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ORIGINAL ARTICLE

Modelling the effect of temperature, water activity and carbon dioxide on the growth of *Aspergillus niger* and *Alternaria alternata* isolated from fresh date fruit

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Abstract

Aims: To quantify and model the combined effects of temperature (T) (10–40°C), water activity (a_w) (0·993–0·818) and CO_2 concentration (9·4–55·1%, v/v) on the growth rate of *Aspergillus niger* and *Alternaria alternata* that cause spoilage during the storage and packaging of dates.

Methods and Results: The effects of environmental factors were studied using the γ-concept. Cardinal models were used to quantify the effect of studied environmental factors on the growth rates. Firstly, the cardinal parameters were estimated independently from experiments carried out on potato dextrose agar using a monofactorial design. Secondly, model performance evaluation was conducted on pasteurized date paste. The boundary between growth and no-growth was predicted using a deterministic approach. *Aspergillus niger* displayed a faster growth rate and higher tolerance to low a_w than *Al. alternata*, which in turn proved more resistant to CO_2 concentration. Minimal cardinal parameters of T and a_w were lower than those reported in the literature.

Conclusions: The combination of the a_w and CO_2 effects significantly affected *As. niger* and *Al. alternata* growth. The γ-concept model overestimated growth rates, however, it is optimistic and provides somewhat conservative predictions.

Significance and Impact of the Study: The developed model provides a decision support tool for the choice of the date fruit conservation mode (refrigeration, drying, modified atmospheric packaging or their combination) using T, a_w and CO_2 as environmental factors.

Introduction

The fruit of the date palm (*Phoenix dactylifera* L.) has considerable cultural and traditional significance in North Africa, the Middle East and in other Arabic countries in general. It has also been increasingly consumed in other parts of the world such as United States, Europe and Asia. The demand for date fruits is consequently increasing steadily. Worldwide date production has increased from 3 431 000 tonnes in 1990 to 7 505 000 tonnes in 2011 (FAOSTAT 2011). Date fruits have a high nutritive value and contain essential nutrients such as many classes of bioactive agents including polyphenols, carotenoids, sterols and vitamins (Baliga *et al.* 2011). The *Deglet-Nour* variety accounts for the majority of cultivated dates in North Africa. Most dates are consumed fully ripe as dried fruits. This stage allows a long shelf life due to a low water content (a_w = 0·60). The North African

populations prefer fresh *Deglet-Nour* dates, consumed at a semi-ripe stage, due to their attractive colour and very pleasant taste: sweet and tender with a higher water content (a_w = 0·80–0·85) rather than dry dates. However, fresh dates are easily contaminated with many biological hazards. Their sugar and water content, soft texture and thin skin make fresh *Deglet-Nour* dates very susceptible to spoilage by fungal development.

Fungi are the main cause of spoilage during the storage of date palm fruit. The fungal date spoilage species of the highest occurrence are *Aspergillus*, *Penicillium* and *Cladosporium* (Shenasi *et al.* 2002b; Al-Sheikh 2009). *Aspergillus niger*, *Aspergillus flavus* and *Aspergillus fumigatus* are among the *Aspergillus* species most commonly reported in date fruit (Shenasi *et al.* 2002b; Atia 2011); they display the highest occurrence at 39–83, 40–49 and 26–40%, respectively (Giridhar and Reddy 2001; Al-Sheikh 2009; Colman *et al.* 2012). The other main fungal spoilage species in date fruit are mould species of the genus *Alternaria* (*Al. alternata* 22–62% and *Al. clamydospora* 24%) (Giridhar and Reddy 2001; Al-Sheikh 2009), *Cladosporium* spp. and *Fusarium* spp.

The preservation of fresh dates during storage and marketing in Africa and Europe requires the control of the development of microbial spoilage flora. Limiting or inhibiting fungal development, taking into account environmental factors, should help to increase the shelf life of products without altering their organoleptic qualities.

The growth of moulds in foods is affected by environmental factors, in particular temperature, a_w, pH, solute concentrations, atmospheric composition and various compounds that stimulate or inhibit growth (Gibson and Hocking 1997). Models such as polynomial equations and the linear Arrhenius–Davey are used to describe the combined effect of temperature and a_w on the radial growth of *Fusarium verticilliodes* and *Fusarium proliferatum* on corn (Samapundo *et al.* 2005). Diverse secondary models were assessed by Yogendrarajah *et al.* (2016) for their ability to describe the growth rate of *As. flavus* and *Aspergillus parasiticus* as a function of individual and the combined effects of temperature and a_w. These authors reported that the extended Gibson model was the best model for describing the combined effect of temperature and a_w on the growth rate of both fungal species in peppercorns. Models associating the γ-concept with cardinal models, describing the influence of environmental factors, have been developed with some success (Emborg and Dalgaard 2008; Judet-Correia *et al.* 2010; Garcia *et al.* 2011). The γ-concept, introduced by Zwietering *et al.* (1992), is based on the principle that environmental factors affect the microbial growth rate independently. For the main environmental factors temperature, pH and a_w, γ functions were developed as cardinal models where the parameters of the model are the cardinal growth values: x_{min}, x_{opt}, x_{max} (Emborg and Dalgaard 2008). These models have been applied successfully to describe and quantify the effects of environmental conditions, temperature a_w and pH, on the development of moulds (Rosso and Robinson 2001; Sautour *et al.* 2001; Marín *et al.* 2009; Nevarez *et al.* 2009).

In 2008, Emborg and Dalgaard had presented a γ function that quantified the inhibitory effect of CO_2 on the growth of *Morganella psychrotolerans*, histamine-producing bacteria in fish. As far as we know, no study has been published on the modelling of the interaction between CO_2 concentration and a_w on mould growth rates using a deterministic approach (γ function with interaction term). On the other hand, few studies have reported the use of polynomial models to describe the CO_2 effect and its interaction with other factors on mould growth (El Halouat and Debevere 1997; Abellana *et al.* 2000; Guynot *et al.* 2003; Samapundo *et al.* 2007). However, Pateraki *et al.* (2007) reported the influence of sulphur dioxide, controlled atmospheres and a_w on the *in vitro* germination, growth and ochratoxin A production by *Aspergillus carbonarius* isolated from grapes. Temperature and a_w effects on the sporulation of *As. flavus* on an artificial substrate (Czapek Dox Agar) and maize stalks were also studied by Giorni *et al.* (2012). Diverse secondary models were assessed by Yogendrarajah *et al.* (2016) for their ability to describe individual and combined effects of a_w and temperature on the radial growth rate of *As. flavus* and *As. parasiticus* isolates in whole black peppercorns (*Piper nigrum* L.).

In North Africa, the *Deglet-Nour* dates marketed locally or intended for export are harvested dry or dried before packaging for good storage stability. They are packed in various types and sizes of packaging: different plastic trays, cardboard boxes, baskets, covered date bunches, etc. These types of package have much more marketing interest and mechanical protection than as a means of conservation or as a maturation-regulator concept such as modified atmosphere packaging (MAP). Dates with 20% moisture or lower can be kept at −18°C for more than 1 year, or at 0°C for up to 12 months, or at 4°C for up to 8 months, or at 20°C for 1 month (relative humidity (RH) should be kept between 65 and 75% in all cases) (Kader and Hussein 2009). The present study is focused on the *Deglet-Nour* date harvested before full maturity (*Rutab* stage), which is popularly consumed as a fresh date. It is available for a short period during the harvest season, because of its vulnerability to postharvest decay. The combination of CO_2 enriched packaging and storage at refrigerator temperature can be a promising technique

to inhibit fungal development, preserve freshness and extend the shelf life of dates. Indeed, the MAP inhibitory effect on the fungal growth is attributed not to the decrease in the O_2 concentration but to the CO_2 enrichment. It would also be interesting to evaluate the effectiveness of the combination of CO_2 enriched MAP (with O_2 concentration fixed at approx. 20%; O_2 concentration in air) and a_w without refrigeration effect (at room temperature). It has been demonstrated that fungal growth is not affected by reduced O_2 levels in a modified atmosphere, but a total inhibition of growth may occur in the strict absence of O_2 (Geysen *et al.* 2005; Pitt and Hocking 2009).

The aim of this work was to evaluate the influence of temperature, a_w and CO_2 on the mycelial growth of *As. niger* and *Al. alternata* isolated from fresh date fruit: (i) quantifying the influence of environmental conditions on mycelial growth kinetic parameters by fitting a mathematical model (primary and secondary modelling); (ii) assessing the combined effects of environmental conditions using the γ-concept; (iii) evaluating the cardinal models performance with kinetic parameters determined from experiments on food matrix (date paste).

Materials and methods

Isolation and identification of used strains

The spores used in this study were obtained from strains of *As. niger* and *Al. alternata* isolated from *Deglet-Nour* date fruit (*Phoenix dactylifera* L.) on potato dextrose agar (PDA) and identified by molecular methods and morphological and cultural observation. Date samples were collected from three different provinces (Biskra, Tolga and Doucen) in the southern region of Algeria during the peak of the harvest period. Total fungal counts for each province were carried out on PDA and the results were expressed as CFU per gram of fresh date, representing the total number of viable fungal spores on this fruit. DNA extraction from isolated fungal strains was carried out according to Karakousis *et al.* (2006) with modifications suggested by El Sheikha and Montet (2011). After mould DNA extraction, a fragment of the 28S rDNA gene approx. 260 bp was amplified by polymerase chain reactions (PCR) with U1 the eukaryotic universal primer and U2 the reverse primer. DNA amplicons were purified using the Wizard PCR Preps DNA purification system (Promega, Madison, WI) according to the manufacturer's instructions, and sent off for sequencing (GATC Biotech, Konstanz, Germany). The obtained sequences were compared with databases in the Genbank of the National Centre for Biotechnology Information using BLAST alignment software.

Inoculum preparation and inoculation

Only the strains (one strain of each species) isolated from date fruits of the Biskra province were studied. In fact, this province is characterized by an important agricultural dynamism particularly in the date-palm production sector. It is the most important province in terms of production (with 30% of national output) and the export of dates in Algeria (Benziouche and Cheriet 2012). Previously isolated cultures of *As. niger* and *Al. alternata* were cultured on PDA with 0·01% chloramphenicol at 25°C for 10 days to obtain heavily sporulating cultures. The spores were collected after washing the culture with sterile physiological saline containing 0·005% Tween 80 and gently scraping the surface with a glass rod. The suspension was filtered to remove hyphal fragments and aggregates. After estimating the spore concentration with a haemocytometer, the suspension concentration was adjusted with sterile physiological saline containing 0·005% Tween 80 to obtain a final spore concentration of 10^6 spores ml^{-1}. Then, Petri plates of PDA medium were inoculated centrally with 5 μl of the prepared spore suspension.

Experimental procedure and setup

The water activity of the PDA growth medium was modified by the addition of glycerol (substituting part of the water with glycerol) to reach a_w values from 0·993 to 0·818. The a_w of each modified medium was checked with an AquaLab Series 4TEV water activity meter (Decagon Devices, Inc., Pullman, Washington, DC). To study the effect of CO_2, the inoculated plates of *As. niger* and *Al. alternata* were enclosed in sealed plastic bags under vacuum using a Multivac C200 (Sepp Haggemuller KG, Wolfertschewenden, Germany). The plastic bags were composed of oriented polypropylene film coated with polyvinylidene chloride/polyethylene (PVdC/PE) (CFS Cellpack Packaging, Illfurth, France), with the following characteristics: O_2 permeability of 20 cm^3 m^{-2} day^{-1} at 23°C and 75% RH; CO_2 permeability of 80 cm^3 m^{-2} day^{-1} at 23°C and 75% RH; water vapour permeability of 3·8 g m^{-2} day^{-1} at 38°C and 90% RH. Three litres of the desired gas mixture was then injected into the sealed bag by means of a syringe needle through a silicone rubber septum stuck on the bag; the O_2 concentration was set at 20% (by reference to the O_2 concentration in air 20·95%) and different levels of CO_2 concentration in the headspace gas were obtained by reducing the N_2 concentration. Gas mixtures were prepared from pure gases (O_2, CO_2 and N_2) using a multi gas flow meter (model G; Aalborg Instruments, Orangeburg, NY). Gases were humidified at the desired a_w, by bubbling through one of

two saturated salt solutions: K_2SO_4 (a_w = 0·967 at 35°C) and $K_2Cr_2O_7$ (a_w = 0·980 at 25°C) for *As. niger* and *Al. alternate*, respectively. The humidified gas mixtures were introduced in to a jar equipped with a fan for stirring the mixture, before their injection in the bags. For each incubation condition, each plate was enclosed separately inside 3 l capacity plastic bags. Three to five replicate plates were used per treatment. The colony diameter was measured by taking pictures of the colonies with a digital camera. The pictures were analysed subsequently using ImageJ software (NIH, Bethesda, MD), and colony diameters were calculated by using the diameter of the Petri dish (d = 85 mm) as scale reference.

The modified atmosphere in this closed system (sealed bag) is considered constant (CO_2 concentration relative variation ± 3%), assuming that there are no significant variations in the gas concentrations of the headspace due to mycelial respiration and/or bag permeability. This hypothesis was verified by comparing some growth curves generated from this system with the growth curves generated from a device in which the temperature, RH and atmosphere were continuously stabilized (controlled system). Comparing the estimated growth rate of the two systems, there were no significant variations in the headspace of the closed system when growth is stopped at colony diameters of <70 mm. Therefore, only the closed system has been developed in the present study, and collected growth data of moulds must be limited to colony diameters of <70 mm. In addition, measurements of CO_2 and O_2 concentrations in the closed system during growth were made and are presented in Fig. 1. This figure shows a steady evolution of the CO_2 and O_2 in the bags, with the exception of two conditions (the most favourable for growth) for which significant increases in CO_2 and decreases in O_2 were noted. This demonstrates that the significant change in gas composition during experiments is not due to the gas permeability of the bags, but is due to fungal growth.

Experimental design

Experimental data sets corresponding to the studied environmental conditions were recorded using a monofactorial design (Baril *et al.* 2012), by altering one environmental factor at a time while maintaining the other factors set at constant values. Nine levels of growth media a_w from 0·993 to 0·818 were studied (Table 1) with incubation at 35 and 25°C for *As. niger* and *Al. alternata*, respectively, in natural air. The temperature values 35 and 25°C were retrieved from the literature (Sautour *et al.* 2001; Leong *et al.* 2006) assumed in our study as optimal values. The effect of temperature on growth rates was studied at eight temperatures ranging from 10 to 40°C (Table 1). Water activities of the plate media were prepared at 0·970 and 0·986 for *As. niger* and *Al. alternata* incubated in natural air. Six levels of CO_2 concentration were studied ranging from 9·4 to 55·1%. In the case of *As. niger* the incubation temperature was 35°C and the medium a_w was set at 0·970. Concerning *Al. alternata* the incubation temperature was 25°C and

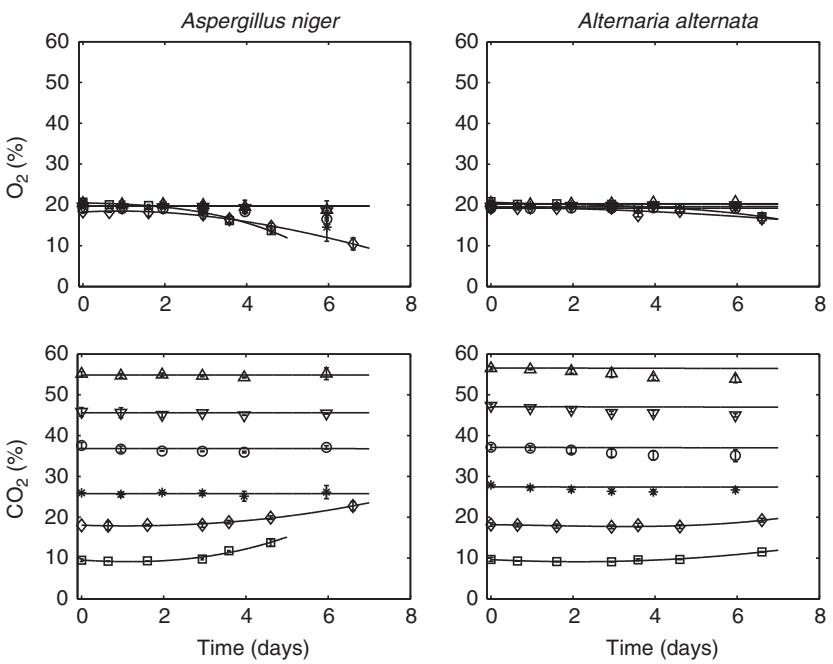

Figure 1 Evolution of headspace CO_2 and O_2 content in the polyethylene bags (closed system). Initial percentages of CO_2/O_2 (%, V/V) for *Aspergillus niger*: □ 9·5/20·6, ◊ 18·0/18·4, ✱ 26·0/20·65, ○ 37·6/19·1, ▽ 45·8/19·6 and △ 55·1/20·4. For *Alternaria alternata*: □ 9·7/20·8, ◊ 18·2/19·3, ✱ 27·9/20·2, ○ 37·2/19·2, ▽ 47·3/19·6 and △ 56·5/20·5. Error bars represent standard deviations (n = 3).

Table 1 Estimated growth rates (μ) of *Aspergillus niger* and *Alternaria alternata* at different temperatures, water activity and CO_2 concentrations. Mean values ± 95% confidence interval

	As. niger			*Al. alternata*		
Environmental factor	μ (mm day^{-1})	λ (days)	RMSE (mm)	μ (mm day^{-1})	λ (days)	RMSE (mm)
Temperature °C (air, 0·970 a_w for *As. niger* and 0·986 a_w for *Al. alternata*)						
10	1·1 ± 0·1	5·5 ± 0·5	0·1	3·7 ± 0·4	2·6 ± 0·3	1·1
15	5·3 ± 0·1	3·5 ± 0·3	0·9	5·5 ± 0·7	1·3 ± 0·3	1·3
20	11·6 ± 0·2	2·3 ± 0·3	1·2	9·6 ± 1·5	1·0 ± 0·2	1·9
25	17·3 ± 0·2	1·7 ± 0·2	0·9	10·8 ± 1·2	0·7 ± 0·3	2·9
30	23·1 ± 0·1	1·5 ± 0·2	1·4	9·6 ± 1·1	0·4 ± 0·2	2·6
35	25·5 ± 0·3	1·3 ± 0·3	1·0	5·8 ± 0·8	0·8 ± 0·3	1·6
37	21·9 ± 2·6	1·2 ± 2·1	4·3	4·3 ± 0·6	1·0 ± 0·3	1·3
40	0	ND	ND	ND	ND	ND
Water activity (air, 35°C for *As. niger* and 25°C for *Al. alternata*)						
0·993	14·5 ± 1·4	0·6 ± 0·1	3·1	11·2 ± 1·2	0·2 ± 0·1	2·8
0·979	25·4 ± 2·4	0·7 ± 0·1	4·3	11·6 ± 1·1	0·2 ± 0·2	3·1
0·952	28·2 ± 3·1	0·8 ± 0·1	4·3	8·4 ± 0·9	0·4 ± 0·2	2·1
0·928	21·4 ± 2·1	0·8 ± 0·1	4·0	5·6 ± 0·7	0·3 ± 0·2	1·5
0·908	15·8 ± 1·9	0·9 ± 0·2	2·8	3·7 ± 0·7	0·8 ± 0·2	0·9
0·871	12·8 ± 2·0	1·1 ± 0·5	2·6	1·4 ± 0·5	0·5 ± 0·5	0·6
0·852	6·3 ± 0·5	1·3 ± 0·2	3·0	0·5 ± 0·4	0·6 ± 0·8	0·6
0·828	3·3 ± 0·3	1·8 ± 0·3	1·6	0·4 ± 0·5	1·1 ± 1·0	0·6
0·818	0·4 ± 0·1	3·5 ± 0·3	0·1	0	ND	ND
CO_2 (%, v/v) (35°C and 0·970 a_w for *As. niger*, 25°C and 0·986 a_w for *Al. alternata*)						
9·4 ± 0·2	22·6 ± 1·7	0·5 ± 0·1	4·0	11·4 ± 1·1	0·8 ± 0·1	2·4
18·1 ± 0·5	16·2 ± 1·5	0·6 ± 0·2	3·7	8·5 ± 1·0	1·0 ± 0·2	1·8
26·3 ± 0·7	11·0 ± 1·3	0·9 ± 0·2	2·7	6·0 ± 0·9	0·9 ± 0·3	1·4
36·4 ± 0·8	7·2 ± 0·6	0·7 ± 0·2	1·7	4·1 ± 0·7	1·1 ± 0·3	0·9
45·7 ± 0·7	4·7 ± 0·6	0·9 ± 0·2	1·2	2·5 ± 0·4	1·0 ± 0·3	0·8
55·1 ± 0·8	3·2 ± 0·6	0·7 ± 0·3	1·1	1·9 ± 0·5	1·1 ± 0·5	0·7

RMSE, root mean squared error between experimental and estimated colony diameters; ND, not determined.

the medium a_w was set at 0·986. The assays were carried out in triplicate for an incubation period of 1 week.

In order to validate the growth/no-growth boundary prediction, for *As. niger*, 25 combinations of a_w (range from 0·80 to 0·88) and CO_2 concentrations (range from 45 to 65%, v/v) were tested. For *Al. alternata*, 36 combinations of a_w (range from 0·82 to 0·92) and CO_2 concentrations (range from 30 to 55%, v/v) were tested. The choice of combinations is based on the range of interest of the fresh *Deglet-Nour* a_w (at around 0·85 a_w). However, the CO_2 concentrations were empirically chosen on the basis of the selected a_w, in such a way to avoid combinations that are not located entirely on one area (growth or no-growth area). The experimental procedure detailed previously was carried out to generate data of the tested combinations; three replicates were made for each combination. The incubation time was 4 weeks for all tested conditions. The visible and not-visible colonies are reported as, respectively, growth and no-growth results, and then plotted with the predicted boundary.

Cardinal models performance

Date paste was prepared from organic *Deglet-Nour* dates, freeze-dried (LYOFAL, Salon de Provence, France) and homogenized in a blender (Waring, New Hartford, CT, USA) using liquid nitrogen. Date pastes with the desired a_w were prepared by adding the appropriate amounts of distilled water to the date powder, based on the sorption isotherm equation determined by Belarbi *et al.* (2000). They were pasteurized in heat-resistant-bags hermetically vacuum-sealed (forming a very fine layer <2 mm and with no water exchange) and then spread in Petri dishes. The Petri plates were then inoculated and conditioned in bags under different CO_2 concentrations as described above. The incubation conditions were (temperature, a_w, CO_2 (%, v/v)): (25, 0·86, 10); (25, 0·86, 0); (15, 0·92, 10); (15, 0·92, 0); (25, 0·90, 20); (25, 0·90, 30). The assays were carried out in triplicate for a maximum period of 6 weeks. The assays were carried out in triplicate for a maximum period of 6 weeks. The diameter of mycelial growth was measured as described previously.

Growth assessment and kinetic modelling

The diameter of the developed mycelia was measured along two perpendicular axes. The mean values of the diameters at any time t was noted $d^{(t)}$, and it was plotted against time and fitted to a two-phase linear model for the estimation of the growth rate μ (mm day^{-1}) and the lag time λ (days) (Gougouli and Koutsoumanis 2010).

$$d^{(t)} = \begin{cases} d^0 & \text{for } t \le \lambda \\ d^0 + \mu(t-\lambda) & \text{for } t > \lambda \end{cases} \quad (1)$$

where d^0 is the diameter of the inoculated spore suspension at $t = 0$ (d^0 = 4–5 mm). The combined effects of environmental factors (temperature, a_w and CO_2), was based on the γ-concept (Zwietering *et al.* 1996; Rosso and Robinson 2001):

$$\mu = \mu_{opt}\gamma(T)\gamma(a_w)\gamma(CO_2) \quad (2)$$

The cardinal model with inflection was adjusted as a secondary model to describe the effect of temperature and a_w on the fungal growth rate (Rosso *et al.* 1993; Sautour *et al.* 2001), whereas the simple cardinal model was adjusted for the effect of CO_2 (Ross and Dalgaard 2004). So, the γ function for the cardinal model with inflexion was

$$\gamma(X) = \frac{(X - X_{min})^2(X - X_{max})}{(X_{opt} - X_{min})[(X_{opt} - X_{min})(X - X_{opt}) - (X_{opt} - X_{max})(X_{opt} + X_{min} - 2X)]} \quad (3)$$

and the γ function for the simple cardinal model was

$$\gamma(CO_2) = \left(\frac{CO_{2\,max} - CO_2}{CO_{2\,opt} - CO_{2\,max}}\right)^2 \quad (4)$$

where X is the studied T or a_w factor; μ_{opt} is the growth rate at optimal environmental factors X_{opt} and $CO_{2\ opt}$; X_{max} and $CO_{2\ max}$ are the level of the factor above which no-growth occurs; X_{min} the level of the factor below which no-growth occurs; here $X_{max} = 1$ for a_w. Because the absence of CO_2 is optimal for fungal growth, the $CO_{2\ opt}$ value is set at 0% (Ross and Dalgaard 2004). In order to take into account interactions between factors, the term ξ (T, a_w, CO_2) describing the quantitative effects of the interactions on fungal growth rate was introduced (Le Marc *et al.* 2002). Then μ can be calculated as follows:

$$\mu = \mu_{opt}\gamma(T)\gamma(a_w)\gamma(CO_2)\xi(T, a_w, CO_2) \quad (5)$$

with

$$\xi(T, a_w, CO_2) = \begin{cases} 1 & , \Psi \le 0{\cdot}5 \\ 2(1-\Psi) & , 0{\cdot}5 < \Psi \le 1 \\ 0 & , \Psi > 1 \end{cases} \quad (6)$$

Ψ being defined as follows:

$$\psi = \sum_i \frac{\varphi_{e_i}}{2\prod_{j \ne i}(1 - \varphi_{e_j})} \quad (7)$$

where φ_{e_i} or φ_{e_j} are the contributions to the interactions of the environmental factors. Contribution of temperature, a_w and CO_2 are assumed to be (Zuliani *et al.* 2007):

$$\varphi_T = [1 - \gamma(T)]^3, \varphi_{a_w} = [1 - \gamma(a_w)]^3 \text{ and } \varphi CO_2 = [1 - \gamma(CO_2)]^2 \quad (8)$$

Parameter estimation and statistical methods

Parameters for fitting eqn (1) were calculated using linear regression with a curve fitting toolbox (MATLAB 6.5; The Math-Works Inc., Natick, MA). For Eqn (2), before parameter identification, μ was transformed by square root of μ to improve the homogeneity of the variance. These parameters were iteratively adjusted to the goodness-of-fit merit function using the 'fminsearch' function of MATLAB software (ver. 6.5; Math-Works). This merit function was the root mean squared error (RMSE) between all experimental and predicted data. The RMSE was chosen as a goodness-of-fit indicator for the estimated parameters. Because the model was non-linear for the parameters, the confidence interval for each parameter was determined via Monte Carlo simulations (Hessler 1997) with 2000 simulations for each kinetic parameter identification.

Growth/no-growth boundary prediction

The boundary between growth and no-growth of *As. niger* and *Al. alternata* was defined as the transition between the growth domain in which $\mu > 0{\cdot}001$ mm day^{-1} and no-growth domain $\mu \le 0{\cdot}001$ mm day^{-1}. This criterion (0·001 mm day^{-1}) has been selected due to the fact that the model does not converge for $\mu = 0$ mm day^{-1} and there was no significant difference between boundaries calculated from $\mu <$ mm day^{-1}, as the contour plots were superimposed. The boundary was determined using a deterministic approach (Le Marc *et al.* 2002). To predict the boundary between the growth and no-growth domains, Eqn (5) was used with the temperature factor fixed at 25 and 35°C for *Al. alternata* and *As. niger*, respectively. The experimental design for the validation of predicted boundaries was described previously.

Model performance on date paste

For each combination of temperature, a_w, and CO_2 concentration used to evaluate model performance, μ_{opt} on date paste was determined and the mean $\bar{\mu}_{opt}$ was calculated (Judet-Correia *et al.* 2010). The accuracy of A_f and the bias B_f factors proposed by Ross (1996) were calculated as follows:

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

$$B_f = 10^{\left(\sum \log(\mu_{predicted}/\mu_{observed})/n\right)} \quad (10)$$

The predicted growth rates $\mu_{predicted}$ were calculated from Eqn (5) using $\bar{\mu}_{opt}$. n is the number of observations.

Results

Identification of the fungal strains

Fresh dates from the Biskra, Tolga and Doucen Algerian provinces yielded total fungal population counts of $2{\cdot}6 \pm 0{\cdot}7$, $2{\cdot}4 \pm 0{\cdot}9$ and $2{\cdot}8 \pm 0{\cdot}8$ $\log_{10}$ CFU g^{-1} of fresh date (standard deviation with $n = 5$, n being the population counted on date samples from one date palm; date weight $13{\cdot}5 \pm 2{\cdot}1$ g, average mass from 30 dates). Three morphologically distinct types of colonies were found in the counted populations, two types of mould and one type of yeast (results not reported). Comparison of the obtained sequences of isolated moulds with databases in the Genbank yielded a > 95% sequence identity with *As. niger* (AB573988.1) and *Al. alternata* (GU048607.1). Both strains were present in the date samples collected from each of the three provinces.

Primary modelling: growth as a function of time

Over the period delimited for measurement, growth was linear after a lag phase defined as the period during which the colony diameters $d^{(t)}$ remained equal to the initial diameter d^0 = 4–5 mm (corresponding to the diameter of the drop of spore suspension deposited on the agar), as shown in Fig. 2. The estimated growth rate (μ) values at different temperatures, water activity and CO_2 concentrations are listed in Table 1. The RMSE values (1·69 mm in average for all trials) proved the goodness-of-fit of the two-phase linear model, indicating that the predictions describe the experimental data satisfactorily. Considering the estimates of μ parameters, the growth of *As. niger* was significantly faster than that of *Al. alternata* in the same tested conditions of temperature ($T \geq 20$°C), a_w and CO_2.

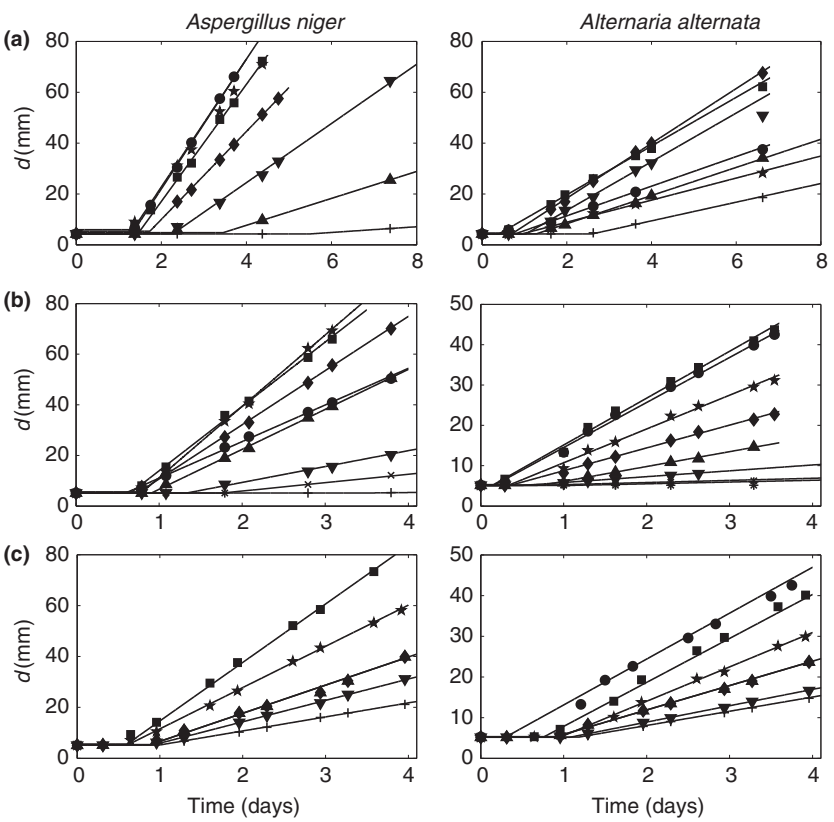

Figure 2 Experimental (symbols) and simulated (solid lines) growth diameter $d^{(t)}$ kinetic of *Aspergillus niger* and *Alternaria alternata* at different temperatures (a: + 10, ▲ 15, ▼ 20, ♦ 25, ■ 30, ● 35, and ★ 37°C; incubated in air/0·970 a_w and air/0·986 a_w for *As. niger* and *Al. alternata* respectively), water activities (b: ● 0·993, ■ 0·979, ★ 0·952, ♦ 0·928, ▲ 0·908, ▼ 0·871, × 0·852, + 0·828 and ✱ 0·818; incubated in air/35°C and air/25°C for *As. niger* and *Al. alternata* respectively) and CO_2 concentrations (c: ■ 9·4, ★ 18·1, ♦ 26·3, ▲ 36·4, ▼ 45·7 and + 55·1%; incubated in 35°C/0·970 a_w and 25°C/0·986 a_w for *As. niger* and *Al. alternata* respectively).

Secondary modelling: effect of temperature, a_w and CO_2 on the growth rate

The growth rate (μ) for both fungal strains decreased when the temperature and a_w dropped away from their optimal values, and continuously decreased with increasing CO_2 concentration. Equation (2) (based on the γ-concept), which couples the cardinal model with inflexion describing the temperature and a_w effect, and the simple cardinal model describing CO_2 concentration effect, was successfully adjusted to the experimental data. The goodness-of-fit of Eqn (2) is expressed by the low RMSE values reported in Table 2 (0·41 mm day^{-1} for *As. niger* and 0·29 mm day^{-1} *Al. alternata*), and the model fitting shown in Fig. 3. The estimated parameters of the environmental factors tested (minimum, optimum and maximum values) in the secondary cardinal model are presented in Table 2. *Aspergillus niger* grows at a faster rate (μ_{opt} = 28·6 ± 2·5 mm day^{-1}) and was more tolerant to a low a_w ($a_{w\ min}$ = 0·786 ± 0·006) than *Al. alternata* (μ_{opt} = 12·1 ± 1·4 mm day^{-1}, $a_{w\ min}$ = 0·808 ± 0·004), whereas *Al. alternata* was more resistant to high CO_2 concentrations than *As. niger*, with respective maximal CO_2 concentrations of 95·1 ± 6·8% and 79·8 ± 4·0%. These maximal cardinal values of CO_2 concentrations were derived by fitting and are extrapolations, since the experimental range of CO_2 concentration did not exceed 55%. Also, the confidence intervals of the two $CO_{2\ max}$ are large and take into account the quality and number of experimental data. All tested CO_2 concentrations in the bag headspace had a significant effect on the growth of both

Table 2 Estimated cardinal growth parameters of *Aspergillus niger* and *Alternaria alternata* on PDA. Mean values ± 95% confidence interval

Parameter	*As. niger*	*Al. alternata*
μ_{opt} (mm day^{-1})	28·6 ± 2·5	12·1 ± 1·4
T_{min} (°C)	4·4 ± 1·5	1·6 ± 3·4
T_{opt} (°C)	34·2 ± 1·5	26·1 ± 1·3
T_{max} (°C)	39·0 ± 1·1	39·4 ± 0·9
$a_{w\ min}$	0·786 ± 0·006	0·808 ± 0·004
$a_{w\ opt}$	0·958 ± 0·006	0·988 ± 0·008
$a_{w\ max}$*	1·000	1·000
$CO_{2\ opt}$* (%)	0·0	0·0
$CO_{2\ max}$ (%)	79·8 ± 4·0	95·1 ± 6·8
RMSE (mm day^{-1})	0·4	0·3

RMSE, root mean squared error between experimental and simulated μ data; PDA, potato dextrose agar.
*Set values.

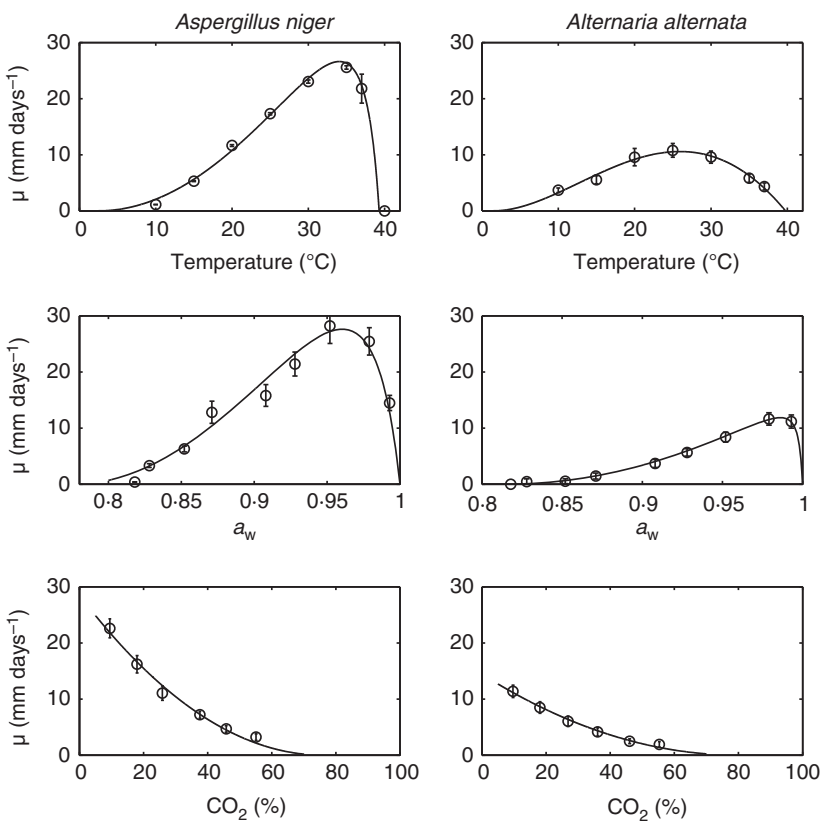

Figure 3 Growth rate (μ) of *Aspergillus niger* and *Alternaria alternata* as a function of temperature (incubated in air/0·970 a_w and air/0·986 a_w for *As. niger* and *Al. alternata* respectively), water activity (incubated in air/35°C and air/25°C for *As. niger* and *Al. alternata* respectively) and CO_2 concentration (incubated in 35°C/0·970 a_w and 25°C/0·986 a_w for *As. niger* and *Al. alternata* respectively). Solid lines are data simulated using a cardinal model with inflection describing the effect of water activity and temperature, and a simple cardinal model describing the effect of CO_2 concentration. Error bars represents 95% confidence interval of estimated growth rate.

strains. In *As. niger* and *Al. alternata*, respectively, μ was reduced by 49 and 53% when exposed to about 26% CO_2, up to 86 and 83% in approx. 55% CO_2. It must be noted that at a high CO_2 concentration (55%) the colonies of *As. niger* were white, compact and wrinkled, with a light brown pigmentation when observed in reverse plates. *Alternaria alternata* colonies became white and cottony, with some acquiring a greenish tinge in the centre.

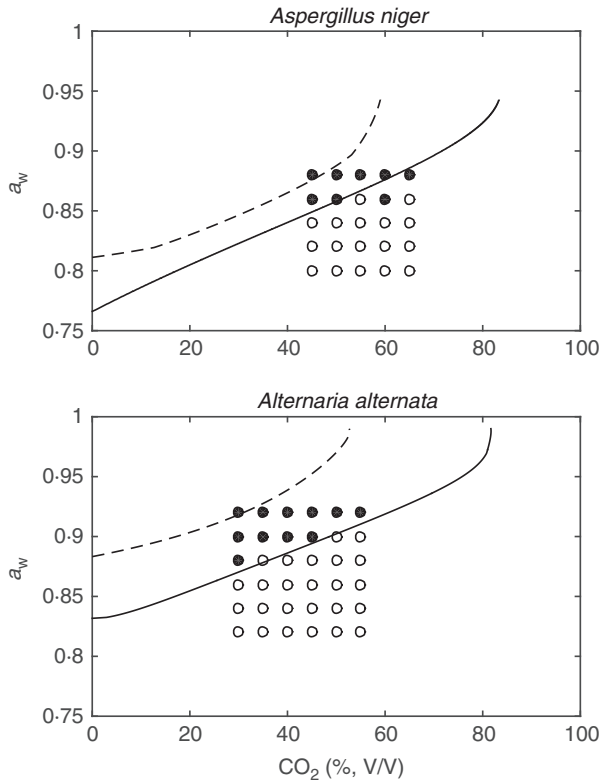

Figure 4 Growth/no-growth interface of *As. niger* and *Al. alternata* (as a function of CO_2 concentration and a_w. Comparison of the observed data for growth (●) and no-growth (○) with the predicted boundary as determined by eqn (5) (- - -: $\mu = 0{\cdot}1$ mm day^{-1}; —: $\mu = 10^{-3}$ mm day^{-1}).

Growth/no-growth interface

The interface defined by the interaction model (Eqn 5) was compared with the new experimental observations of growth/no-growth in Fig. 4. So, the model we used correctly describes the interactions between a_w and CO_2 concentration effect, as well as the growth/no-growth boundaries, in both *As. niger* and *Al. alternata.* In the case of *Al. alternata*, all the experimental observations were correctly predicted, and only two observations were wrongly predicted among all experimental observations regarding *As. niger* (growth at 0·86 a_w/60% CO_2 and at 0·88 a_w/65% CO_2).

Cardinal models performance

The evaluation of cardinal models performance was carried out directly in pasteurized *Deglet-Nour* date paste. The bias (B_f) and the accuracy (A_f) factors were calculated from Eqn (5) (model with interaction term ξ) for both strains with six conditions. B_f is 1·18 and 1·03, and A_f is 1·72 and 1·23 for *As. niger* and *Al. alternata*, respectively. B_f value was close to 1 for *As. niger*, and greater than 1 for both strains, indicating that, in general, the γ-concept model predicted slightly higher growth rates values than the observed ones. The A_f value was greater than 1 for both strains, and a large value was obtained for *As. niger*. The A_f values indicate, on average, the predictions deviated from the observations by 72% for *As. niger* and by 23% for *Al. alternata*. The graphical comparison of the observed and predicted growth rates of the two strains is presented in Fig. 5.

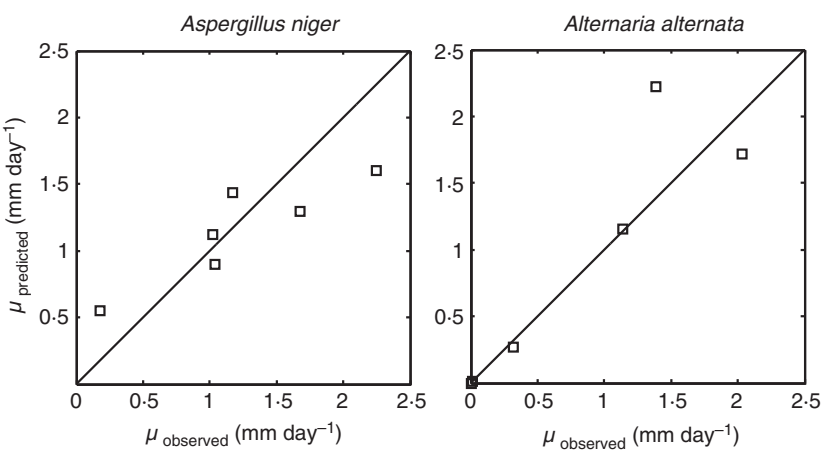

Figure 5 Comparison of predicted growth rates of *Aspergillus niger* and *Alternaria alternata* using eqn 5, and those observed on date paste at 6 different conditions of temperature, a_w and CO_2 concentration.

Discussion

The total mould counts in fresh *Deglet-Nour* dates were lower than the mould concentrations determined by Shenasi *et al.* (2002a) and Habibi Najafi and Haddad Khodaparast (2009) on other fresh date varieties, but were similar to the findings of Moore *et al.* (2002). *Aspergillus niger* and *Al. alternata* were the only mould species isolated from the samples of fresh *Deglet-Nour* date. However, other mould spoilage species can be present on date fruit, such as *Penicillium*, *Cladosporium* (Hamad 2008), *Rhizopus* and *Fusarium* (Gherbawy *et al.* 2012). Our fresh *Deglet-Nour* date samples having been collected directly from the date palm in sterile conditions, *As. niger* and *Al. alternata* species may have been present in date fruits beforehand. The Djerbi (1983) FAO report stated that *Aspergillus* spp. and *Alternaria* spp. are major causative agents of date fruit spoilage.

The cardinal models have been satisfactory applied to describe the effect of these factors on the growth of both strains. The cardinal parameter values can be compared with data published in the literature, except the T_{min} for *As. niger* and the $a_{w\ min}$ for *Al. alternata* which were significantly lower. The estimated T_{min} (4·0 ± 1·6°C) was lower than values reported in the literature (6–10°C) (Pitt and Hocking 2009; Gougouli and Koutsoumanis 2010; Astoreca *et al.* 2012). However a T_{min} value of 4·0 ± 1·1°C was estimated by Gougouli and Koutsoumanis (2012) using a lag time for germination as the kinetic response. A higher μ value (1·13 ± 0·04 mm day^{-1}) was observed at 10°C for *As. niger*, compared with that of Bellí *et al.* (2004) (0·14–0·61 mm day^{-1} at 0·98 a_w). The T_{max} (39·8 ± 1·1°C) and T_{opt} (34·2 ± 1·4°C) were relatively close to values given by some authors: T_{max} = 40–43·1°C and T_{opt} = 30–35°C (Sautour *et al.* 2001; Vats and Banerjee 2002; Leong *et al.* 2006; Pitt and Hocking 2009; Gougouli and Koutsoumanis 2010; Astoreca *et al.* 2012). With regard to *Al. alternata* the estimated T_{opt} (26·1 ± 1·3°C) was in agreement with the values determined experimentally (25·0°C) by Sautour *et al.* (2001) and Oviedo *et al.* (2011). The estimated $a_{w\ opt}$ (0·988 ± 0·008) and $a_{w\ min}$ (0·808 ± 0·004) were, respectively, close to and clearly higher than the values found in the literature (0·980–0·987 and 0·883–0·922) (Sautour *et al.* 2001; Torres *et al.* 2003; Pose *et al.* 2009; Oviedo *et al.* 2011). In the case of *As. niger*, $a_{w\ opt}$ and $a_{w\ min}$ values (0·958 ± 0·006/0·786 ± 0·006) were slightly different compared with those estimated by Parra and Magan (2004) (0·97/0·80) and those reported by Tassou *et al.* (2007) (0·962 ± 0·004/0·826 ± 0·037). Although *Al. alternata* is known to be a non-xerophilic fungus, both strains were able to grow at a_w < 0·85 (xerotolerant behaviour) when temperatures and CO_2 factors are at their optimum level for growth. This behaviour is probably due to the environmental nature on which the studied strains were isolated: a_w of dates and the arid ecosystem of the sampling provinces. Indeed, water activity in date fruits varies from about 0·65 (dry-date) to 0·85 (soft-date such as fresh *Deglet-Nour*). It is important to emphasise that the water activity of fresh dates exceeds the reported $a_{w\ min}$ values. Thus, the low a_w of fresh dates is not enough to prevent fungal spoilage, hence the interest in including an inhibitory environmental factor such as CO_2.

The few studies published that deal with the modelling of the CO_2 effect on fungal growth mostly used polynomial modelling. Using a quadratic polynomial equation, Samapundo *et al.* (2007) studied the influence of modified atmospheres and their interaction with a_w on the growth of *Fusarium verticillioides* and *F. proliferatum* on corn. They observed that both a_w and CO_2 concentration had significant and synergistic effects on growth, but a_w had the largest effect. The use of a multivariate statistical method (PLS) revealed that a_w, CO_2, and their interaction were the main factors significantly affecting fungal growth (Abellana *et al.* 2000; Guynot *et al.* 2003). It should be noted, however, that no cardinal modelling approach was found in the literature describing the effect of CO_2 on fungal growth, although the effect of CO_2 on bacterial growth has been described using a cardinal model (Ross and Dalgaard 2004), applied to the growth of *Morganella psychrotolerans* (Emborg and Dalgaard 2008). In our study, the CO_2 cardinal model was applied successfully to the fungal growth rate, as shown in Fig. 3 (bottom). For the two fungal species studied, the $CO_{2\ max}$ concentration can be compared with the $CO_{2\ max}$ values given for other species in other studies. The growth of *Eurotium chevalieri* and *Xeromyces bisporus* was totally inhibited in an 80% CO_2/20% O_2 atmosphere, but only delayed in the case of *Fusarium oxysporum*, *Aspergillus flavus*, *Penicillium commune* and *Byssochlamys fulva* (Taniwaki *et al.* 2010). El Halouat and Debevere (1997) found that 80% CO_2 and 20% N_2 or 60% CO_2 and 40% N_2 completely inhibited germination on plates of *Eurotium amstelodami* and *F. oxysporum*, but similar conditions did not prevent the germination and growth of *As. niger* and *Penicillium chysogenum*.

The benefits of including interactions between environmental parameters to predict the bacterial growth/no-growth boundary has been underlined in many studies (Augustin 1999; Le Marc *et al.* 2002; Mejlholm and Dalgaard 2007; Zuliani *et al.* 2007). Cairns-Fuller *et al.* (2005) highlighted a clear interaction between a_w and CO_2 concentration effects on fungal growth: for *Penicillium verrucosum* on wheat grain at the optimum a_w (0·95 a_w) and 1% of O_2 concentration, growth rates decreased to 40% when the mould was exposed to a 25% CO_2 atmosphere and by 75% in 50% CO_2. At 0·90 a_w and

50% CO_2, the growth rate was reduced by 90%. In this study, only the interactions between a_w and headspace CO_2 concentration were studied, however, the temperature was set at 35 and 25°C for *As. niger* and *Al. alternata*, respectively. This choice was made in order to evaluate the possibility of storage at room temperature. Given the reality that room temperature can vary considerably, fixing the temperature factor at the most favourable level to growth was required to be the 'worst case scenario'. Any rise in a_w needs to be compensated for by an increase in the CO_2 concentration in order to reach the growth/no-growth limit (Fig. 4). For example, for *As. niger*, when increasing a_w from 0·82 to 0·85, the CO_2 concentration needs to be stepped up from 28 to 46%, i.e. an 18% increase. For *Al. alternata*, an increase of 18% in CO_2 (from 30 to 48%) would be necessary for the same increment in a_w (0·03), i.e. an increase from 0·87 to 0·90.

The evaluation of the cardinal models performance was conducted directly on reconstituted freeze-dried date instead of the whole fruit. This matrix model is a real food matrix that enables moulds to be grown on a homogeneous and flat substrate with the desired a_w. Determining the growth rate on date paste was a way to include the possible effects of environmental factors and their interactions, which were not taken into account in this study carried out on PDA media. The prediction quality of the γ-concept model improved when the interaction term ξ was added. Indeed, the calculated B_f and A_f values from Eqn (5) (with ξ) were respectively lower and closer to 1 compared with those calculated from Eqn (2) (without ξ). The obtained B_f and A_f values indicate that the model was optimistic and provides somewhat conservative predictions (Ross 1996); it predicts better the growth rates for *Al. alternata* than *As. niger*. Figure 5 shows that data points for *As. niger* are distributed around the diagonal line. However, the used model significantly underestimate the growth rate on date paste under the conditions at 25°C/0·90 a_w/20% CO_2, and overestimate it at 15°C/0·92 a_w/10% CO_2. For *Al. alternata*, the dispersion of data points was less, except the point at 15°C/0·92 a_w/00%, CO_2 having a clear deviation from the diagonal line, suggesting that the model could be considered relatively suitable for the prediction of *Al. alternata* growth.

The models developed in the present study can contribute to assessing the risk of fungal spoilage and predicting the shelf life of fresh *Deglet-Nour* dates. In fact, an arbitrary parameter 'rejection-time' (i.e. the time required for a mycelium to be visible on a contaminated date) can be used to express the shelf life of this fragile fruit (Dantigny *et al.* 2006; Gougouli *et al.* 2011). It should be noted that the lag time duration is a significant part of the total rejection time. However, because of the difficulty in the development of secondary models to predict λ, only growth rates were modelled in our study; the influence of the physiological state of the inoculum causing wide variability of the estimated λ. Gougouli and Koutsoumanis (2010) reported that the product $\mu \times \lambda$ was relatively constant for the growth of *Penicillium expansum* and *As. niger* at the different tested temperatures. In our study, the product $\mu \times \lambda$ was found to be relatively constant (low correlation between μ and $1/\lambda$) for each studied factor. This approach simplifies the modelling of lag time and the growth rate by a single growth model. The effect of the inoculum size on the lag time (Baert *et al.* 2008; Gougouli *et al.* 2011) and the relation between the germination time and the lag time of mycelial growth of individual spores (Gougouli and Koutsoumanis 2013) must be taken into consideration for an effective shelf life evaluation of this fruit.

Aspergillus niger and *Al. alternata* are moulds responsible for the post-harvest decay of date fruit. Both were isolated from fresh *Deglet-Nour* dates. Our results show a significant effect of temperature (T), water activity (a_w) and CO_2 concentration on the growth rate of both strains. The deterministic approach (Le Marc *et al.* 2002) described the boundary between the growth and no-growth in both strains regarding the combined effect of a_w and CO_2 concentration. The advantage of using γ-concept with the interaction term ξ is that it does not require additional experimentation to estimate growth/no-growth boundary. In addition, it is defined only by the cardinal parameters already determined that have a simple microbiological significance. Under the conditions tested on date paste, the γ-concept model was more efficient for growth rate predictions of *Al. alternata* compared with those of *As. niger*. However, the predictions were optimistic for both strains. The results of the models developed could be applied advantageously to optimize quality preservation using combinations of T, a_w and CO_2 in packaging in such a way that the growth of *As. niger* and *Al. alternata* is inhibited.

Conflict of Interest

No conflict of interest to declare.

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