of temperature fluctuations on the TTD for all the studied isolates and inoculum levels. Isolate variability increased as the growth conditions became more stressful and with a lower inoculum level. Inoculum level affected the intraspecies variability but not the repeatability of the experiments. In dynamic conditions, isolate responses depended both on the temperature shift and, predominantly, the final temperature level. AFB₁ production was highly variable among the isolates and depended on the inoculum level. This suggests that, from an ecological point of view, the potential isolate variability and interaction with dynamic conditions should be taken into account in developing strategies to control growth and predicting mycotoxin risks by mycotoxigenic fungi. This type of study could also be useful practically in predicting relative risks in colonisation and contamination with AFB₁ in staple stored food commodities.

Keywords intraspecies variability; *Aspergillus flavus*; growth; aflatoxin; dynamic temperature; inoculum level

1. Introduction

Fungal growth and mycotoxin contamination of food products represent an important food safety issue for the food industry. *Aspergillus* species are particularly important because they are xerophilic and able to colonise a range of food matrices, resulting in spoilage problems and mycotoxin contamination. This causes significant economic losses of staple food crops. Many factors can influence fungal growth in food products including nutritional composition, temperature, pH, water activity (a_w), atmospheric composition, presence and concentration of preservatives, different fungal communities, as well as storage times. Inter- and intra-species differences have been shown to be an important source of variability in terms of fungal growth and mycotoxin production (Abbas et al., 2005; Astoreca et al., 2007; Belli et al., 2004; Garcia et al., 2011a, 2011b; Lahouar et al., 2016; Romero et al., 2007; Santos et al., 2002; Singh et al., 2015; Yogendrarajah et al., 2016).

Usually, spoilage by filamentous fungi is visible to the naked eye in the form of characteristic colonies on the surface of food products, especially bakery goods. In general, spoilage has been evaluated by physically measuring the rate of colonisation on the food surface. However, the assessment of filamentous fungal activity is complex because they grow in three dimensions and are able to colonise a greater substrate surface area than yeasts or bacteria (Dantigny et al., 2005). The measurement of hyphal extension rates, usually reported as radial growth rate, is probably the simplest and most direct method to measure fungal growth. Nevertheless, as stated by Medina et al. (2012), these measurements do not account for the true representation of the three-dimensional nature of fungal growth, although there is a relationship between radial growth and fungal biomass (Trinci, 1971). In addition, the whole process is time consuming and requires significant inputs of time and consumables. Methods based on spectrophotometry (turbidimetric measurements) have been widely used for bacterial growth but rarely for examining ecophysiology of filamentous fungi. Spectrophotometric assays provide fast results that are expressed in Optical Density (O.D.) units. Only a few authors have used this kind of approach for mycological studies (Medina et al., 2012; Mohale et al., 2013; Rossi-Rodrigues et al., 2009; Samsudin et al., 2016). The use of a semi-solid agar medium has been effectively utilised to examine relative growth in relation to environmental factors and also in relation to different anti-fungal compounds (Medina et al., 2012).

There is interest in understanding the relationship between initial inoculum size and how this affects the relative growth rate in relation to environmental conditions (Aldars-García et al., 2016; Baert et al., 2008; Barberis et al., 2012; Burgain et al., 2013; Garcia et al., 2010; Gougouli et al., 2011; Morales et al., 2008). In addition, how does initial inoculum size affect the capacity for mycotoxin production? This could be important as the initial inoculum load in a food matrix may influence, or indeed determine, how much mycotoxin is produced. In this study, we used *Aspergillus flavus* as the target mycotoxigenic species because it colonises a range of cereals, nuts and spices and contaminates them with aflatoxins, especially aflatoxin B₁ (AFB₁) which is a class 1A carcinogen (IARC, 1993). Thus, it is important to try and model the effects of ecophysiological factors on the activity of mycotoxigenic species, especially with differing inoculum loads. In nature, the fungal community consists of a range of species as well as isolates of the same species. There have been few studies which have focused on different inoculum sizes of isolates of the same species in relation to steady state temperatures and temporal shifts in this factor.

Thus, the objectives of this study were to (i) compare the impact of different steady state temperatures (15-35 °C) on relative initial growth of 10 isolates of *A. flavus* at two initial inoculum levels (log₂, log₃); (ii) examine the effect of four temperature shifts (between 15-30 °C) and inoculum size on rates of growth using the Bioscreen C; (iii) evaluate the effect of initial inoculum size and steady state and temperature shifts on AFB₁ production and (iv) examine what

impact these environmental conditions/shifts have on within-isolate variation using a secondary metabolite conducive medium. The ten tested isolates were divided into two groups, based on their AFB₁ production ability: 5 high (range of production) and five low AFB₁ producers.

2. Material and methods

2.1. *A. flavus* isolates

Ten isolates of *A. flavus* isolated from chilli powder, maize grains and pistachio nuts were used in this study (Table 1). There were five high and five low AFB₁ producers.

2.2. Inoculum preparation, culture medium and inoculation

Isolates were sub-cultured on Malt Extract Agar (Sigma-Aldrich, Dorset, UK) at 25 °C for 7 days to obtain heavily sporulating cultures. After incubation, spores were collected by scraping the surface of the plates, diluting them in sterile water adjusted to 0.92 a_w with glycerol containing Tween 80 (0.05% v/v), and filtered through sterile glass wool into a tube. The total spore concentrations (per ml) were determined and decimal dilutions (in sterile water adjusted to 0.92 a_w with glycerol, containing Tween 80 (0.05% v/v)), were prepared to obtain the two different spore concentrations: 10⁵ and 10⁴ spores/mL for each isolate. The basic medium used in this study was a semisolid Yeast Extract Sucrose (YES) agar, whose protocol of preparation was previously optimised by Medina et al. (2012) and adjusted to 0.92 a_w. Spore suspensions were used to inoculate semi-solid YES medium. 100 µL of the spore suspension was pipetted into 9.9 mL of semi-solid YES agar, for each spore concentration, resulting in two final concentrations of 10³ and 10² spore/mL in the semisolid YES agar were prepared for each isolate.

2.3. Growth assessment

Growth was studied at 15, 20, 25, 30, 35, 37 °C and four temperature shift treatments from 15 to 25 °C (F1), 15 to 30 °C (F2), 20 to 25 °C (F3) and 20 to 30 °C (F4), after 48 hours at the lowest temperature.

Optical densities, which are directly related to the fungal biomass of *A. flavus* (Medina et al., 2012) were recorded using a Bioscreen C Microbiological Growth Analyser (LabSystems, Helsinki, Finland). 100-well microtitre plates specifically manufactured for this machine were loaded with the 10³ and 10² spore/mL semisolid YES agar of each isolate; one plate per spore concentration was used. The wells of the microplate were filled with 300 µL of the inoculated medium, thus ca. 300 and 30 spores were inoculated in each well for the 10³ and 10² spores/mL inocula, respectively. For each temperature condition two inoculum levels were set with 9 replicates (well) per isolate (10 isolates x 9 replicates=90 wells plus 10 empty wells. Overall, one plate per inoculum level and temperature condition was used.

The O.D. was recorded every 30 min using the 600nm filter over a 7 days period, except for 20 °C and 15 to 25 °C (F1), where 14 and 9 days respectively, were needed to reach the growth threshold set for the experiments. Data were recorded using the software Easy Bioscreen Experiment (EZExperiment) provided by the manufacturer and then exported to a Microsoft® Excel® Professional 2010 (14.0.4756.1000) (Microsoft Corporation, Redmond, Washington, USA) sheet for further analysis.

2.4. Aflatoxin B₁ assessment

Following incubation, the well contents was decanted in order to analyse the mycotoxin concentration. AFB₁ extraction was carried out as follows: the contents of 3 wells was collected for each isolate and temperature condition, in triplicate. AFB₁ was extracted with 0.8 mL of chloroform, shaken for 1 min and left stationary for 20 min. The chloroform phase was separated and the aqueous phase re-extracted twice with 0.8 mL of chloroform. The organic extracts were combined and evaporated to dryness. The residues were derivatized using trifluoroacetic acid as described by the AOAC (2000) and transferred to a HPLC vial. All derivatized samples were analysed by HPLC (Agilent 1200 series HPLC (Agilent, Berkshire, UK)). Chromatographic separations were performed on a stainless steel C18 column (Phenomenex Luna ODS2 150 x 4.6 mm, 5 µm). Methanol: water: acetonitrile (30:60:10) was used as the mobile phase at a flowrate of 1mL/min. AFB₁ derivative fluorescence was recorded at excitation and emission wavelengths of 360 and 440 nm respectively. Standard curves were constructed with different levels of AFB₁. Aflatoxin B₁ recovery assays were performed to ensure the analytical quality of the results. This showed that for all standards the recovery rate was >87%.

2.5. Data analysis

2.5.1. Total Time to Detection (TTD) for static temperatures

Raw datasets obtained from the Bioscreen C were subjected to further analysis. Before analyses, the average of the measurements for each well during the first 60 min was calculated and automatically subtracted from all subsequent measurements in order to remove the different signal backgrounds. Then, the TTD for an O.D. of 0.1 was obtained using a Microsoft® Excel® template (kindly provided by Dr. R. Lambert), which used linear interpolation between successive O.D. readings.

In order to stabilise the variance, for the TTD comparisons, a square root transformation was used. However, raw data are presented in Tables and Figures. The Kruskal–Wallis test was used to establish the differences among median TTD values of the 10 isolates under the different treatment levels at $p < 0.05$.

Finally, based on the TTD, a polynomial model that described the TTD as a function of the temperature was fitted. The general expression of the polynomial model used was:

$$TTD = a_0 + a_1 T + a_2 T^2 \quad (1)$$

a_i are the constants to be estimated, T is the independent variable (temperature) and TTD is the response variable. This model was chosen as only temperature levels in the range 15-37 °C were tested, which were not enough to fit models used for the whole range of growth temperatures. Statistical analysis was carried out with Statgraphics® Centurion XVI.I (Statpoint, Inc., Maryland, USA).

2.5.2. Prediction of TTD under fluctuating temperatures

The TTDs obtained under the static temperatures were used to design the experiments for estimating the TTDs under fluctuating temperature conditions. The time-temperature scenarios studied included a single abrupt shift from a low to an upper temperature. The TTD under fluctuating temperature was predicted through the model fitted at constant temperature, and compared to the experimental results generated under the temperature changing scenarios (experimental TTD). These experiments were carried out in the same way as for the static conditions.

The TTD of each isolate for the changing temperature (total TTD=TTDT) treatments consisted of a single abrupt temperature shift from an initial temperature (TI) to a final temperature (TF) at a time t_s . Thus, Fig. 1 was calculated using the following equation:

$$TTDT = \begin{cases} TTDI & \text{if } ts > TTDI \\ TTDF + (TTDI - TTDF) * \left(\frac{TF - TI}{TF} \right) * \left(\frac{ts}{TTDF} \right) & \text{if } ts < TTDI \end{cases} \quad (2)$$

Where TTDI and TTDF were calculated by substituting the corresponding temperature in the polynomial equation (Eq. (1)). That is, when the temperature shift occurs before the end of TTDI, after TTDF is consumed, the remaining TTD is a percentage of the interval (TTDI-TTDF), which was assumed to be proportional to (i) the temperature shift, and (ii) the timing of the temperature shift.

3. Results

3.1. Time to Detection (TTD) at constant temperature conditions: isolate variability and inoculum size effects

In the first part of the study, the objective was to determine the influence of temperature on the relative initial growth of the 10 *A. flavus* isolates and address their differences. Thus, TTDs of 0.1, indicating initial growth of the spores, were calculated. In this study, all experiments were carried out at constant temperature conditions. TTDs were further fitted to a second order polynomial equation (Eq. (1)) in order to predict the TTD as a function of the temperature for each isolate. The developed polynomial models accurately predicted the influence of temperature on the TTD, with goodness of fit (r^2) ranging from 0.980 to 0.999. Both temperature and its quadratic term had significant effects on TTD (Table 2).

All *A. flavus* isolates grew under all steady state temperature profiles tested, except for 15 °C. According to the Kruskal-Wallis test, there were significant differences ($p < 0.05$) in TTD among isolates. However, these differences depended on the temperature profile tested. TTD values followed the time sequence 37 < 35 < 30 < 25 < 20 °C in all cases. This pattern is shown in Fig. 2. In general, all isolates showed similar TTD values at the same temperature and inoculum level. The TTDs at 30, 35 and 37 °C did not revealed much significant differences between isolates, at the 95% confidence level.

At 25 °C, differences between the 10 isolates increased (higher coefficient of variation (CV %)) which could suggest more within-isolate variability as temperature became more marginal for growth. Isolate responses at 20 °C were highly variable, not only among isolates but also within replicates of the same isolate. Furthermore at 20 °C in some replicates, conidia failed to initiate grow at all. At the five steady state temperatures, the isolate UdL-TA 3.327 had the lowest TTDs, and isolates UdL-TA 3.244 and UdL-TA 3.332 the highest. No differences in growth pattern were found between the low and high 6AFB₁ producer groups.

Statistical analysis showed a clear difference between the TTD for the two initial inoculum levels for all temperatures examined (Fig. 2). In addition, differences among isolates' TTD increased for the lower initial inoculum size (10² spores/mL), i.e., the difference in time was greater at the lower inoculum level. The differences between the 2 inoculum levels were greater at 20 °C. For example, for isolates UdL-TA 3.270 and UdL-TA 3.332, the differences at 30 °C were 0.72 and 3.54 hours for the high and the low inoculum level treatments respectively. At 20 °C these differences were 18.74 and 22.48 hours, respectively, for the low and the high inoculum levels. From Figure 2 it was also observed that the TTD was significantly affected by the spore concentration, and these differences were more marked as conditions became more marginal for conidial germination. In general, within-isolate variability was more affected by the marginal conditions (20 °C) than by the inoculum level. Increasing the conidial inoculum size from 10² to 10³ spores/mL, when temperature was 20°C (a realistic practical storage temperature for agricultural products), had a profound effect on the ability of *A. flavus* to initiate growth. At this

temperature, a difference in the prediction of growth initiation of more than 2 days occurred depending on the initial inoculum level.

3.2. Prediction of relative initial growth times (TTD) as affected by temperature shifts

Generally, under ambient transport conditions temperature is not a fixed value and fluctuates during distribution and the length of the food chain. Thus, it is important to better understand the effect of such fluctuations on fungal growth due to temperature or indeed inoculum size impacts on relative level of risk from growth or toxin contamination. Thus, an approach to predict the effect of temperature shifts on the ability of fungi to grow (in our case by means of the TTD) was developed taking into account the results from the static temperature studies.

In order to evaluate the suitability of the model to predict the TTD of *A. flavus* conidial germination and growth under changing temperature scenarios, the predicted TTDs derived from equation 2 were compared to experimental TTDs obtained by carrying out the experiment at set shifting temperature profiles.

The four temperature shifts were imposed prior to the TTD. Agreement between the model predictions and experimental data results was assessed by plotting predicted TTD versus observed TTD (Fig. 3). The observed response values agreed well with the predicted response values except for the temperature shift from 15 to 30 °C where a clear overestimation was obtained (approx. 50 hours).

Nonetheless, accurate predictions were possible under the two profiles with 25 °C as the final temperature, with a mean underestimation of only 4 hours made. Also, good agreement between the experimental and predicted TTD for an initial temperature of 20 °C and a final temperature of 30 °C (Fig. 3) was obtained, with a mean underestimation of 9 hours.

Results for isolate UdL-TA 3.244 at 10^2 spores/mL are summarised in Figure 4 which represents the TTD for the four shifting temperature scenarios and TTD of the static temperatures involved in the dynamic profiles. At the same temperature profile and inoculum concentration level the 10 tested isolates did not differ much in their TTDs. TTDs followed the time sequence $F4 < F2 < F3 < F1$ except for isolate UdL-TA 3.327 which was $F4 < F3 < F2 < F1$ for both inoculum levels (Fig. 5). The highest difference among isolates was observed in the 20 to 25 °C temperature regimes. The harshest temperature shift to initiate growth was from 15 to 25 °C, which took approximately 167.67 and 141.67 hours to reach the TTD, for the low (ca. 30 spores) and higher inoculum (300 spores) concentration, respectively. However, the differences observed in this temperature profile (F1) were more marked within isolate than among isolates (see large error bars). The standard deviation was always greater for those profiles where the initial temperature was 15 °C.

3.3. Effect of inoculum level, temperature profiles and intra-species variability on aflatoxin B₁ production

The amount of AFB₁ produced after 7 days growth (except for 20 °C and 15 to 25 °C which were 14 and 9 days respectively due to the slow growth rates) was determined for all isolates and both inoculum levels (Fig. 6). Firstly, AFB₁ was not detected at 15 °C, when tested as a single constant temperature treatment. The amount of AFB₁ production depended on the temperature, and followed the profile $35 > 30 > 25 > 37 > 20$ °C, for the higher inoculum level and the profile $35 > 30 > 25 > 20 > 37$ °C, for the lower inoculum level. For some isolates the optimum production was at 30 °C, for some others at 35 °C.

The inoculum level had different effects on the AFB₁ production depending on the temperature. AFB₁ production was in general higher at the higher initial inoculum level, except for the 37 °C steady state treatment, and the temperature shift from 20 to 30 °C. This pattern was more marked for the high AFB₁ producer isolates. For example, at 37 °C, isolate UdL-TA 3.270 produced 282.97 and 40.45 µg/kg for the 10^2 and 10^3 spores/mL inocula, respectively, or to a

lesser extent isolate UdL-TA 3.268 which produced 86.45 and 1.44 µg/kg for the 10² and 10³ spores/mL inocula, respectively. The same pattern was found in the temperature shift from 20 to 30 °C (detailed later). At 35, 30 and 25 °C, no differences were observed among inocula. Only at the constant temperature of 20 °C was more AFB₁ produced at the higher inoculum level for all isolates. However, some isolates were not able to produce the toxin at this temperature.

Looking at the low producing isolates, no AFB₁ was produced at 35 °C, and very low amounts at 37 °C. At 25 °C some isolates of this group produced more AFB₁ than the ones in the high producer group, namely isolates UdL-TA 3.267, UdL-TA 3.269 and UdL-TA 3.325 at both initial inoculum levels. A similar behaviour was found at 20 °C where isolates UdL-TA 3.325, and UdL-TA 3.269, and the UdL-TA 3.325 for the 10² and 10³ spores/mL inocula respectively, produced more AFB₁ than some isolates of the high producer group at the same inoculum level.

In order to determine the effect of temperature shifts, the theoretical amount of AFB₁ produced taking into account the time periods at each temperature was calculated as follows:

$$\text{Calculated AFB}_{1TI} = \frac{(AFB_{1TI} * ts) + (AFB_{1TF} * tf)}{ts + tf} \quad (3)$$

Where AFB_{1TI} and AFB_{1TF} correspond to the amount of toxin produced at the initial and final temperature, respectively, under the single steady state scenarios, and ts and tf are the time periods for the initial and final temperature respectively for each shifting temperature profile. Table 3 shows both theoretical and experimental AFB₁ concentrations.

No clear pattern was found where the shifting temperature treatments were used. For the changing scenario F1 (15 to 25 °C) a dramatic increase in AFB₁ production was detected, compared to the low levels detected at 15 and 25 °C, under steady state conditions. The same trend was observed for the shift from 20 to 30 °C, where in general a 3-4 fold increase in AFB₁ production was observed, when compared to the calculated AFB₁. In contrast, the shift from 20 to 25 °C resulted in a lower AFB₁ production regarding when compared with that produced in the steady state same two temperatures treatments. More variability was found for the shift from 15 to 30 °C, where for some isolates there was an overestimation and for others an underestimation of AFB₁ production.

4. Discussion

4.1. Intraspecies variability for growth and AFB₁ production

Generally, food products are stored at suboptimal conditions to minimise mould growth and this may influence the intraspecies variability in both germination and initial colonisation and potential for mycotoxin production (Astoreca et al., 2007; Bellí et al., 2004; Garcia et al., 2011a, 2011b; Lahouar et al., 2016; Mohale et al., 2013; Mugrabi de Kuppler et al., 2011; Pardo et al., 2004; Parra and Magan, 2004; Romero et al., 2007; Tassou et al., 2009; Tauk-Tornisiello et al., 2007; Yogendrarajah et al., 2016).

This study has utilised a suboptimal a_w level, representing environmental stress, to examine and quantify effects of steady state and shifting temperatures on growth of groups of high and low AFB₁ producing *A. flavus* isolates for the first time. The parameter studied was the TTD, which is the time in which mould growth is detected at a certain biomass level as demonstrated previously by Medina et al. (2012). These values are a very good approximation to understand the growth of fungal colonies in a 3D space and at very low biomass levels. The data were based on spectrophotometric measurements, thus if a full model was developed and applied to realistic food products, thorough validation would be necessary. The present study has shown that it is possible to predict TTD under steady state and some shifting temperatures. The results obtained in the first part of the study (steady state temperatures) showed that as temperature became more marginal for conidial germination and mycelial growth, intraspecies variability increased. This trend has been observed using other criteria by some authors for other fungi.

For example, Romero et al. (2007) evaluated the effects of a_w (0.80-0.95) and temperature (15-35 °C) on lag phase of four *Aspergillus carbonarius* isolates, and found the greatest difference at limiting conditions. Garcia et al. (2011a) working with 79 isolates of *Penicillium expansum* reported coefficients of variation for the lag phase of 12.7 and 14.3% at 20 and 1 °C, respectively. This suggests that intraspecies variability is dependent on the environmental conditions, and is higher when conditions are closer to the boundaries for activity. In the present study, we have focused on steady state temperatures and shifting temperatures but under a fixed water stress condition. In this situation, under marginal conditions isolate variability was found to be higher.

For shifting temperature scenarios, intraspecies variability did not appear to be significant, with only a few isolates, among the 10 studied, behaving differently from the others. The final temperature had the major effect on intraspecies variability. Within isolate variability was more affected by the initial temperature than by the final temperature, since %CV was higher for those profiles which were set initially at 15 °C.

Longer TTDs were observed when low inoculum levels were used. Conceptually, TTD should parallel lag phases prior to growth. Of course, the latter parameter has been studied many times under different inoculum levels (Aldars-García et al., 2016; Baert et al., 2008; Burgain et al., 2013; Chulze et al., 1999; Morales et al., 2008; Sautour et al., 2003). Such studies have shown that changes in the inoculum size affected and increased the length of the lag phases prior to growth when the inoculum size decreased. Moreover, the inoculum level was also found to be a critical factor in TTD intraspecies variability: as inoculum decreased, intraspecies variability increased.

Considering the effect of temperature on AFB₁ production, the amount produced was highly variable among the 10 isolates. Santos et al. (2002) studied the production of patulin and citrinin by 10 isolates of *P. expansum*, and showed that patulin production was isolate dependent. Aldars-García et al. (2015) predicted the probability of growth and AFB₁ production of *A. flavus* using a cocktail inoculum of 25 isolates and an inoculum with a single isolate. Different results in terms of growth behaviour were obtained for both inocula but not for AFB₁ production which gave very similar probabilities, highlighting the possibility of a homogeneous boundary of AFB₁ production among isolates, although the amount produced by isolates was different. The variability in the amount of mycotoxin produced will be influenced by nutritional substrate, interacting environmental conditions, source, age and whether wild or sub-cultured on rich artificial laboratory media (Garcia et al., 2011a; Romero et al., 2010; Yogendrarajah et al., 2016). Of course, prevention is better than cure. However, the present work perhaps provides some insight into ways to minimise mycotoxin production by reducing inoculum load or controlling germination of conidia of such fungi.

4.2. Predicting relative initial growth (TTD) and AFB₁ production under shifting temperature scenarios

In order to measure the effect of a temperature shift on the time needed to initiate growth, *A. flavus* isolates were subjected to sudden temperature upshifts. Adaptation to environmental stresses is usually explained by biological mechanisms in the cell, which requires a certain amount of time depending on the cells' physiological state and the new environmental conditions (Brooks et al., 2011; Swinnen et al., 2005). Many studies have been carried out under fluctuating temperatures for bacterial pathogens (Bovill et al., 2000, 2001; Kim et al., 2008; Koseki and Nonaka, 2012; Muñoz-Cuevas et al., 2010; Zwietering et al., 1994) and to a lesser extent for fungi (Aldars-García et al., 2015; Gougouli and Koutsoumanis, 2012, 2010). In some of them when models included germination or growth rates, instantaneously adaptation to the new environment was assumed for these rates (Gougouli and Koutsoumanis, 2012) and in other

cases, when primary observations were modelled, for example, visible growth, inclusion of a 'memory parameter' in the models was required for acceptable predictions (Aldars-García et al., 2015).

Muñoz-Cuevas et al. (2010) developed a dynamic growth model for a *Listeria monocytogenes* isolate. They found that growth behaviour depended not only on the magnitude of the change between the previous and current environmental conditions but also on the current growth conditions. Similarly, we found that TTD under dynamic temperature depended mainly on the final temperature and to a lesser extent on the magnitude of the change and initial temperature. Figure 4 illustrates an example of this dependence for isolate UdL-TA 3.244 at 10^2 spores/mL, in which all TTD for the changing temperature scenarios are around the TTD at 25 °C. This behaviour may suggest that 48 hours at a restrictive temperature could not be enough to slow the cells' metabolism to a point which prevents them from quickly adapting to better growth conditions.

AFB₁ production under dynamic temperature conditions was enhanced under some scenarios and inhibited under others. Several authors have described that abiotic stress is involved in the activation of mycotoxin biosynthetic genes (Jurado et al., 2008; Kohut et al., 2009; Schmidt-Heydt et al., 2009). Then, the stress induced by the temperature shift may have a similar effect, triggering AFB₁ production. Nevertheless, in some cases, the temperature shift appeared to inhibit AFB₁ production.

Garcia et al. (2012) studied mycotoxin production by *Fusarium* spp. under 3 changing temperature scenarios (15 to 20 °C, 15 to 25 °C and 25 to 30 °C). They also found that for some profiles the mycotoxin production was enhanced and for others inhibited. Furthermore, this pattern was different depending on the mycotoxin studied. Ryu and Bullerman (1999) studied the production of deoxynivalenol and zearalenone on rice with cycling temperatures, finding that mycotoxin production was stimulated under the temperature shifts.

Studies on the effect of inoculum revealed different outcomes on mycotoxin production; direct relationship between the amount of mycotoxin produced and the inoculum size was reported by Aldars-García et al. (2016) and Chulze et al. (1999). On the other hand, Morales et al. (2008) reported that colonies from conidial suspensions of 10^6 spores/mL produced lower amounts of patulin (in apples) than those from the 10^4 spores/mL suspensions. These results may suggest a possible inhibition of germination, and thus mycotoxin production, when spore concentration is too high. This certainly occurs in soil fungi where fungistasis can limit the number of fungal spores germinating to ensure survival under stress conditions (e.g. *Fusarium* species). Thus, mycotoxin production may be enhanced as inoculum size decreases. A possible explanation of such behaviour is that when fewer spores colonise a niche, there is more nutrient availability for the fungus, and then more energy could be utilised for secondary metabolite production. Further research on this area is required to understand how inoculum size affects the biosynthesis of mycotoxins.

As conclusion, taking into account isolate variability and inoculum size in mycological studies would give more realistic results, since in a real scenario contamination we may encounter different isolates in a food product. Furthermore, it becomes evident that temperature shifts have an important effect on fungal behaviour, and that there is potential for modelling and predicting toxigenic mould behaviour under steady state and fluctuating temperatures. The data generated in the present study is useful for a better understanding of the behaviour of isolates under dynamic temperature scenarios, in order to improve our understanding of mycotoxin contamination of food matrices, and thus help in the development of approaches to improve shelf-life of products prone to fungal spoilage, improve shelf-life and improve food safety.

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Table 1. Description of the isolates used in the present study

Isolate code*	Origin	AFB ₁ production on PDA at 25 °C for 7 days (ng/g)
UdL-TA 3.244	Chilli	20.5
UdL-TA 3.267	Pistachio nuts	25.9
UdL-TA 3.269	Pistachio nuts	28.3
UdL-TA 3.324	Maize grains	5.4
UdL-TA 3.325	Maize grains	1.5
UdL-TA 3.268	Pistachio nuts	471.2
UdL-TA 3.270	Pistachio nuts	114.8
UdL-TA 3.327	Maize grains	178.5
UdL-TA 3.331	Maize grains	547.2
UdL-TA 3.332	Maize grains	2114.6

*Isolate codes are the names of the cultures held in the Food Technology Department Culture Collection of University of Lleida, Spain.

1 Table 2. Parameter estimation of the polynomial models for TTDs of the 10 *A. flavus* isolates at both inoculum levels.

Inoculum level	Variable	Coefficient estimated value ^a									
		UdL-TA	UdL-TA	UdL-TA	UdL-TA	UdL-TA	UdL-TA	UdL-TA	UdL-TA	UdL-TA	UdL-TA
		3.244	3.267	3.269	3.324	3.325	3.268	3.270	3.327	3.331	3.332
10 ² spores/mL	Constant	77434.80 ± 5282.23	66100.10 ± 2336.32	75437.91 ± 3320.37	61534.18 ± 2424.18	65000.17 ± 2643.21	70988.46 ± 3070.81	64260.80 ± 3259.81	59395.60 ± 1958.30	60100.50 ± 4172.01	67532.80 ± 3684.03
	T	-4329.09 ± 374.55	-3643.88 ± 168.53	-4348.05 ± 240.76	-3372.45 ± 175.77	-3569.84 ± 191.66	-4096.93 ± 222.66	-3532.86 ± 236.37	-3383.57 ± 141.99	-3292.88 ± 302.51	-3777.97 ± 260.32
	T ²	63.48 ± 6.43	52.89 ± 2.92	65.29 ± 4.19	48.99 ± 3.05	51.57 ± 3.34	61.71 ± 3.87	51.25 ± 4.11	50.97 ± 2.47	47.72 ± 5.26	55.74 ± 4.45
	r ²	0.93	0.98	0.97	0.98	0.98	0.96	0.96	0.98	0.93	0.95
10 ³ spores/mL	Constant	62490.8 ± 3171.44	51181.3 ± 2514.44	60660.88 ± 2836.99	51837.57 ± 1772.97	52029.60 ± 2182.80	59022.71 ± 2360.32	54020.90 ± 2557.70	45936.10 ± 1254.28	48765.90 ± 2865.00	64245.00 ± 4101.45
	T	-3470.54 ± 228.74	-2774.71 ± 182.32	-3488.48 ± 205.71	-2842.81 ± 128.56	-2835.64 ± 158.27	-3398.32 ± 171.15	-2964.15 ± 185.46	-2571.03 ± 90.95	-2643.11 ± 207.74	-3646.82 ± 295.85
	T ²	50.592 ± 3.97	39.8439 ± 3.17	52.43 ± 3.58	41.3474 ± 2.24	40.76 ± 2.75	51.17 ± 2.98	42.96 ± 3.23	38.40 ± 1.58	38.04 ± 3.62	54.18 ± 5.13
	r ²	0.96	0.96	0.96	0.98	0.98	0.97	96.87	0.99	0.95	0.94

2 ^a mean values ± sd

3 *All coefficient estimates were significant at P < 0.05

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7 Table 3. Experimental and calculated amount of AFB₁ produced by the 10 isolates tested (in µg/kg in YES medium) at the four dynamic scenarios
8 assayed, and at both inoculum levels.

Dynamic temperature		15 to 25 °C		15 to 30 °C		20 to 25 °C		20 to 30 °C	
		Calculated AFB ₁	Experimental AFB ₁	Calculated AFB ₁	Experimental AFB ₁	Calculated AFB ₁	Experimental AFB ₁	Calculated AFB ₁	Experimental AFB ₁
10 ³ spores/mL	UdL-TA 3.244	10.05	348.41	224.57	21.71	9.23	1.20	224.57	154.89
	UdL-TA 3.267	79.01	246.21	437.89	117.92	72.75	0.73	438.08	560.09
	UdL-TA 3.269	317.75	461.84	1227.65	394.23	296.00	6.13	1231.83	1506.65
	UdL-TA 3.324	0.46	71.83	3.75	0.93	0.69	0.14	4.02	52.95
	UdL-TA 3.325	147.43	1639.31	1434.11	242.69	145.93	41.92	1444.65	1967.94
	UdL-TA 3.268	27.34	904.74	797.56	320.56	27.82	32.25	800.27	3003.27
	UdL-TA 3.270	14.02	728.72	1052.71	2197.34	15.84	12.53	1055.67	3253.55
	UdL-TA 3.327	21.04	824.82	598.63	1467.84	26.83	21.62	606.13	3069.58
	UdL-TA 3.331	31.16	484.30	571.24	541.71	28.65	1.79	571.27	297.59
	UdL-TA 3.332	14.46	818.21	625.43	19.07	26.19	16.98	638.34	2759.85
10 ² spores/mL	UdL-TA 3.244	0.22	594.31	121.42	2.79	0.20	0.00	121.42	164.94
	UdL-TA 3.267	27.81	492.69	487.17	81.80	25.54	0.00	487.17	82.73
	UdL-TA 3.269	159.54	720.58	904.19	81.78	146.59	0.00	904.27	1041.60
	UdL-TA 3.324	3.39	117.15	19.65	3.41	3.11	0.00	19.65	11.68

UdL-TA 3.325	5.30	4606.23	885.59	131.34	6.39	0.00	887.11	2023.25
UdL-TA 3.268	20.17	944.26	826.20	1304.90	18.68	0.12	826.36	5820.10
UdL-TA 3.270	4.47	0.00	1045.16	1195.52	4.16	0.00	1045.21	5811.20
UdL-TA 3.327	13.55	696.70	672.71	1587.49	14.16	2.86	674.42	5930.62
UdL-TA 3.331	18.02	612.17	727.45	1209.34	16.59	0.00	727.49	416.03
UdL-TA 3.332	0.82	389.94	214.84	11.30	0.80	0.00	214.89	5571.19

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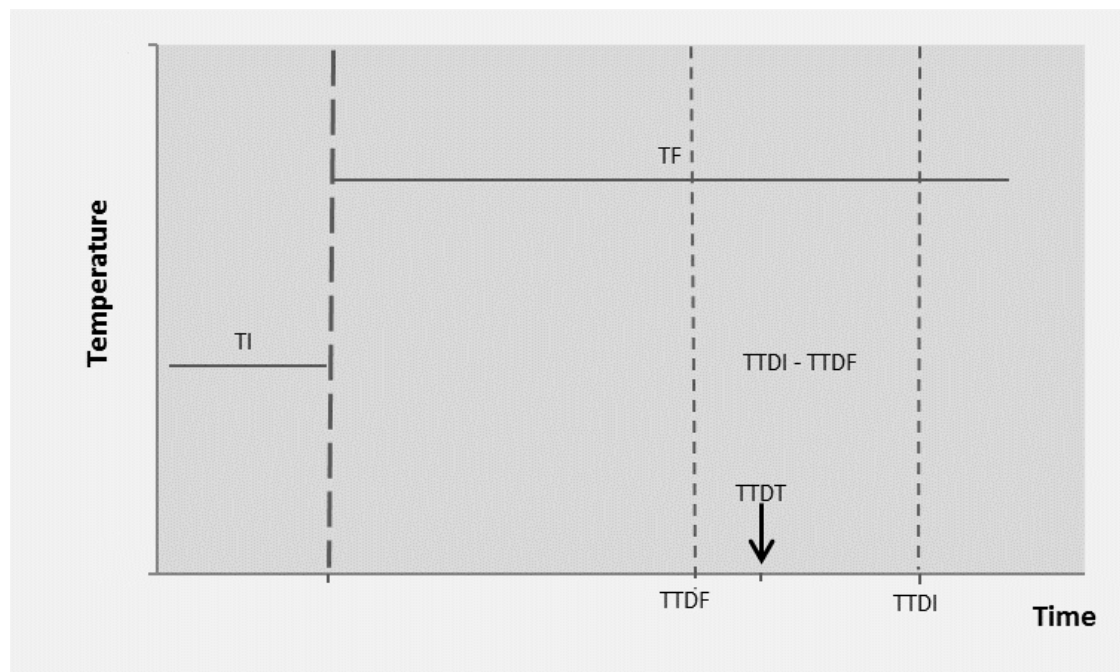


Figure 1. Schematic representation of TTD as affected by the temperature shift.

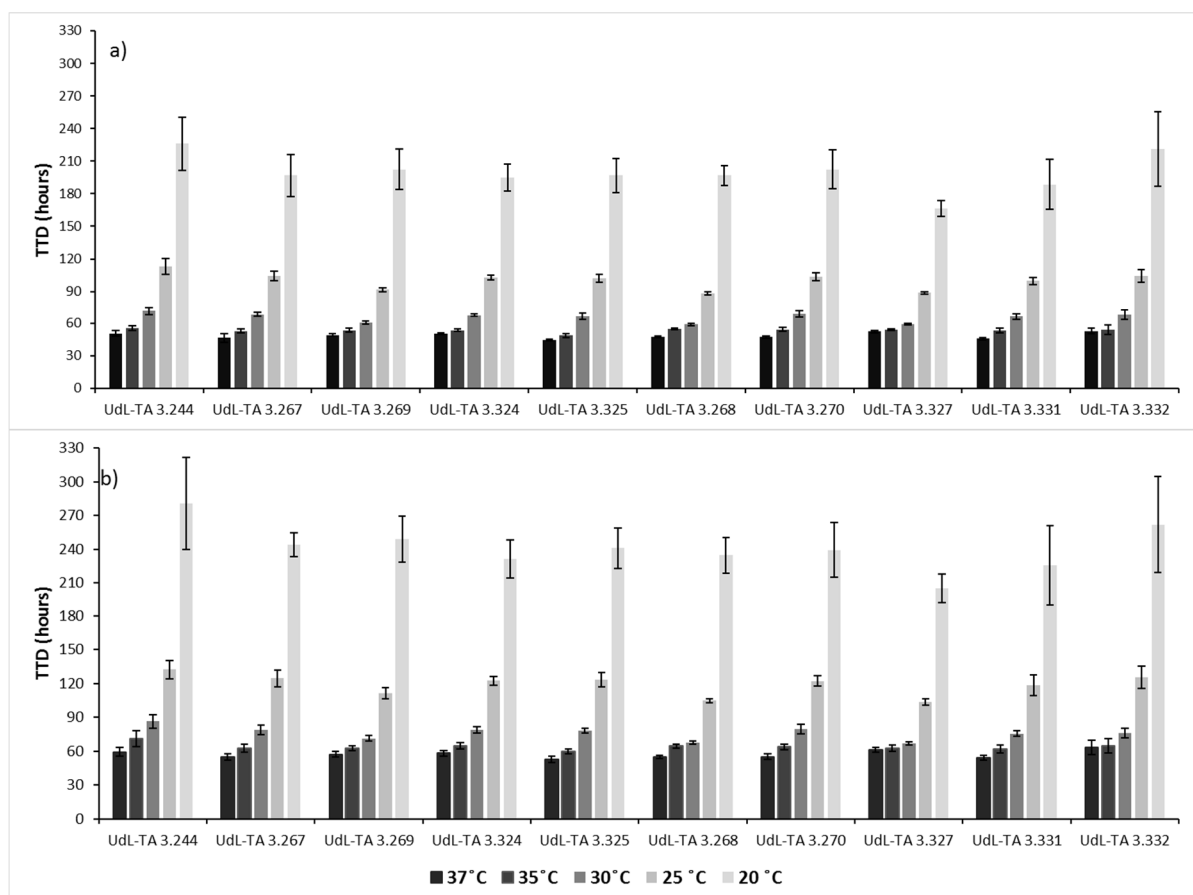


Figure 2. TTDs at 37, 35, 30, 25 and 20 °C for the ten isolates studied at a) 10³ and b) 10² spores/mL.

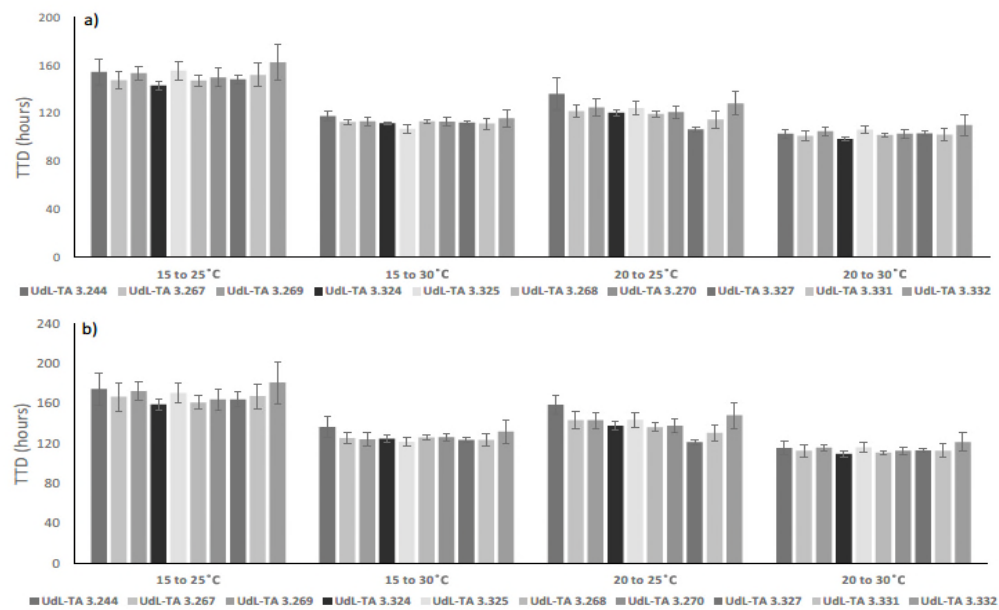


Fig. 3. Experimental TTD obtained under the dynamic temperature profiles: 15 to 25 °C (black bars), 20 to 25 °C (dark grey bars), 15 to 30 °C (white bars) and 20 to 30 °C (light grey bars) for the a) 10^3 spores/mL inoculum and the b) 10^2 spores/mL inoculum for the 10 isolates tested. Error bars are standard deviations ($n = 9$).

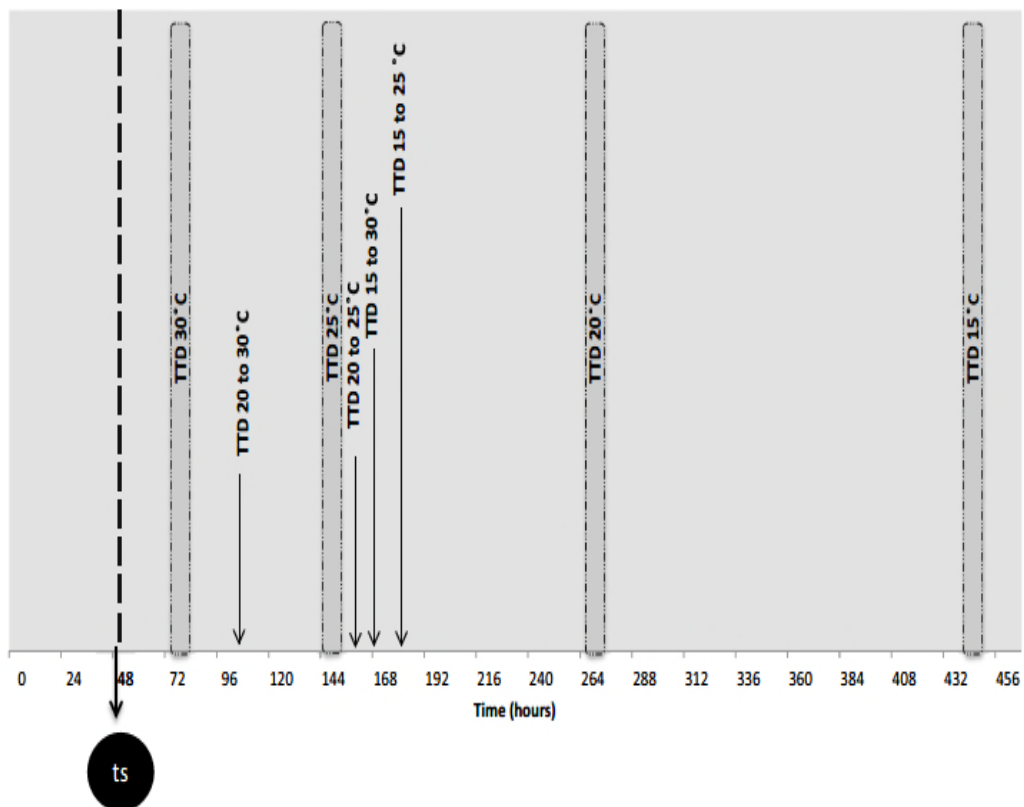


Fig. 4. Comparison among the TTD under both static and dynamic temperature profiles for isolate UdL-TA 3.244 at 10^2 spores/mL.

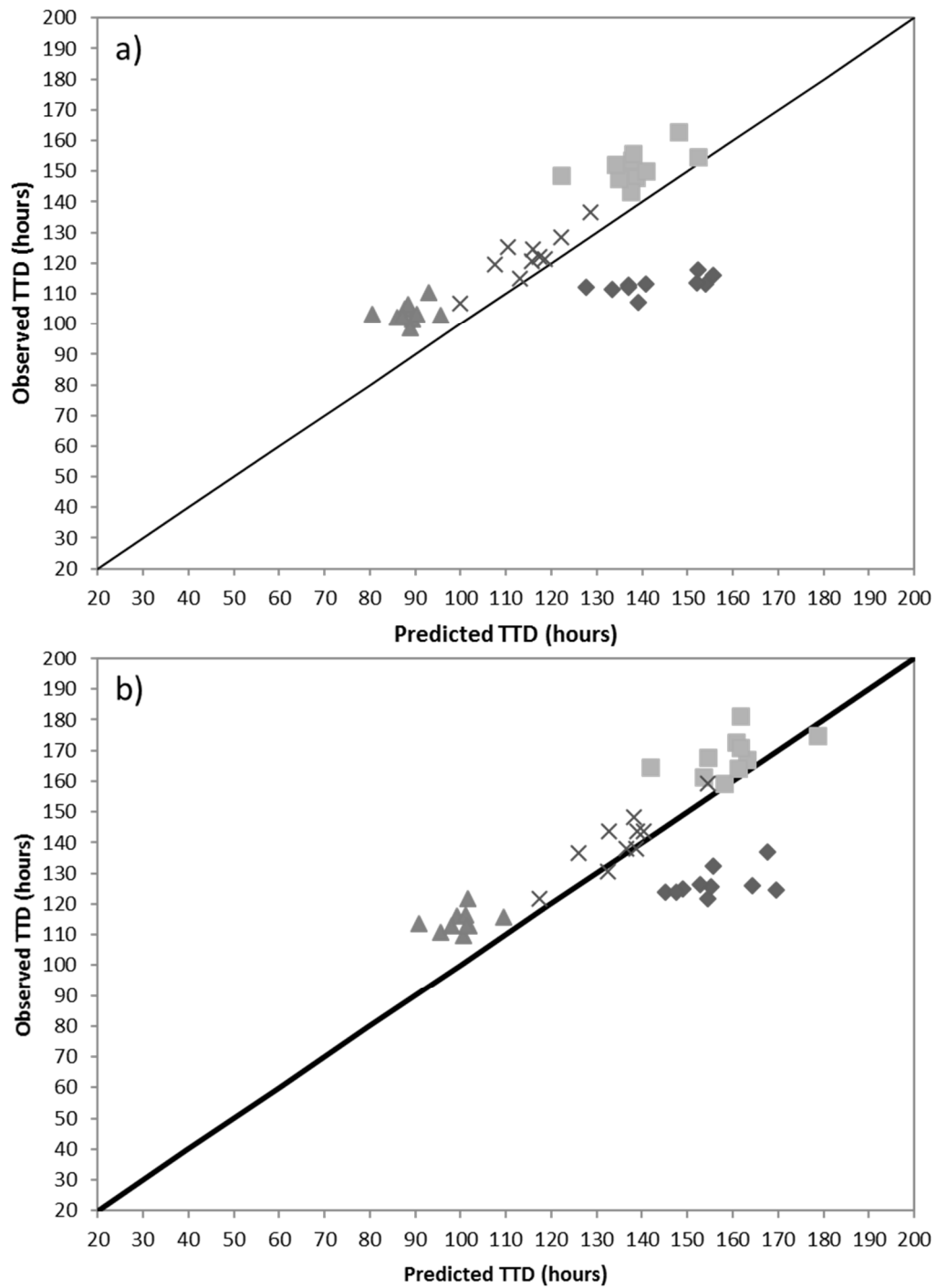


Figure 5. Predicted TTD versus observed TTD at the four changing temperature for the ten isolates studied at a) 10^3 and b) 10^2 spores/mL and the four dynamic temperature profiles: 15 to 25 °C (■), 20 to 25 °C (×), 15 to 30 °C (◆) and 20 to 30 °C (▲).

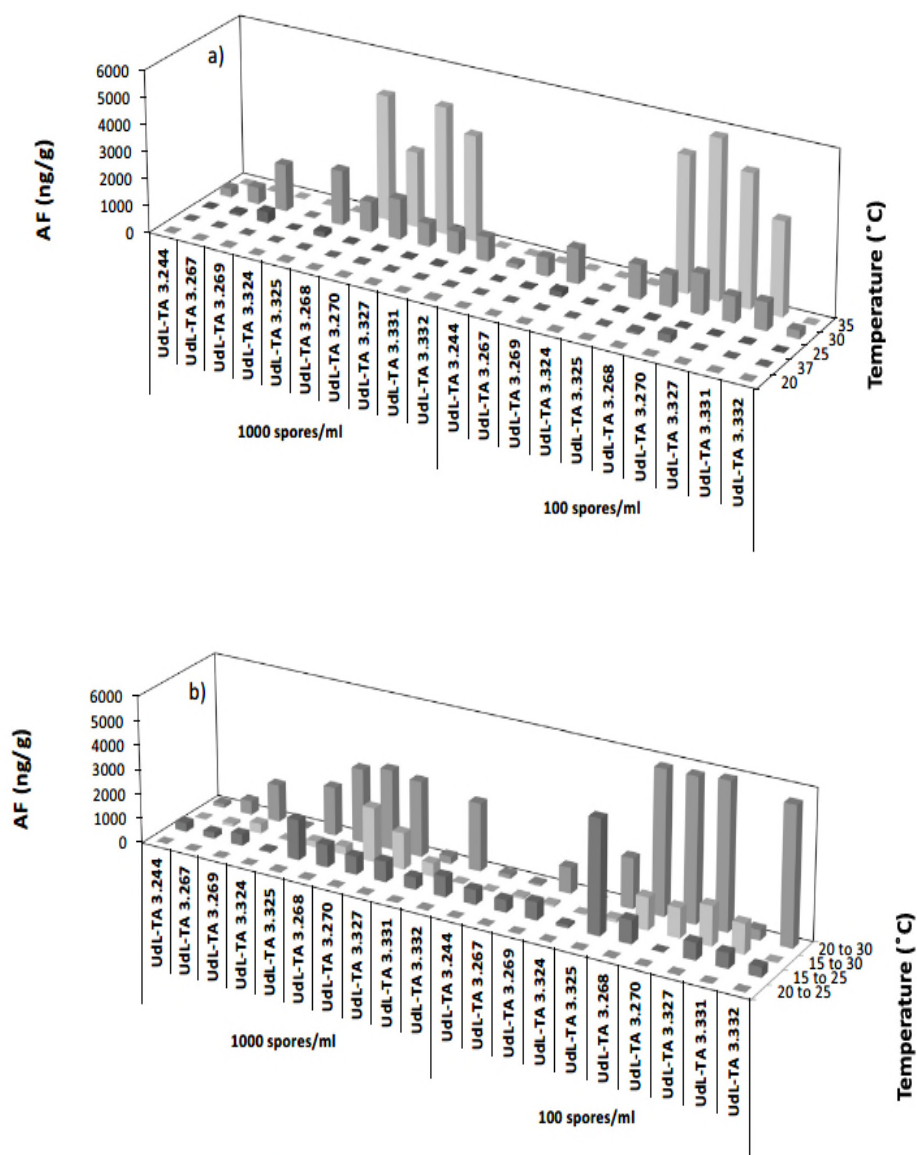


Fig. 6. Aflatoxin B1 production (ng/g) of 10 *A. flavus* isolates under a) five constant temperature levels and b) four dynamic scenarios at both inoculum levels.



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Assessment of intraspecies variability in fungal growth initiation of *Aspergillus flavus* and aflatoxin B1 production under static and changing temperature levels using different initial conidial inoculum levels

Aldars-García, Laila; Marín, Sonia; Sanchis, Vicente; Magan, Naresh; Medina, Angel

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