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Effect of sugar concentration (°Brix) and storage temperature on the time to visible growth of individual ascospores of six heat-resistant moulds isolated from fruit products

Juliana Lane Paixão dos Santos[a,*], Simbarashe Samapundo[a], Jan Van Impe[b], Anderson S. Sant'Ana[c], Frank Devlieghere[a]

[a] Research Unit Food Microbiology and Food Preservation, Department of Food Technology, Safety and Health, member of Food2Know, Faculty of Bioscience Engineering, Ghent University, Belgium
[b] Chemical and Biochemical Process Technology and Control (BioTec+), Department of Chemical Engineering, University of Leuven, Belgium
[c] Department of Food Science, Faculty of Food Engineering, University of Campinas, Brazil

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ABSTRACT

Heat-resistant moulds (HRMs) pose great challenges to processors of fruit-based products due to their thermal resistance and ability to grow across a broad range of conditions. Therefore, the quantification of the effect of inhibitory factors (conditions) on the growth of HRMs is very important to be used to prevent spoilage during shelf-life. This study assessed the minimum temperature and maximum sugar content (°Brix) for the growth of six HRMs (*Byssochlamys* spp. and *Neosartorya* spp.) previously isolated from fruit products. In addition, the time to form a visible colony (t_v, days) was determined to assess biological variability of individual ascospores within same population. Heat activated ascospores (10 min at 80 °C) were spread plated (± 100 spores) on acidified Potato Dextrose Agar (aPDA, pH 3.5) plates from which the °Brix was adjusted with fructose-glucose (1:1) to levels between 44 and 59 °Brix followed by incubation at 30 °C. To assess the effect of temperature, inoculated plates of aPDA were incubated at 4, 7, 8, 10, 12 and 14 °C. Three replicates (= 3 aPDA plates) were prepared per condition evaluated. The number of visible colonies were counted daily for up to two months. Probability distribution functions were then fitted in @Risk to the cumulative t_v's. With regards to cold tolerance, *B. nivea* was the most cold sensitive as it had the least ability to germinate and form visible colonies at low temperatures (no growth when T ≤ 10 °C). On the other hand, *N. hiratsukae* was the most cold tolerant, being able to form visible colonies at temperatures ≥ 7 °C. Likewise, *B. nivea* was the most sensitive to increased °Brix values, whilst *N. udagawae* was able to grow out at the highest °Brix evaluated (59°/a_w = 0.86). The tolerance of potential spoilage HRMs to high sugar levels and their ability to grow under chilled conditions represent a challenge for the microbial stability of high-sugar fruit products. Differences in individual t_v's were mostly observed under conditions at the growth/no growth regions. For instance, individual t_v's of *B. nivea* ascospores ranged from 24 to 46 days at 12 °C and those of *N. udagawae* ranged from 20 to 45 days at 59°Brix. The t_v's data from each HRM and condition evaluated were then fitted to different statistical distributions (Exponential, Normal, Lognormal, Weibull, Logistic or Pareto) to allow the use of the obtained data in further Quantitative Microbial Spoilage Risk Assessment work for pasteurized fruit products.

1. Introduction

Heat-resistant moulds (HRMs) are well known as specific spoilage microorganisms (SSOs) of thermally treated fruit products worldwide. Their classification as SSOs of these products is due to their high occurrence in fruits, their ability to withstand pasteurization treatments usually applied to fruit products and to overcome the hurdles typically applied by fruit processors (Pitt & Hocking, 2009, pp. 1–519; Rico-Munoz, 2017; Samson, Houbraken, Thrane, Frisvad, & Andersen, 2010; Santos et al., 2018; Tournas, 1994). These hurdles include, for instance, the development of robust formulations by lowering the water activity (a_w, often by increasing the sugar content), the presence (addition) of organic acids (low pH), by limiting the amount of available oxygen inside the packages, by adding antifungal preservatives and by storing

* Corresponding author. Faculty of Bioscience Engineering, Department of Food Technology, Safety and Health, Coupure Links 653, Gent, 9000, Belgium.
E-mail address: Juliana.lanepsantos@ugent.be (J. Lane Paixão dos Santos).

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the food product under chilled conditions (Berni, Tranquillini, Scaramuzza, Brutti, & Bernini, 2017; King, Michener, & Ito, 1969; Panagou, Chelonas, Chatzipavlidis, & Nychas, 2010; Sant'ana, Rosenthal, & Massaguer, 2009; Tournas, 1994; Tremarin et al., 2015). The application of intense heat treatments and hurdles such as those listed above are usually enough to ensure the total inhibition of most spoilage microorganisms. However, these treatments and conditions may not fully prevent the outgrowth of HRMs. If ascospores persist post-pasteurization on the products and encounter favorable conditions, they may germinate and grow out. Their growth is accompanied by visible mycelium formation on the foods surface and organoleptic deterioration, resulting in rejection by consumers and consequent economic losses. Furthermore, many HRM species are known to produce mycotoxins (Frąc, Jezierska-Tys & Yaguchi 2015; Houbraken, Samson, & Frisvad, 2006; Tournas, 1994).

Many studies have focused on the effect of environmental factors on the growth of HRMs through the estimation of fungal growth rates and lag times (Panagou et al., 2010; Santos et al., 2018b; Tremarin et al., 2015) often of high inoculum. However, less attention has being given to these effects on the time to visible growth of HRMs, i.e., the time required for the colony to develop to a size that consumers can detect (Berni et al., 2017; Roland & Beuchat, 1984). Nevertheless, this parameter is of great interest of fruit processors as it can be translated into a predicted time of rejection, i.e., the mould-free shelf-life of the product (Burgain, Bensoussan, & Dantigny, 2013; Dantigny, 2016; Huchet et al., 2013). Contamination of food products by HRMs is most likely to occur by only one or a few spores (Berni et al., 2017; Pitt & Hocking, 2009, pp. 1–519; Santos et al., 2018; Tranquillini, Scaramuzza, & Berni, 2017). Therefore, the incorporation of individual ascospore growth response (= biological variability) as a stochastic process needs to be taken into account in studies aiming to determine mould-free shelf-lives.

The biological variability inherent of single fungal spores belonging to the same population has been explored by some authors (Dagnas, Gougouli, Onno, Koutsoumanis, & Membré, 2017; Garcia, Ramos, Sanchis, & Marín, 2010; Gougouli & Koutsoumanis, 2013; Samapundo, Devlieghere, De Meulenaer, & Debevere, 2007), who highlighted the importance of performing growth kinetics studies at the individual spore level. These studies focused on the effect of environmental factors on the distribution of individual lag times, while to date there are no available data on the time to visible growth of individual ascospores.

Therefore, this study aimed to assess the inhibitory levels of °Brix and temperature on the growth of six different species of HRMs. In addition, the time to form visible colony (t_v) by individual ascospores (= biological variability) for each HRM specie and condition was assessed and further fitted to statistical distributions.

2. Material and methods

2.1. HRMs strains

Six heat-resistant moulds (HRMs) strains previously identified and isolated from raw and processed fruit products (Santos et al., 2018) were investigated in this study: *Byssochlamys nivea* (Bysso nivea 76-1) and *Neosartorya laciniosa* (Neosart laciniosa 67-2) - isolated from pasteurized strawberry puree, *Byssochlamys fulva* (Bysso fulva 56-2) and *Neosartorya hiratsukae* (Neosar hiratsukae 77-5)- isolated from strawberries, *Neosartorya udagawae* (Neosar udagawae 54-3) - isolated from sieved strawberry puree, and *Neosartorya fischeri* (Neosar fisheri 95-1)- isolated from extracted orange juice. The isolates were maintained in the culture collection of the Laboratory of Applied Mycology (MYCOLAB, Department of Food Technology, Safety and Health, Ghent University, Belgium).

2.2. Preparation of ascospore suspensions

HRMs strains were grown for 30 days at 30 °C on 10 plates of Malt Extract Agar (MEA, Oxoid, UK) to ensure ascospores formation. Subsequently, ascospores suspensions were prepared by flooding each Petri dish with 8 ml of sterile 0.1% Tween 80 (Sigma-Aldrich, USA), followed by filtration and centrifugation for three times at 8.000 g for 15 min at 4 °C. The final suspension were then obtained by adding 10 ml of sterile distilled water (SDW) to the pellet. Sonication (amp: 45%, 30s-interval) was applied for up to 4 min to separate the ascospores clusters of *Byssochlamys* strains. Ascospores suspensions concentration were determined after spread plating serial decimal dilutions (in sterile 9 ml-distilled-water tubes) on MEA after heating shock at 80 °C for 10 min. Enumeration was done after incubation of the plates for 5–7 days at 30 °C. The final suspensions were further standardized according to the subsequent test, kept at 2 °C and used up to 7 days after harvesting.

2.3. Effect of °Brix on the time to visible growth

The °Brix effect was assessed on aPDA (pH 3.5, HCl 6N) from which the °Brix was adjusted with fructose (F-0127, Sigma) and glucose (G-8270, Sigma) (1:1) to 44–59 °Brix (= a_w 0.86–0.91) (see Table 1). The values of °Brix (i.e. the value (%) of total sugar content) were checked by means of a pocket refractometer (HR25/800 Kruess, Germany). Before inoculation of the aPDA was done, the ascospores suspensions were standardized to 10^2 -10^3 spores by serial decimal dilutions in sterile 9 ml-glycerol solutions tubes adjusted to the same a_w-values as the media to be inoculated. After activating the suspensions in a water bath at 80 °C for 10min, aliquots of 100 µL of standardized suspensions were spread plated (± 100 spores) in triplicate on aPDA plates and incubated at 30 °C. All Petri dishes were then sealed with Parafilm® to avoid dehydration after which plates with the same °Brix were placed together in sealed plastic boxes. Non-inoculated plates with adjusted aPDA were incubated together with inoculated plates with similar a_w-values. a_w measurements were performed as a_w is a more useful parameter in food microbiology as it objectively describes the amount of water available whilst °Brix is universally applied in the fruit processing industry. The a_w media was checked monthly by means of an a_w meter (Sprint TH500, Novasina Thermoconstanter, Switzerland). The plates were checked periodically for visible growth (colony diameter = 2 mm) for up to 60 days. Growth/no growth data, time to visible growth (t_v, days) and the number of visible colonies were assessed. The experiment was performed twice, with a total of three plates checked per condition and experiment (n = 6).

2.4. Effect of temperature on the time to visible growth

For the experiments assessing the effect of temperature, the suspension of each HRM was initially standardized to 10^2 -10^3 spores/ml in sterile acidified phosphate buffer solution prepared by dissolving 68 g/L of potassium dihydrogen phosphate (Sigma Aldrich) in 1000 ml of distilled water acidified to pH 3.5 with phosphoric acid (Merck).

Table 1
Formulation of sugar-based growth media.

Fructose (g)	Glucose (g)	PDA[a] (g)	Distilled water (g)	°Brix	a_w ± SD
105	105	19.5	290	44	0.904 ± 0.003
110	110	19.5	280	47	0.902 ± 0.007
115	115	19.5	270	50	0.894 ± 0.007
120	120	19.5	260	53	0.883 ± 0.005
125	125	19.5	250	56	0.869 ± 0.006
130	130	19.5	240	59	0.860 ± 0.002

[a] Potato Dextrose Agar.

After being heat activated (80 °C for 10 min) aliquots of 100 μL of standardized suspensions were spread plated (± 100 spores) in triplicate on acidified Potato Dextrose Agar (aPDA, pH 3.5, HCl 6N) plates. After inoculation, the plates were sealed with Parafilm® and incubated at 4, 7, 8, 10, 12 and 14 °C. The plates were periodically checked for visible growth (colony diameter ≥ 2 mm) for up to 60 days. Growth/no growth data, t_v's, and the number of visible colonies were assessed. The experiment was performed twice with a total of three plates checked per condition and experiment (n = 6).

2.5. Statistical analysis

Statistical distributions were fitted to the data, i.e., number of observed colonies vs. time, for each condition by means of @Risk 7.0 software for Excel (Palisade Corporation, NY, EUA). The probability distributions available in @Risk were visually compared (adjusted-fit plot, Probability-Probability (P–P) plots and Quantile-Quantile (Q-Q) plots) and ranked based on the Bayesian Information Criterion (BIC). The distributions with the best fits were chosen and their estimates for important parameters such as the mean, 95% confidence interval (CI), and standard deviation, were obtained. Significant differences (at $\alpha = 0.05$) between t_v's were determined by comparing their 95% CI's for overlap or lack thereof.

3. Results

The six °Brix levels evaluated (expressed as the total sugar content, %), were selected based on data in literature (Berni et al., 2017; Beuchat & Toledo, 1977) and our own preliminary studies (data not shown). In general, visible mycelia (i.e., fungal colony diameter ≥ 2 mm) were observed within 2–45 days at 30 °C, depending on the sugar content (°Brix) and isolate (Table 2). *B. nivea* was the least tolerant to elevated sugar levels and was unable to grow at sugar concentrations ≥53°Brix (aw ≤ 0.883). The other five isolates evaluated grew out at sugar concentrations equal to 56°Brix within the two month incubation period. Of these, *N. udagawae* was the most tolerant to elevated sugar levels as it was the only isolate able to develop visible colonies at 59°Brix ($a_w = 0.86$) within the incubation period evaluated. At the lowest sugar concentration evaluated, 44°Brix ($a_w = 0.904$), very short t_v's (of 2–12 days) were observed for all HRMs evaluated. Overall, increase in sugar concentration from 44 to 56°Brix resulted in significantly longer t_v's ($p < 0.05$) for the *Neosartorya* strains. In addition, a significant increase ($p < 0.05$) in the t_v of *N. udagawae* was observed when the sugar concentration increased from 56° (9–20 days) to 59°Brix, in which visible colonies were observed after 20–45 days. On the other hand, *N. fischeri* growth was less affected by increasing sugar concentration, with t_v's being not significantly different ($p > 0.05$) from 44 to 53°Brix. Unlike *B. nivea*, the response of *B. fulva* towards changes in the °Brix was similar to that exhibited by the *Neosartorya* strains. Increase in sugar concentration in the range of 44–53°Brix did not result in significant differences ($p > 0.05$) in the t_v's of *B. fulva* and the *Neosartorya* strains.

The minimum growth temperatures (cold tolerance) of the six HRMs strains were evaluated on aPDA ($a_w = 0.99$/4°Brix). The t_v's ranged from 6 to 46 days, depending on the incubation temperature and species (isolate) (Table 3). *B. nivea* was the most cold-sensitive as it was not able to germinate and form visible colonies at low temperatures (≤10 °C) and it presented visible growth at 12 °C after up to 46 days of incubation. On the other hand, *N. hiratsukae* was the most tolerant to low temperatures and was able to form visible colonies at temperatures ≥7 °C after 27–40 days, while *B. fulva*, *N. fischeri*, *N. laciniosa* and *N. udagawae* were able to form visible colonies at temperatures ≥10 °C. The growth of these HRMs was significantly delayed ($p < 0.05$) at 10 °C, with visible growth being exhibited only after 19–38 days. On the other hand, visible outgrowth at 14 °C occurred just after 6–13 for all the *Neosartorya* strains and after 8–23 days and 18–35 days for *B. fulva* and *B. nivea*, respectively.

The results of this study show that ascopores t_v's originating from the same population are highly variable. Therefore, this parameter would be better described by probability distributions describing variabilities and/or uncertainties. In this study it was assumed that each visible colony detected after a determined period originated from a single ascospore. Thus, the ca. 100 t_v values (from ca. 100 ascospores) were obtained for each isolate at each experimental condition. These t_v's were fitted to statistical distributions in @Risk. The best fitting distributions were identified (see Tables 2 and 3). Fig. 1 shows the cumulative t_v's of ascospores from the HRMs isolates evaluated in this study on aPDA at sugar concentrations ranging from 44 to 59°Brix at 30 °C. Most of the data regarding the effect of °Brix was best represented by right-skewed exponential curves bounded at the estimated parameter "Risk shift", i.e. a shift value from 0, and described by a single scale parameter. The most likely values from exponential curves were concentrated in the lower boundary, which in this study indicate the shortest t_v's observed for the majority of the ascospores in a population (= single HRM). Moreover, in some cases, different types of distributions were selected, according to the goodness of fit (Table 2). For instance, the *N. udagawae* t_v data at 56 and 59°Brix were best represented (described) by lognormal and normal distributions, respectively. Likewise, *N. laciniosa* ascospores t_v's data were best represented by a Weibull distribution at 56°Brix (Fig. 1). These differences in distributions were also associated with the growth/no growth regions in which wider t_v ranges were observed implying greater biological variability among ascospores from the same population under these conditions.

Fig. 2 shows the cumulative t_v's of *Byssochlamys* and *Neosartorya* spp. arising from ascospores inoculated on aPDA and stored at temperatures ranging from 7 to 14 °C. Reduction of temperature in the investigated range (14 °C–7 °C) appears to have a more pronounced effect on the distribution and spread of individual t_v's compared to the effect of sugar content (°Brix) in the evaluated range (44–59°Brix). This can be also deduced from the large distances between the cumulative t_v curves at different temperatures (Fig. 2). Most of the curves were determined to be exponential right-skewed, most t_v-values concentrated in the lower boundary (=shorter times). Besides, *N. hiratsukae*, which were the most low-temperature tolerant HRM strain evaluated in this study, had individual ascospores t_v's expressed by right-skewed Pareto distributions at 12 and 14 °C. These are characterized by a shape and scale parameter (α, β): (4.59, 9) and (4.87, 6), respectively. These curves are similar to an exponential distribution, with its density decreasing from its mode in α at a rate β. In difference, the curves obtained for the *Byssochlamys* isolates were represented by logistic, normal and lognormal distributions (Table 3) and characterized by larger t_v ranges (Fig. 2). Whilst logistic curves are similar to normal curves, they distinguished by generating most likely values at extreme conditions (tails).

4. Discussion

Fungi have, in general, relatively simple nutritional requirements for growth. Dormancy is usually broken after the ascospores are exposed to an external trigger such as heat, high pressure or chemicals (Dijksterhuis, 2007; Wyatt, Wösten, & Dijksterhuis, 2013). The transition from a dormant to an active metabolic state is characterized by the degradation of compatible solutes, decrease in the viscosity of the cytoplasm and disruption of the thick cell wall, allowing nutrient uptake by the activated ascospores and initiation of the germination process. This comprises formation and elongation of the tube, followed by hyphal extension and branching (Burgain et al., 2013; Gougouli et al, 2013; Wyatt et al., 2013). The t_v is often defined as the time at which the diameter of the mycelium is equal to 2–3 mm, which corresponds to the lag time and the beginning of the linear growth (Gougouli, Kalantzi, Beletsiotis, & Koutsoumanis, 2011). Therefore, there is a trend to replace the lag time for t_v in studies aiming to predict fungal spoilage, as

Table 2
Estimated parameters of cumulative distributions fitted to individual times to growth (t_v, days) data obtained on aPDA (pH = 3.5) plates adjusted to different °Brix (44–59) stored at 30 °C up to 60 days. No growth means that no visible mycelium was observed in any of the replicates.

HRM	°Brix	a_w ± SD	Distribution	t_v (days) ± SD	5th percentile	95th percentile
N. hiratsukae	44	0.904 ± 0.003	RiskExpon(0,29641; RiskShift(5,99911))	6.3 ± 0.69	6	8
	47	0.902 ± 0.007	RiskExpon(0,65418; RiskShift(3,99811))	4.6 ± 1.07	4	7
	50	0.894 ± 0.007	RiskNormal(5,64211; 0,88313)	5.6 ± 0.88	4	6
	53	0.883 ± 0.005	RiskExpon(0,47244; RiskShift(5,99814))	6.5 ± 1.01	6	8
	56	0.869 ± 0.006	RiskExpon(2,9192; RiskShift(8,9705))	11.9 ± 3.57	9	20
	59	0.860 ± 0.002	No Growth	-	-	-
N. udagawae	44	0.904 ± 0.003	RiskExpon(0,47368; RiskShift(2,99644))	3.5 ± 0.73	3	5
	47	0.902 ± 0.007	RiskExpon(1,2256; RiskShift(2,9908))	4.2 ± 1.72	3	7
	50	0.894 ± 0.007	RiskExpon(1,8684; RiskShift(2,9902))	4.8 ± 1.67	3	9
	53	0.883 ± 0.005	RiskExpon(2,8246; RiskShift(4,9835))	7.8 ± 2.87	5	16
	56	0.869 ± 0.006	RiskLognorm(10,134; 3,2801; RiskShift(1,9221))	12.1 ± 3.3	9	20
	59	0.860 ± 0.002	RiskNormal(31,1053; 8,0195)	31.1 ± 8.01	20	45
N. laciniosa	44	0.904 ± 0.003	RiskExpon(1038; RiskShift(1,9934))	3.0 ± 1.26	2	7
	47	0.902 ± 0.007	RiskExpon(0,69427; RiskShift(2,99558))	3.7 ± 1.91	3	7
	50	0.894 ± 0.007	RiskExpon(0,88889; RiskShift(3,9924))	4.9 ± 1.59	4	8
	53	0.883 ± 0.005	RiskExpon(0,4661; RiskShift(5,99605))	6.5 ± 1.45	6	10
	56	0.869 ± 0.006	RiskWeibull(1,6185; 9,4512; RiskShift(11,5347))	20 ± 5.36	14	31
	59	0.860 ± 0.002	No Growth	-	-	-
N. fischeri	44	0.904 ± 0.003	RiskExpon(0,21698; RiskShift(2,99795))	3.2 ± 0.5	3	4
	47	0.902 ± 0.007	RiskExpon(1,1282; RiskShift(2,9904))	4.1 ± 2.19	3	12
	50	0.894 ± 0.007	RiskExpon(1,7699; RiskShift(2,9843))	4.8 ± 3.1	3	16
	53	0.883 ± 0.005	RiskExpon(1,3883; RiskShift(3,9865))	5.4 ± 2.64	4	9
	56	0.869 ± 0.006	RiskExpon(3,4066; RiskShift(9,9626))	13.5 ± 2.78	10	19
	59	0.860 ± 0.002	No Growth	-	-	-
B. nivea	44	0.904 ± 0.003	RiskExpon(2,7045; RiskShift(4,9385))	7.7 ± 2.20	5	12
	47	0.902 ± 0.007	RiskExpon(2,8966; RiskShift(6,9001))	9.9 ± 3.38	7	16
	50	0.894 ± 0.007	RiskExpon(2,1639; RiskShift(7,9645))	10.2 ± 2.58	8	16
	53	0.883 ± 0.005	RiskExpon(6,9211; RiskShift(14,8179))	21.9 ± 5.26	15	35
	56	0.869 ± 0.006	No Growth	-	-	-
	59	0.860 ± 0.002	No Growth	-	-	-
B. fulva	44	0.904 ± 0.003	RiskExpon(1,2286; RiskShift(1,9649))	3.2 ± 1.30	2	7
	47	0.902 ± 0.007	RiskExpon(0,63636; RiskShift(2,94215))	3.6 ± 1.28	3	7
	50	0.894 ± 0.007	RiskExpon(1; RiskShift(3,9804))	5.0 ± 2.11	4	10
	53	0.883 ± 0.005	RiskExpon(1,8103; RiskShift(5,9688))	7.8 ± 4.67	6	24
	56	0.869 ± 0.006	RiskExpon(1,1455; RiskShift(9,9792))	11.1 ± 3.93	10	20
	59	0.860 ± 0.002	No Growth	-	-	-

the last is of much more applicability for this type of approach (Berni et al., 2017; Burgain et al., 2013; Dantigny, 2016; Gougouli & Koutsoumanis, 2017; Santos, Chaves, & Sant'Ana, 2017).

Water activity (a_w) is the most important factor influencing fungal germination and outgrowth. As a result, this parameter has been assessed by the majority of available studies focused on HRMs growth (Berni et al., 2017; Panagou et al., 2010; Roland & Beuchat, 1984; Samson et al., 2010). However, while a_w is an useful parameter among food microbiologists, the effect of sugar concentrations (°Brix) is universally applied in the fruit processing industry. Despite this, scarce data are currently available regarding the effect of °Brix on the HRMs growth (Berni et al., 2017; Beuchat & Toledo, 1977).

Berni et al. (2017) recently determined the sugar concentration limiting conditions (°Brix) of *Neosartorya* strains (*N. hiratsukae, N. pseudofischeri, N. glabra*) on fruit-based media. The authors observed that *Neosartorya* spp. could tolerate sugar concentrations from 49 to 56°Brix, dependent on the species. These results were slightly below those observed in our study (53–59°Brix). In contrast to our findings, *N. hiratsukae* was only able to grow at Brix values ≤ 50°Brix in fruit-based media. This could be a result of differences in the media (aPDA vs. fruit based media) and strains used. Beuchat and Toledo (1977) investigated the behavior of *B. nivea* ascospores in fruit products supplemented with sucrose (20–60% soluble solids). The authors reported that the majority of fruit products containing 60% soluble solids did not support the growth of *B. nivea*, whilst visible mycelia were observed on fruit products with 40% sucrose after 3–13 days (at 30 °C) and 4–34 days (at 21 °C) (Beuchat & Toledo, 1977). In contrast to our results, Panagou et al. (2010) did not observe growth when *B. nivea* and *B. fulva* were inoculated on non-acidified Malt Extract Agar whose a_w had been adjusted with glycerol to values ≤ 0.88, regardless of the storage temperature (10-45 °C).

HRMs can be classified as thermo-tolerant microorganisms, i.e., which are able to tolerate (=to growth) temperatures as high as 45–50 °C and lower than 20 °C (Mouchacca, 2007; Panagou et al., 2010; Pitt & Hocking, 2009, pp. 1–519; Samson et al., 2010). In agreement with our results, *B. nivea* was inhibited in diverse fruit products incubated at 7 °C (Beuchat & Toledo, 1977). Whilst the majority of HRMs have not been reported to growth at chilled conditions, one of the evaluated strains, *N. hiratsukae*, was able to grow out at 7 °C after almost one month. Although much less attention is given to *N. hiratsukae* in literature compared to other HRMs, it has been isolated from soil, fruit, fruit juice, spoiled tea-based beverage and indoor environments (Berni et al., 2017; Samson et al., 2007; Santos et al., 2018).

It is known that t_v may be highly dependent on some factors, including media composition, temperature and inoculum size (Burgain et al., 2013; Dagnas et al., 2017; Valík & Piecková, 2001; Walker & White, 2005; Zimmerman et a., 2011; Zimmerman et al., 2013). Berni et al. (2017) observed shorter times for detection of visible mycelium of *Neosartorya* strains at lower °Brix values. As an example, while the visible growth of *N. glabra* took place after 6–8 days at 47–51°Brix, visible colonies were only detected after 20–38 days at 56°Brix. It has also been demonstrated in some studies that similar growth parameters are obtained when HRMs were inoculated on distinct (different types of) fruit matrices with the same a_w-values (Berni et al., 2017; Zimmerman et al., 2011; Zimmerman et al., 2013). This is contradictory to the findings of Beuchat and Toledo (1977) who observed discrepancies on the time required for growth when *B. nivea* ascospores were inoculated in different fruit juices and nectars with similar a_w-

Table 3
Estimated parameters of cumulative distributions fitted to the individual times to growth (t_v, days) data obtained on aPDA (pH = 3.5) plates stored at temperatures ranging from 7 °C to 14 °C up to 60 days. No growth means that no visible mycelium was observed in any of the replicates.

HRM	Temperature (°C)	Distribution	t_v (days) ± SD	5th percentile	95th percentile
N. hiratsukae	7	RiskExpon(4,4902; RiskShift(26,9707))	32 ± 4.05	27	40
	8	RiskExpon(4,2432; RiskShift(21,9771))	25 ± 3.88	22	33
	10	RiskExpon(4,1735; RiskShift(9,9787))	14 ± 3.91	10	22
	12	RiskPareto(4,5953; 9)	12 ± 6.13	9	27
	14	RiskPareto(4,8743; 6)	8 ± 2.46	6	10
N. udagawae	7	No Growth	-	-	-
	8	No Growth	-	-	-
	10	RiskExpon(2654; RiskShift(18,9908))	22 ± 3.34	19	30
	12	RiskExpon(2,1294; RiskShift(10,9875))	13 ± 3.28	11	19
	14	RiskExpon(0,93469; RiskShift(8,99618))	10 ± 3.24	6	10
N. laciniosa	7	No Growth	-	-	-
	8	No Growth	-	-	-
	10	RiskExpon(5,0704; RiskShift(21,9745))	27 ± 3.88	22	35
	12	RiskExpon(4,6838; RiskShift(11,9828))	17 ± 3.68	13	25
	14	RiskExpon(0,76891; RiskShift(8,99677))	10 ± 3.39	9	13
N. fischeri	7	No Growth	-	-	-
	8	No Growth	-	-	-
	10	RiskExpon(2,75; RiskShift(19,9596))	23 ± 3.08	20	29
	12	RiskExpon(4,1546; RiskShift(10,9572))	15 ± 4.43	11	24
	14	RiskExpon(0,90909; RiskShift(7,97934))	9 ± 0.86	8	11
B. nivea	7	No Growth	-	-	-
	8	No Growth	-	-	-
	10	No Growth	-	-	-
	12	RiskLogistic(40,9177; 3,7612)	40 ± 7.28	24	46
	14	RiskNormal(27,5886; 5,7337)	28 ± 5.73	18	35
B. fulva	7	No Growth	-	-	-
	8	No Growth	-	-	-
	10	RiskLognorm(6,7016; 3,0758; RiskShift(23,1762))	30 ± 3.40	27	38
	12	RiskNormal(22,6957; 2,5287)	23 ± 2.53	16	27
	14	RiskExpon(4,1075; RiskShift(7,9558))	12 ± 5.14	8	23

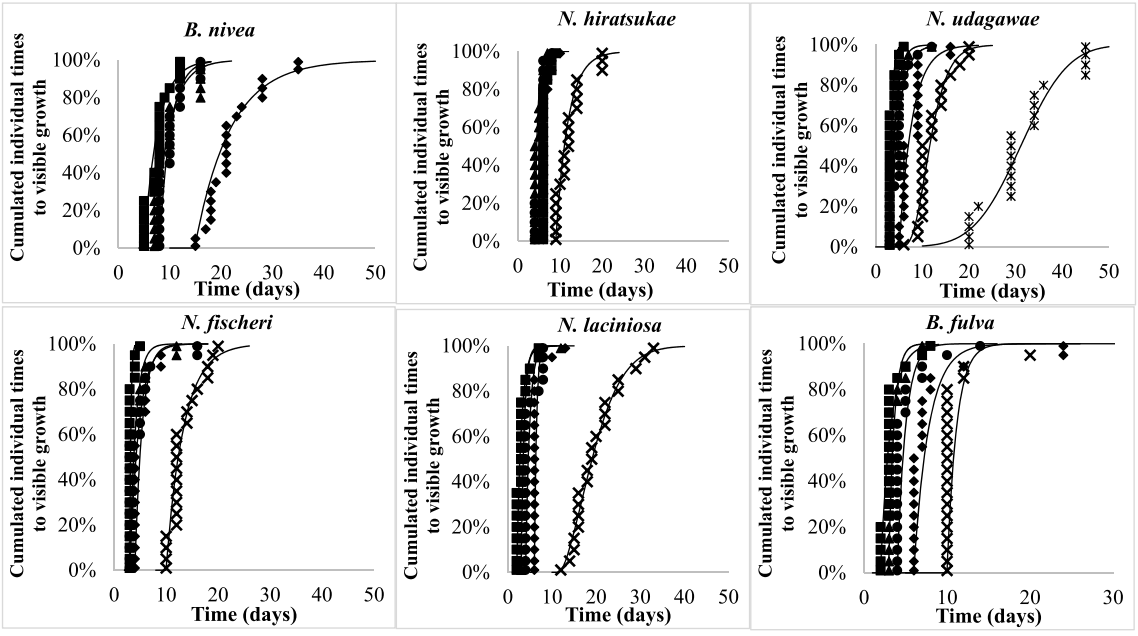

Fig. 1. Cumulated individual times to visible growth of spores of *Byssochlamys* and *Neosartorya* strains on acidified PDA (pH = 3.6) adjusted to 44°Brix (■), 47°Brix (▲), 50°Brix (●), 53°Brix (♦), 56°Brix (✕) and 59°Brix. (✳) at 30 °C.

values. As an example, when *B. nivea* was inoculated in apple juice (a_w = 0.92 and 40% sucrose) it was able to form visible mycelium after 17 days at 21 °C, while in grape juice it took only four days for the appearance of the first mycelium. In contrast, cranberry juice stored at 21 °C did not support the growth of HRMs when the °Brix was as low as 48.5 (a_w = 0.94) (Beuchat & Toledo, 1977). The lower values for maximum °Brix for HRMs growth observed in fruit-based medium compared to our results may be a result of compositional differences. Fruits contain phenolic compounds and organic acids that may have a significant effect on the outgrowth of HRMs (Amaeze, 2013; Beuchat & Toledo, 1977; Panagou et al., 2010; Valík & Piecková, 2001; Zimmermann, Massaguer, Maria, & Aragão, 2013).

The interval (spread) of individual t_v's corresponds to their natural biological variability and therefore cannot be reduced by increasing the number of performed experiments. Larger spreads of the t_v's of individual ascospores were observed as the conditions became more sub-

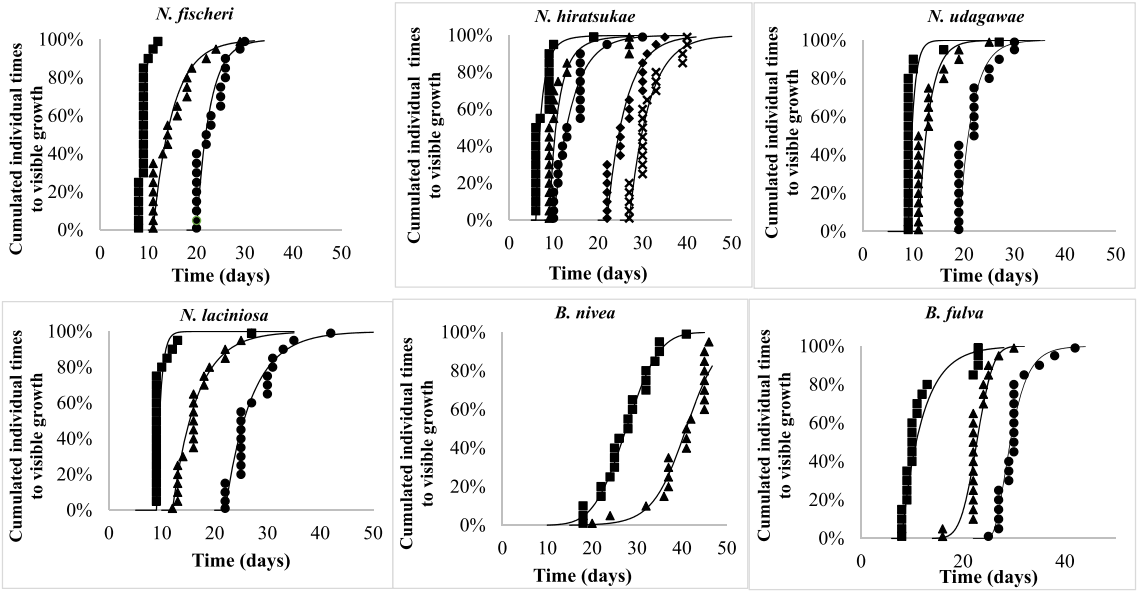

Fig. 2. Cumulated individual times to visible growth of spores of *Byssochlamys* and *Neosartorya* strains on acidified PDA (pH = 3.6) stored at 14 °C (■), 12 °C (▲), 10 °C (●), 8 °C (♦) and 7 °C (×).

optimal, suggesting that greater variability occurs when fungal growth is stressed. While no data are currently available regarding the biological variability of ascospore, such variability has been reported for individual heat sensitive spores (conidia) (Dagnas et al., 2015, 2017; Garcia et al., 2010; Gougouli & Koutsoumanis, 2013; Samapundo et al., 2007). Garcia et al. (2010) studied the effect of a_w and temperature on the growth of heat sensitive fungi at various inoculum size and observed that low inoculum levels and suboptimal conditions lead to high variability of estimated lag times. Dagnas et al. (2017) quantified the inhibitory effect of a_w and storage temperature on lag times of single spore of moulds isolated from spoiled bakery products and concluded that stressful conditions result in discrepancy between individual and population lag time. Likewise, Samapundo et al. (2007) assessed the effect of a_w and temperature on the individual lag times of moulds associated with the spoilage of maize and observed larger variabilities (spread) on growth parameters at suboptimum conditions. Nevertheless, uncertainties referring to possible variations within replicates may also have contributed to the spread of the t_v's.

The t_v was defined in this study as the time required for visual detection of mycelium, i.e., the time taken for the colonies to grow to a diameter ≥2 mm. This threshold has also been used by other authors (Zimmerman et al., 2013), while a threshold of 3 mm has been applied in other studies (Dantigny, 2016; Gibson, Baranyi, Pitt, Eyles, & Roberts, 1994; Gougouli et al., 2011; Valík & Piecková, 2001). It is worth mentioning that the t_v's observed in this study may underestimate the real time required before rejection time of fruit products. Despite the aPDA mimic low pH found in most of fruit products, it does not take into account the presence of other fruit compounds, such as organic acids and preservatives, reduced oxygen content and other stress factors which may potentially contribute to longer t_v's. Moreover, the t_v-values observed are much shorter than the typical shelf-lives of fruit products stored at ambient temperature of a few to several months. At the same time, it is important to emphasize that the ascospores inoculated in this study were not submitted to high intensities pasteurization (as done for pasteurized fruit products), which can sub-lethally injure the ascospores resulting in longer times to visible growth. Ultimately, the t_v's of large inoculums as used in this study (*ca.* 100 ascospores per plate), are mostly represented by ascospores with relatively short germination and lag times and may highly differ than contamination by one or a few spores (Burgain et al., 2013; Gougouli et al., 2011). Hence, it is important to validate such data in real fruit products. Nevertheless, our data represents worst case scenarios, which may be very useful for developing fail safe predictive (shelf-life) models.

5. Conclusions

The study of environmental factors (intrinsic and extrinsic) preventing the growth of spoilage microorganisms is crucial to maintain and/or increase the microbial stability of food products. In this study growth/no growth limits for temperature and °Brix were established for six different HRM species isolated from fruit and fruit based products. Moreover, the variability inherent of individual ascospores regarding their individual time to form visible growth was quantified and described as parametric statistics distributions. Large ranges of individual times to form visible mycelia were mainly observed under conditions at the growth/no growth regions. Ultimately the generated data will be used in predictive and microbial risk assessment studies aiming the prevention of the spoilage of fruit based products by HRMs.

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