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Growth parameters of *Penicillium expansum* calculated from mixed inocula as an alternative to account for intraspecies variability

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ABSTRACT

The aim of this work was to compare the radial growth rate (μ) and the lag time (λ) for growth of 25 isolates of *Penicillium expansum* at 1 and 20 ºC with those of the mixed inoculum of the 25 isolates. Moreover, the evolution of probability of growth through time was also compared for the single strains and mixed inoculum. Working with a mixed inoculum would require less work, time and consumables than if a range of single strains has to be used in order to represent a given species. Suitable predictive models developed for a given species should represent as much as possible the behavior of all strains belonging to this species.

The results suggested, on one hand, that the predictions based on growth parameters calculated on the basis of mixed inocula may not accurately predict the behavior of all possible strains but may represent a percentage of them, and the median/mean values of μ and λ obtained by the 25 strains may be substituted by the value obtained with the mixed inoculum. Moreover, the predictions may be biased, in particular, the predictions of λ which may be underestimated (fail-safe). Moreover, the prediction of time for a given probability of growth through a mixed inoculum may not be accurate for all single inocula, but it may represent 92% and 60% of them at 20 and 1 ºC, respectively, and also their overall mean and median values.

In conclusion, mixed inoculum could be a good alternative to estimate the mean or median values of high number of isolates, but not to account for those strains with marginal behavior. In particular, estimation of radial growth rate, and time for 0.10 and 0.50 probability of growth using a cocktail inoculum accounted for the estimates of most single isolates tested. For the particular case of probability models, this is an interesting result as for practical applications in the food industry the estimation of t_{10} or lower probability may be required.

1. Introduction

There is a need to ensure the microbiological quality and safety of food products, and this has stimulated interest in the use of mathematical models for quantifying and predicting microbial behavior (Lahlali et al., 2005). In particular, prediction of spoilage and patulin accumulation by *Penicillium expansum* is required in cold stored fruits, as well as during transport and intermediate storage steps. As one of the most important aspects of model development is ensuring that predictions made by the model are applicable to real situations, it is important that such models account for the behavior of most strains in a microbial species.

Many studies have been published on the effects of the most important abiotic factors, temperature and water activity (a_w) on mold growth. Usually, less than 5 isolates belonging to a given species were used in those studies. Most authors reported significant intraspecific variability on mold growth when several strains were included in their studies (Arroyo et al., 2005; Astoreca et al., 2007, 2010; Bellí et al., 2004; Pardo et al., 2004, 2005b; Parra and Magan, 2004; Romero et al., 2007; Tassou et al., 2009), while others did not find differences among strains of the same species (Bellí et al., 2005; Pardo et al., 2005a).

The use of cocktail inocula of different isolates as an 'average' representation within a species has been proposed (e.g. Hocking and Miscamble, 1995; Patriarca et al., 2001; Pose et al., 2009; Romero et al., 2007, 2010). This concept was introduced for physiological studies on foodborne bacterial pathogens, particularly in acquisition of data for predictive modeling studies, as a way of determining the extremes of growth limits for particular species (Buchanan et al., 1993; Gibson et al., 1987). The use of bulked spore suspensions was applied for the first time to study the a_w tolerances of fungi by Hocking and Miscamble (1995). Although this methodology can be criticized because of the loss of information regarding the responses of individual strains of a species, it is accepted as a legitimate method of achieving a "worst case" scenario (Hocking and Miscamble, 1995). Besides, the use of the cocktail inoculum reduces the use of materials and laboratory work.

García et al. (2012) analyzed the minimum number of single isolates of *P. expansum* and replicates that would lead to equivalent growth parameter estimates to those obtained with a high number of strains

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(n = 62). This work revealed that increasing the number of isolates is much more effective than increasing the number of replicates. In particular, 25–30 isolates of *P. expansum* led to the same mean growth parameters as the total 62 (p = 0.05); all the isolates were from a reduced geographical area.

For this reason, the aim of this work was to compare the growth parameters of 25 isolates of *P. expansum* at two different temperature conditions with those of the mixed inoculum of the 25 isolates. In addition, the evolution of probability of growth through time was also compared for the single strains and mixed inoculum. In apple derived product industries it can be important to predict safe cool and deck storage times, and this was the starting point of the work.

2. Materials and methods

2.1. Fungal isolates and preparation of inoculum

This work was carried out on twenty-five isolates of *P. expansum* previously isolated from apples in Lleida (Spain) during the 2004 and 2005 seasons. All of them are maintained in the culture collection of the Food Technology Department of Lleida University, and had previously been identified according to Pitt and Hocking (1997) and proven to be patulin producers (Morales et al., 2008a). The isolates were subcultured on malt extract agar (MEA: Malt extract 20 g, peptone 1 g, dextrose 20 g, agar 15, in 1 L) plates and incubated at 25 ºC for 7 days to enable significant sporulation. After incubation, a sterile inoculation loop was used to remove the conidia from MEA plates and they were suspended in 5 mL of H_2O/glycerol solution with 0.98 water activity level. After homogenizing, the 25 individual suspensions were enumerated using a Thoma counting chamber (BLAUBRAND®, Germany) after which the final concentration was adjusted to 1–5 × 10^4 spores/mL. A mixed inoculum was prepared with all isolates according to Hocking and Miscamble (1995) at a total concentration of 1–5 × 10^4 spores/mL, where all strains had equal weight.

2.2. Medium preparation and water activity modification

Growth was determined on Apple Concentrate Agar Medium (ACAM, apple concentrate: water 1:7 and agar 15 g/L). Commercial apple concentrate was provided by Indulleida S.A. (Alguaire, Spain). The initial a_w was 0.99 and was adjusted to 0.98 (= a_w of apples) by addition of glycerol. Medium was autoclaved and poured into 9 cm sterile Petri dishes. The final a_w of the medium was checked with an AquaLab Series 3 (Decagon Devices, Inc., WA, USA) to an accuracy of ±0.003.

2.3. Inoculation, incubation and growth assessment

Petri dishes were inoculated centrally with 5 μL of the spore's suspension and enclosed in polyethylene bags in order to maintain a constant water activity. Incubation was done at 1 °C and 20 °C, which represented sub-optimal and near optimal growth temperatures, respectively. 10 replicates were prepared per condition evaluated. Fungal growth was observed on a daily basis for a maximum period of 60 days or until the colony reached the edge of the Petri dish by measuring the colonies perpendicular diameters with a ruler.

2.4. Statistical analyses

Radiuses of growing colonies were plotted against time, and the Baranyi and Roberts (1994) model was fitted to the growth curves of the individual strains and also that of the mixed inocula to estimate maximum radial growth rate (μ, mm/day) and time to growth (λ, d) for each condition (20 ºC and 1 ºC) and inocula. Regressions were made for a given strain both replicate by replicate and pooling all replicate data in a single regression run. When no asymptotic trend was observed in the radius data, lag-linear model (Baranyi's model without asymptote) was used. Moreover, logistic regression was applied to binary values along time (0 = no growth; 1 = growth), in order to model the increase in probability of growth through time. From the regression curves, the time to reach 0.10 (t_{10}), 0.50 (t_{50}) and 0.90 (t_{90}) probability was estimated. As the estimated growth parameters (μ and λ) and probability times were not normally distributed (χ^2 test, Kolmogorov–Smirnov test), Kruskal–Wallis tests were used to assess the significance of the differences among single inocula and mixed one ($p < 0.05$). Statgraphics® Plus version 5.1 (Manugistics, Inc., Maryland, USA) was used for all statistical procedures.

3. Results

3.1. Estimated growth parameters: single strains vs. mixed inoculum

The Baranyi and Roberts (1994) model was fitted to the growth data of the individual strains and the mixed inoculum. Resulting growth curves are shown in Fig. 1. At 20 ºC, the colonies reached the edge of the Petri dishes within 10 days, no asymptotic trend was observed, and lag-linear model (Baranyi's model without asymptote) was used; for the 25 single strains, μ values ranged from 4.46 ± 0.09 to 5.72 ± 0.05 mm/day and λ values from 0.27 ± 0.04 to 1.61 ± 0.04 days (r^2 = 0.997–0.999, MSE = 0.194–1.463). The mixed inoculum had a μ of 5.21 ± 0.05 mm/day and a λ of 0.46 ± 0.05 (r^2 = 0.998, MSE = 0.476). In contrast to 20 °C at 1 ºC after 60 days colony radiuses reached at least 30 mm, but from about 40 days they showed decreasing μ. The individual inocula had μ values that ranged from 0.79 ± 0.02 to 1.11 ± 0.03 mm/day and λ values which ranged from 8.14 ± 0.51 to 13.75 ± 0.50 days (r^2 = 0.987–0.998, MSE = 0.117–2.090). The mixed inoculum had a μ of 0.87 ± 0.02 mm/day and a λ of 8.96 ± 0.56 days (r^2 = 0.991, MSE 1.593).

The overall median μ and λ for the 25 strains were not significantly different ($p > 0.05$, Kruskal–Wallis tests) from that for the mixed inoculum. The estimated μ values for the mixed inoculum were located in percentile 0.60 at 20 ºC and percentile 0.30 at 1 ºC of the values obtained by the 25 single inocula, while the estimated λ for the mixed inoculum was lower than the mean and median values obtained for the 25 strains (percentile 0.14 at 20 ºC and percentile 0.25 at 1 ºC) (Fig. 2).

Using the replicated μ and lag phases obtained for each inoculum, Kruskal–Wallis tests were performed for paired comparison of medians between single and mixed inocula. Significant differences were found in the estimated μ at 20 ºC for 17 strains out of 25, and at 1 ºC for 9 out of 25 strains (58% of them showing higher values than the mixed inoculum, and 42%, lower), while the estimated λs for the single inocula were

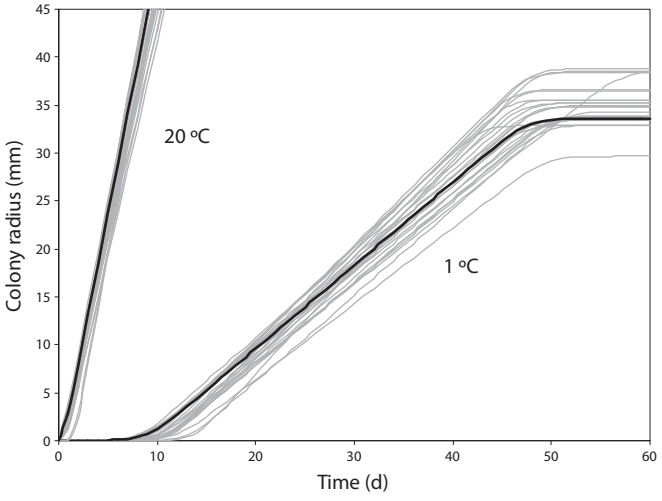

Fig. 1. Growth curves of 25 *Penicillium expansum* strains (——) and a mixed inoculum of them (—) growing on ACAM at 1 and 20 °C.

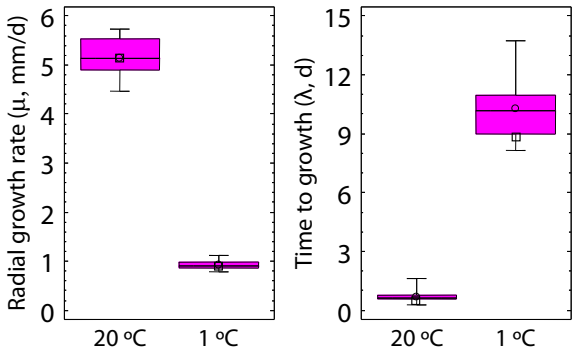

Fig. 2. Distribution of radial growth rates and times to growth for 25 strains of *Penicillium expansum* (Box-and-whisker plot with whiskers = 1.5 × interquartile range). ○, mean value for the 25 strains; □, value for the mixed inoculum.

significantly different from those of the mixed inoculum in 16 cases at 20 ºC and in 15 cases at 1 ºC (they where always longer).

This suggests that the predictions based on growth parameters calculated on the basis of mixed inocula may not accurately predict the behavior of all strains, but they may be representative for some. However, the mean and median values of the estimated μ and λs obtained for the 25 strains may be substituted by the values obtained for the mixed inoculum. The predictions may be biased, in particular, the predictions of λ which may be underestimated (fail-safe), as all the strains which were significantly different from the cocktail had longer λs.

3.2. Estimated probability of growth through time: single strains vs. mixed inoculum

At 20 ºC, 92% strains (23/25) showed visible growth (about 2 mm of diameter) after 24 h of incubation, while two took one more day. In similarity to the results observed for most of the single inocula, growth of the colonies of the mixed inoculum became visible after one day of incubation at 20 °C. This was the result of the narrow distribution of λ (0.3–1.6 days). This infers that mixed inocula can potentially be used for the development of probability models.

At 1 °C, growth on the plates inoculated with single inocula became visible after 8–15 days. Growth of only 12 of the 25 (48%) strains became visible after 9–11 days of incubation (Fig. 3), as well as the mixed inoculum. The reason was that, in this case, the distribution of λ, as shown in the previous section was wider (8.1 to 13.7 days).

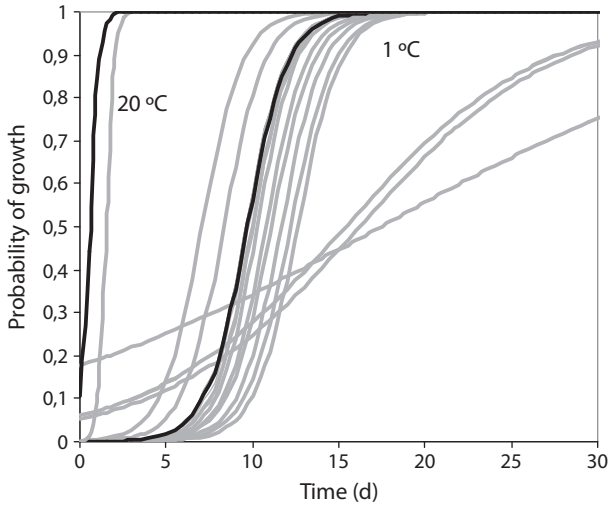

Fig. 3. Growth probability curves of 25 *Penicillium expansum* strains (——) and a mixed inoculum of them (▬) growing on ACAM at 1 and 20 °C.

Logistic regression analysis led to adjusted r^2 of 0.85–0.92 (MSE 0.000–0.001) at 20 ºC and r^2 of 0.60–0.88 (MSE 0.00–0.03) at 1 ºC for the 25 single inocula, while for the mixed one, at 20 ºC adjusted r^2 was 0.85 (MSE 0.001) and at 1 ºC r^2 was 0.85 (MSE 0.01).

Fig. 4 shows the distribution of the estimated values for the time to reach 0.10, 0.50 and 0.90 probability values. The overall medians of the estimated t_{10}, t_{50} and t_{90} for the 25 strains were not significantly different (Kruskal–Wallis tests, $p < 0.05$) from those for the mixed inoculum, both at 20 ºC and at 1 ºC. At 20 ºC the mean and the median of the estimated time values for the 25 strains equaled the estimated values using the mixed inoculum, while at 1 °C the estimated mean values of the 25 strains were higher than those of the mixed inoculum, although the latter were quite close to the median values of the 25 strains individually (percentiles 0.40, 0.28 and 0.30, for t_{10}, t_{50} and t_{90}, respectively). As observed for λ, this suggests that mixed inoculum could be used to assess the worst scenario.

Using the estimated t_{10}, t_{50} and t_{90} values obtained for each inoculum replicate, Kruskal–Wallis tests were performed for paired comparison of medians between single and mixed inocula: significant differences were found in the t_{10}, t_{50} and t_{90} values at 20 ºC for 2 strains out of 25, and for 6, 10 and 10 strains out of 25, for t_{10}, t_{50} and t_{90} values, respectively, at 1 ºC. This suggests that although the predicted time to achieve a given probability of growth for a mixed inoculum may not be accurate for all strains, it is still representative for most.

4. Discussion

In predictive mycology, kinetic growth models are built with the aim to estimate μ and λ, and probability models in order to estimate the probability of growth reached at a given time or, conversely, the time to reach a given probability, e.g. 0.10, 0.50 and 0.90, as presented in the present work. In any case, such estimations may depend on the number of isolates/strains chosen to represent a given fungal species. If the cocktail inoculum technique is to be applied, the number of strains in the cocktail should also be standardized. Recently, García et al. (2012) compared the estimated growth parameters obtained for *Aspergillus carbonarius* and *P. expansum* by using 30 and 62 isolates, respectively. For *P. expansum* the mean μ and λ estimated with 62 isolates was not significantly different from the mean value obtained with 25–30 isolates. As working separately with such large number of isolates may still be tedious, the possibility of using a mixed inoculum containing 25 isolates was considered in this work. In the present study, we tried to know if the use of a mixed inoculum (containing 25 strains) for data generation could replace the need for n = 25 repeated experiments with 25 strains in order to try and obtain results which are representative at species level. Obviously, with a mixed inoculum we loose the information on the distribution of growth parameters of the individual species. The best option would be working with as many strains as possible and describe the statistical distribution of their growth parameters. However, from the practical point of view if we want to model

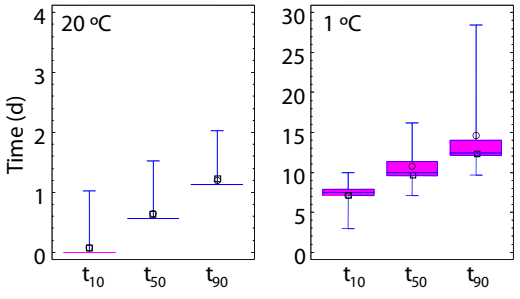

Fig. 4. Distribution of time for 0.10, 0.50, and 0.90 growth probability for 25 strains of *Penicillium expansum* (Box-and-whisker plot with whiskers = 1.5 × interquartile range). ○, mean value for the 25 strains; □, value for the mixed inoculum.

P. expansum behavior to adjust suitable storage times, and duration of particular steps in fruit processing, building models based on mixed inocula would be of limited cost.

Regarding the growth parameters, it was observed that the mixed inoculum accounted for the mean and median values of μ calculated with the 25 strains ($p < 0.05$), but not for those strains with particular divergent behavior, due to either higher (58% of cases) or lower (42%) μ values. None of the mean and the median values of λ obtained for the 25 strains were different from those of the mixed inoculum. In this case those strains which behaved different to the mixed inoculum always showed longer λ. In a mixed inoculum, one may expect that the faster germinating conidia will be characterized by a larger lag time for growth than the same strain in pure culture, due to a "dilution" with the slower germinating conidia. However, this point could not be confirmed in the present study. On the other hand, a shorter λ may be expected for a mixed inoculum as it includes all strains (although at a lower inoculation rate) and those with a major potential will lead the behavior of the overall inoculum (Morales et al., 2008b). Consequently, although the estimations would be biased in terms of λ, using the mixed inoculum would lead to a 'worst scenario' situation.

The particular effects of intraspecies interactions were not assessed in this study, as equivalent inoculum sizes were not assayed for a given strain growing in single and mixed conditions. However, interactions probably occurred in the mixed inoculum which might have affected the results presented here. In general, published results on fungal interactions reported some sort of fungistasis, including reduced germination and number of germ tubes, coiling of hyphae and formation of specialized structures similar to hooks, appressoria and papillae (Martinez et al., 2004; Wagacha et al., 2012). However, most studies dealt with biocontrol agents, thus fungal inhibition was seeked for. The kind of interactions between two strains of the same species could in part be attributed to the type of secondary metabolites a strain produces and whether such metabolites play any role in the infection process (Llorens et al., 2006). In our case, no longer lag times or reduced growth rates were observed in the mixed inoculum, thus fungal inhibition due to competition was unlikely to occur, and there is no base in our experimental design to support an intraspecies stimulation hypothesis. A previous work by Morales et al. (2008b) with two strains of *P. expansum* in apples reported also a significantly shorter λ in the mixed inoculum at 20 ºC, while at 1 ºC μ was significantly smaller in the mixed inoculum than in the two single species, suggesting some sort of fungal inhibition. Interestingly, they analyzed patulin levels in the apples, and lower patulin accumulation was detected in the mixed inoculum.

Cocktail inocula have been used before including 4–5 strains of *Alternaria alternata* or *A. carbonarius* (Pose et al., 2009, 2010; Romero et al., 2007, 2010). In particular, Romero et al. (2010) reported that the mixed inoculum yielded an average μ, which represented the tendency of the individual strains behavior in optimal growth conditions. However, and opposite to what was observed in the present study, in their case the lag phase for the mixed inoculum was the same that the highest obtained for individual strains, suggesting mycelial interactions between the isolates.

Regarding μ and λ values, our 25 strains were in the ranges μ [4.5, 5.7] mm/day and λ [0.3, 1.6] days at 20 ºC and μ [0.8, 1.1] mm/day and λ [8.1, 13.7] day at 1 ºC. μ of a strain of *P. expansum* isolated from grape was lower in Potato Dextrose Agar than ours (<0.1 mm/day at 1 ºC and about 2.7 mm/day at 20 ºC) (Judet-Correia et al., 2010), while similar values were reported by Baert et al. (2007) when working with isolates from apple (μ < 0.9 mm/day at 1 ºC, and 3.0–8.5 mm/day at 20 ºC; λ > 6.2 days at 1 ºC, and 0.8–2.3 days at 20 ºC in apple puree agar medium (APAM)). Moreover, Baert et al. (2007), working with 8 *P. expansum* strains showed that 13–23 days were required to reach a colony surface of 45 cm^2 at 20 ºC, and 115–204 days at 1 ºC in APAM, while in our case 8–9 days were required at 20 ºC, and >55 days at 1 ºC (the experiment lasted for no more than 60 days).

Previous studies reported that the intraspecies variability could be higher under marginal growth conditions (Baert et al., 2007; García et al., 2011a,b). In particular, García et al. (2011b) working with 79 isolates of *P. expansum* reported coefficients of variation (CV) of 13.5 and 17.8% at 20 and 1 ºC, respectively, for μ, and 12.7 and 14.3% at 20 and 1 ºC, for λ. This point was not confirmed in this study as the CV was less than 5% for μ and λ at 1 ºC, and 7% and 44%, at 20 ºC. In fact, in later studies by García et al. (2012), a higher percentage of the data variability was due to replicates under optimal conditions, while under marginal conditions (1 ºC for *P. expansum* and 0.90 a_w for *A. carbonarius*) the contribution to variability of intraspecies difference was more important than that of replicates. As a consequence, the number of replicates/isolates required for an accurate estimation of the growth parameters was not lower under optimal conditions of growth. In the present study only replicates were taken into account; there is still room for improvement as independently repeated experiments could be carried out in order to compare reproduction variability to isolate variability and draw more meaningful conclusions than only using replicate experiments. An elegant study was conducted by Giorni et al. (2007) with 40 strains of *Aspergillus* section *Flavi* inoculated on Czapek agar medium and incubated at different temperatures and water activity values for 14 days in the dark. They showed a higher dispersion in the distribution of colony diameters and ln(aflatoxin B1) produced by the strains when the values of these two variables were higher, thus under suitable conditions for growth, the dispersion of data was wider in absolute terms (not corrected by the mean/median values).

Regarding probability models, similarly to growth models, it was observed that the mixed inoculum accounted for the mean and median estimated t_{10}, t_{50} and t_{90} values of the 25 strains ($p < 0.05$), but not for those strains with particular divergent behavior, although their number was lower than in the case of growth models. For conditions suitable for growth, as 20 ºC in this study, the variability among strains in a daily basis was very low and the mixed inoculum followed the trend of the 92% single strains, while for λ (range 0.27–1.61 days) only 36% were not significantly different to the mixed inoculum. The reason for this is that time to growth calculation was based on discrete daily observations, while estimated λ is a continuous variable. At 1 ºC, however, t_{10}, t_{50} and t_{90} mean/median values were higher than those for the mixed inoculum, suggesting that the initiation of growth was faster for the mixed inoculum, which is in accordance with what was explained above for the λ case. The difference between mean/median values for single and mixed inocula increased with the probability and was higher for t_{90}, with a lower value for the mixed inoculum. t_{10} values were similar but the difference was wider for t_{90}, which suggests that the mixed inoculum had a narrower frame from the first replicate that showed growth to the last that did so (from 9th to 11th day, 2 days), whereas for single inocula it lasted from 1 to 5 days. Lower values of t_{50} and t_{90} in the mixed inoculum may mean that recorded visible growth was due to faster germinating strains, while the higher single inocula means are due to slow germinating strains.

As conclusion, mixed inoculum could be a good alternative to estimate the mean or median values of high number of *P. expansum* isolates, but not to account for those strains with marginal behavior. In particular, estimation of μ, t_{10} and t_{50} through the cocktail inoculum represented a major proportion of the single isolates tested. For the particular case of probability models, it is an interesting result as for practical applications in the food industry the estimation of probabilities even lower than t_{10} may be required. Twenty-five isolates were used in this study based on a previous work on μ and λ estimation, not on probability parameters estimation, thus it is uncertain whether more or less than 25 strains would be required for a good estimation of the species behavior. However, as the intraspecies variability of the probability parameters seems to be lower than that for the growth parameters (μ and λ), it could be hypothesized that a lower number of isolates might be required to estimate probabilities of growth. Probability models are based on discrete 0/1 observations which may be more

consistent across isolates than continuous measurements of growing colonies. These results cannot be directly extrapolated to other fungal species, as they depend on the particular intraspecies distribution of the growth parameters.

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