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HEAT RESISTANCES OF *CANDIDA APICOLA* AND *ASPERGILLUS NIGER* SPORES ISOLATED FROM DATE FRUIT SURFACE

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ABSTRACT

Candida apicola and *Aspergillus niger* strains were isolated and identified from *Deglet-Nour* dates, and their loads were determined on the fruit surface. The thermal inactivation of their spores was evaluated between 42 and 70C. The estimated D values for *C. apicola* and *A. niger* were $D_{50} = 2.3 \pm 0.4$ min and $D_{50} = 22.1 \pm 2.1$ min, respectively. The z_T values obtained were 9.4 ± 2.6 and 9.6 ± 1.0C for *C. apicola* and *A. niger*, respectively. The experimental survival spores obtained on the date surface during non-isothermal treatment were compared with the simulated data based on the Bigelow method and on pasteurization value calculations. *C. apicola* and *A. niger* spores can be inactivated by relatively low temperatures. Validation shows that this model and its parameters can be used to optimize pasteurization of these fungal spores on the surface of fruits using the temperature–time profile data. These thermal treatments can pasteurized date surface while minimizing the color date degradation.

PRACTICAL APPLICATIONS

This study contributes to the design and control of an effective heat treatment against the fungi that cause particular safety and spoilage problems during the preservation and packaging of fragile fresh fruits such as fresh dates.

INTRODUCTION

In high sugar content dried fruit, the higher the water content, the more pleasant the taste. This is the case for dates with intermediate moisture (water activity close to 0.8) and soft flesh. This product has a fresh fruit taste. Their preservation at room temperature (or at 4C) requires sealed packaging to prevent development of microorganism. Date fruits could contain harmful spoilage microorganisms even when protective measures are taken during the cropping stage. In addition, some fungal strains isolated from dates other than the *Deglet-Nour* variety are reported to produce ochratoxin A. The genera with the highest occurrence are *Aspergillus*, *Penicillium* and *Cladosporium* (Ahmed *et al.* 1997; Ragab *et al.* 2001; Shenasi *et al.* 2002b). Processes that can control harmful microorganisms and delay deterioration of fresh fruit include washing, coating, chemical formulation, heat/cooling treatements, high pressure water, partial vaccum and modified atmosphere packaging (MAP). The combination of different preservation techniques such as heat treatment, MAP and cold storage (Rosnes *et al.* 2007) is the most promising approach.

Postharvest heat treatment helps to control pathogen levels and insect pests and to prevent fungal rot (Ferguson *et al.* 2000). It can be a cost-effective alternative to the postharvest application of chemical treatments (Lurie 1998). Currently, it is widely used commercially for the quality control of fresh fruits (Ferguson *et al.* 2000). Air heating alone provided a satisfactory level of disinfestation of Zeller larvae inside dates while limiting quality damage (Ben-Lalli *et al.* 2011).

However, a precise knowledge of the inactivation of fungal spores is very important for designing the thermal treatments that will effectively ensure food safety and food

quality. In almost every standard food technology handbook, the decrease in number of microorganisms is based on the assumption of first-order kinetics.

Heat-resistant fungal spores ranging from $D_{50} = 0.9$ min for *Monilinia fructigena* to $D_{90} = 6.2$ min for *Talaromyces flavus* are mentioned in the literature (Török and King Jr 1991; King 1997; Kotzekidou 1997; Marquenie *et al.* 2002; Shearer *et al.* 2002; Salomão *et al.* 2007; Sant'ana *et al.* 2009). Thermal processes (pasteurization or sterilization) reduce the microbial flora by exposing the microorganisms to high temperatures, but are also liable to affect product quality. In this respect, fresh *Deglet-Nour* dates are particularly sensitive to heat treatment due to their high sugar content, attractive color, soft texture and delicate skin. To reach the best possible compromise between the positive and negative effects of the heat treatment, it is crucial to get a detailed picture of the intrinsic thermal susceptibility of the target microbiological flora. The design and/or optimization of any microorganism thermal destruction or inactivation process are based on the kinetic models sufficiently reliable to provide an estimation of pasteurization parameters guaranteeing the quality and safety of a product.

The aims of this work were (1) to isolate and identify fungi associated with the spoilage of fresh *Deglet-Nour* dates due to the strains *Aspergillus niger* and *Candida apicola*; (2) to estimate the heat resistance parameters (D and z_T values) of their survival spores, following their heat treatments in suspension; (3) to validate the parameter values by experimental quantifying survival spores on date surface (non-isothermal treatments); (4) and to discuss the advantage of using heat treatements to reduce fungal flora while minimizing the thermal impact on color related to date quality.

MATERIALS AND METHODS

Isolation and Identification of the Strains

The spores used in this study were obtained from strains of *A. niger* and *C. apicola* isolated from *Deglet-Nour* date fruits (*Phoenix dactylifera* L.) and identified by molecular methods. Date samples were collected from three provinces of the southern region of Algeria (Biskra, Tolga and Doucen). Samples were kept at approximately 4C and transported in ice-cooled boxes to preserve their original flora. Total fungus and osmophilic yeast counts were carried out on potato dextrose agar and oxytetracycline glucose yeast agar supplemented with 20% (dry weight) sucrose, respectively. *A. niger* and *C. apicola* colonies were isolated from the total fungus count and osmophilic yeast count, respectively.

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 DNA extraction, the fragments of the 28S and 26S rDNA genes were amplified by polymerase chain reactions (PCR) with U1/U2 and NL1GC/LS2 primers for mold and yeast strains, respectively. DNA amplicons were purified using the Wizard PCR Preps DNA purification system (Promega, Madison, WI) according to the manufacturer's instructions and sent for sequencing (GATC Biotech, Konstanz, Germany). The obtained sequences were compared with databases in the GenBank of the National Centre for Biotechnology Information using the BLAST alignment software.

Isothermal Heat Treatment Procedure

A. niger and *C. apicola* were cultured in their respective media at 25C over 10 days to obtain high spore concentration in the cultures. The spores were collected after washing each culture with a sterile physiological saline containing 0.005% Tween 80 and gently scraping the surface with a glass rod. The suspension was filtered to remove the hyphal fragments of mold and aggregates. After estimating the spore concentration with a hemocytometer, the suspension was adjusted with the sterile physiological saline containing 0.005% Tween 80 to obtain a final spore concentration of 10^8 spores/mL.

The isothermal treatment was carried out as follows: Pyrex flask containing 99 mL of the same solution used for preparing the spore suspension was submerged in a temperature-controlled water bath with continuous stirring (sterile magnetic stirrer inside the Pyrex flask). The temperature of both the solution and the water bath was monitored throughout the treatment using two thermocouples (type K, NiCr–Ni sensor, ref. ZA 9020-FS Thermo E4, Ahlborn, Holzkirchen, Germany), one inserted at the center of the Pyrex flask and one in the water bath, and connected to a data logger (ALMEMO 2890-9 V2.3, Ahlborn, Holzkirchen, Germany). When the solution in the Pyrex flask had stabilized at the desired temperature, 1 mL of the spore suspension was inoculated in the flask so that the dilution rate was 1/100 to obtain an initial population of 10^6 spores/mL. At regular time intervals, 1 mL of the heated spore suspension was transferred to sterile test tubes containing 9 mL of sterile physiological saline, previously immersed in ice water. Surviving spore populations (cfu) were counted at regular time intervals. There were three replicates of each heat treatment.

Date Fruit Inoculation and Non-Isothermal Heat Treatment Procedure

The dates used in this study were regularly shaped dates of the *Deglet-Nour* date variety at the *Rutab* stage also called "soft ripe" stage (penultimate stage of maturity). The fruits were disinfected with hot air (80C with 80% relative humidity) for 15 min in a climatic chamber (BIA, Conflans Sainte

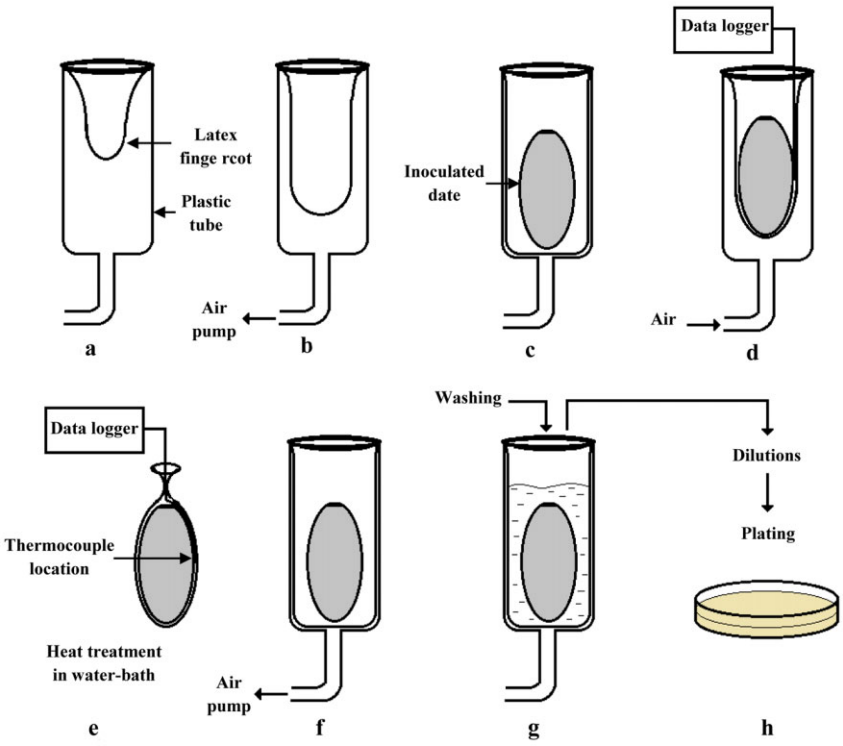

FIG. 1. SCHEMATIC DESCRIPTION OF THE PROTOCOL APPLIED TO THE SURFACE-INOCULATED DATE FRUITS BEFORE (A, B, C, D) AND AFTER (F, G, H) THE HEAT TREATMENT (E)

Honorine, France), then cooled and dried in a laminar flow hood for 1 h. After disinfection, each date was artificially contaminated on the surface with 60 μL of the spore suspension (10^8 spores/mL) with 3 drops (5 μL) on each of the four sides of the date. The drops of the spore suspension were spread lengthways with a sterile Pasteur pipette. The fungal load of the contaminated dates was assessed at 1.8×10^6 cfu/date and 2×10^6 cfu/date for *A. niger* and *C. apicola*, respectively, on control fruits. The setup used for the non-isothermal heat treatment of the inoculated date fruit is shown in Fig. 1. The temperature of the heating bath used for the validation of the heat inactivation kinetic parameters of whole dates was set at 70C in order to shorten the procedure and to limit thermal impact on food quality to an acceptable level. To prevent any washing of the inoculum, the inoculated dates were enclosed within a latex envelope (latex finger cot) and sealed with a metal clamp (Fig. 1a–c). The enclosed dates were then immersed in a temperature-controlled water bath (Polystat, Fischer Bioblock Scientific, Illkirch, France). Just the latex envelope was in contact with water bath, acting as a sterile barrier. The date surface temperature was monitored every second using a 0.5-mm-thick K-type micro-thermocouple (TC S.A., Dardilly, France) inserted between the date surface and the latex covering (Fig. 1d,e) and connected to a data logger (Almemo 2890-9 V2.3, Ahlborn). The latex-encased dates were removed at appropriate time intervals from the bath and cooled in ice water before being rinsed with 20 mL of sterile physiological saline and undergoing the subsequent stages of diluting, plating and counting the viable spores (Fig. 1f–h).

Kinetic Modeling of the Thermal Inactivation

The diminution of the surviving population ($\log_{10} N$) with the increasing duration of the heat treatment may be described by the following first-order equation:

$$\log_{10} N^{(t)} = \log_{10} N^{(0)} - \frac{t}{D_T} \tag{1}$$

where D_T is the decimal reduction time, i.e., the time required to obtain 1 log reduction in the spore population at temperature T. The influence of temperature on spore heat resistance was determined according to the following linear relationship:

$$\log_{10} D_T = \log_{10} D_{T_{ref}} - \left(\frac{T - T_{ref}}{z_T} \right) \tag{2}$$

In which z_T is a parameter corresponding to the temperature increase that produces a 10-fold reduction of the D_T value. The reference temperature T_{ref} for the calculation of the pasteurization value is 70C for fruit juice thermal treatments.

Parameter Estimation and Statistical Methods

Kinetic parameters (D_T and z_T) were computed from the isothermal heat treatment data (isothermal heat treatment procedure) using a reference temperature chosen from the middle of the studied temperature range. They were adjusted using the "*cftool*" function of the MATLAB software (vers. 6.5, MathWorks, MathWorks Inc, Natick, MA, USA).

Non-Isothermal Prediction

To predict the inactivation of *A. niger* and *C. apicola* spores on the surface of date fruits in non-isothermal conditions, the evolution of pasteurization values (noted *PV*) during the date heat treatment was calculated using the following Bigelow model and the recorded temperature kinetics:

$$PV = \int_{t_0}^{t_f} 10^{\left(\frac{T-T_{ref}}{z_T}\right)} dt \quad (3)$$

The *PV* value is the time–temperature history that corresponds to the equivalent isothermal treatment duration at the reference temperature (T_{ref}) taking into account the thermal sensitivity z_T of the target fungus spores. The evolution of the target fungus spore populations ($\log_{10} N$) in non-isothermal conditions was calculated for different heat treatment durations using the following equation:

$$\log_{10} N^{(t)} = \log_{10} N^{(0)} - \frac{PV}{D_{T_{ref}}} \quad (4)$$

RESULTS

Fungal Strains

The counted populations of the total fungi and osmophilic yeasts are shown in Table 1. The pulp was analyzed to check for the possible presence of microorganisms and was found uncontaminated. We therefore considered that the fungal flora counted in this study concerned the date fruit surface. Comparison of the obtained sequences with databases in the GenBank yielded a sequence identity >95% with *A. niger* (AB573988.1) and *C. apicola* (CBS4076). *A. niger* was the main fungus species found in all samples taken from the three districts sampled in Algeria (Biskra, Tolga and Doucen), the yeast *C. apicola* was found in samples from two of these three districts (Biskra and Tolga). These spoilage microbial species are the target for heat treatment optimization.

Thermal Spores Inactivation

The survival spores of *A. niger* and *C. apicola* are log-linear, as shown in Fig. 2, and the heat resistance of these fungal spores is classically quantified by their D_T values, as presented in Table 2. Decimal reduction times at 50C D_{50} were 22.1 ± 2.1 min for *A. niger* and 2.3 ± 0.4 min for *C. apicola*, showing that *A. niger* spores are more heat-resistant than *C. apicola* spores. The influence of the heating temperature on the *D* values is quantified by the parameter z_T (Fig. 3). The z_T values estimated for *A. niger* and *C. apicola* spores are similar (9.6 ± 1.0 and 9.4 ± 2.6C, respectively). The root mean squared error (*RMSE*) of $\log_{10} N$ values is less than 0.06 (i.e., 1.1 min).

Heat Treatment Surface of Date

The protocol described in Fig. 1 allowed the heat treatment of the surface of whole dates inoculated with a known microbial load and the monitoring of load reduction. Figure 4 compares the experimental survival spores obtained on the date surface during heating at 70C with those computed using pasteurization and D_{70} values. The estimated parameters (z_T and D_T) and the initial population for the two species studied were used in the calculations. In the case of the *A. niger* strain, the experimental $\log_{10} N$ values are equivalent to those calculated: as predicted by the model, no surviving spore is detected after 300 s of heat treatment. In the case of the *C. apicola* strain, the experimentally obtained $\log_{10} N$ values are slightly lower than those calculated, but the simulations remain secure because the model underestimates the efficiency of heat treatment.

DISCUSSION

The total fungal count (Table 1) was lower than that determined by Shenasi *et al.* (2002a) and Habibi Najafi and Haddad Khodaparast (2009) on other date varieties but

TABLE 1. TOTAL FUNGI AND OSMOPHILIC YEAST POPULATIONS IN *DEGLET-NOUR* DATE FRUITS FROM THREE DIFFERENT ALGERIAN DISTRICTS (BISKRA, TOLGA AND DOUCEN)

	Total fungi		Osmophilic yeasts	
District	10^2 cfu/g*	10^2 cfu/cm^2†	10^2 cfu/g*	10^2 cfu/cm^2†
Biskra	4.2 ± 1.2	1.8 ± 0.5	0.24 ± 0.10	0.11 ± 0.02
Tolga	2.4 ± 0.9	1.0 ± 0.4	0.16 ± 0.04	0.07 ± 0.02
Doucen	5.8 ± 1.9	2.5 ± 0.8	–	–

Mean value ± standard deviation with $n = 5$ (*n*: population counted on date samples from one date palm, with 5 date palms per province).
* Date weight: 13.5 ± 2.1 g (mean weight from 30 dates).
† Date surface area: 31.8 ± 3.8 cm^2 (mean surface area estimated from 30 dates).

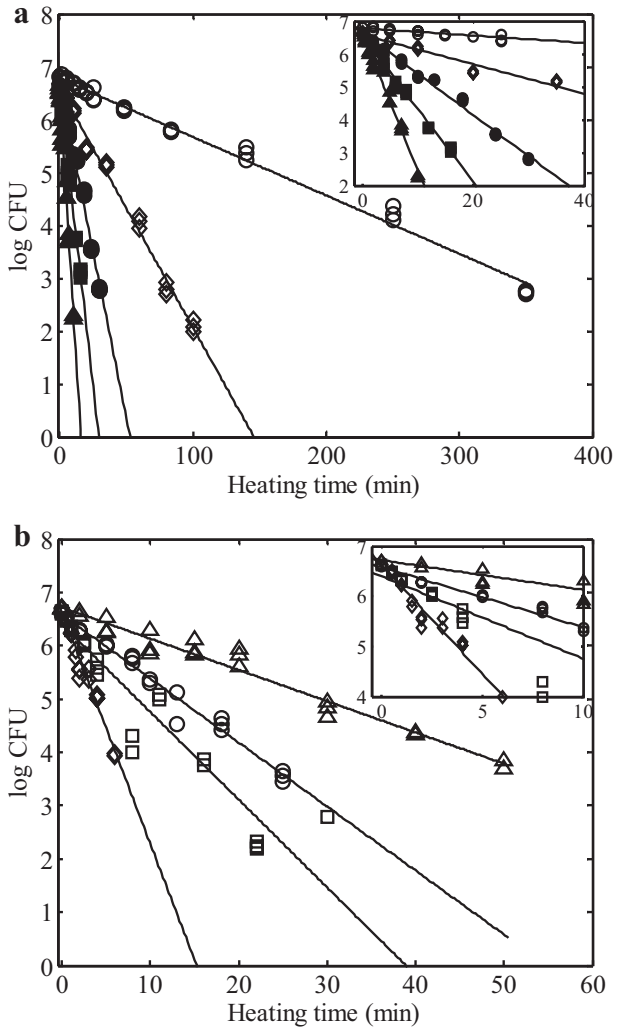

FIG. 2. EXPERIMENTAL (SYMBOLS) AND SIMULATED (SOLID LINES) SURVIVING POPULATIONS LOG_{10} *N* OF *ASPERGILLUS NIGER* (A) AND *CANDIDA APICOLA* (B) SPORES AFTER *T* MINUTE TIMES AT VARIOUS TEMPERATURES (42C △, 45C ○, 47C □, 50C ◇, 55C ●, 57C ■, 60C ▲)

similar to that reported by Moore *et al.* (2002). *A. niger* (mold) is the most commonly reported fungus species in stored foods, particularly in dried products. It may be considered as the worst-case scenario in this study. The behavior of this species regarding heating is within the wide range of heat resistance reported in the literature (Fujikawa and Itoh 1996b; Shearer *et al.* 2002; Reveron *et al.* 2005). In addition, this strain was much more present compared with the total fungal load in all samples. *A. niger* (83–39%) displayed the highest occurrence in date fruits compared with *A. flavus* (49–40%) and *A. fumigatus* (40–26%) (Giridhar and Reddy 2001; Al-Sheikh 2009; Colman *et al.* 2012). *A. niger* strain is responsible for postharvest decay (Pitt and Hocking 2009) and it may present a risk to human health because it has been reported as ochratoxin-producing species (Abarca *et al.* 1994). The yeast *C. apicola* is not considered as the worst-case scenario in terms of temperature resistance. However, this yeast has very specific characteristics: xerotolerant (can grow in low a_w conditions), osmotolerant (can grow in high-sugar concentrations), and it has an extensive fermentative metabolism (alcohol and carbon dioxide). It is often isolated from high-sugar products such as honey (Rosa *et al.* 2003) and high-sugar grape must (Tofalo *et al.* 2009). It follows that *C. apicola* spores are liable to cause serious spoilage in preserved and packaged sugar-rich fruits such as date (total sugar content 70–80% of

TABLE 2. ESTIMATION OF THE DECIMAL REDUCTION TIME (D_T) AT TEMPERATURE *T* OF SPORES (MEAN VALUES ± 95% CONFIDENCE INTERVAL)

	Candida apicola		*Aspergillus niger*	
T (C)	D_T (min)	*RMSE*	D_T (min)	*RMSE*
42	17.1 ± 2.8	0.17	ND	ND
45	8.3 ± 1.1	0.14	90.5 ± 6.0	0.11
47	6.1 ± 1.7	0.47	ND	ND
50	2.3 ± 0.4	0.16	22.1 ± 2.1	0.17
55	ND		8.0 ± 0.7	0.13
57	ND		4.5 ± 0.4	0.12
60	ND		2.4 ± 0.3	0.21

ND, not determined; *RMSE*, root mean squared error between experimental and simulated data ($\log_{10} N$).

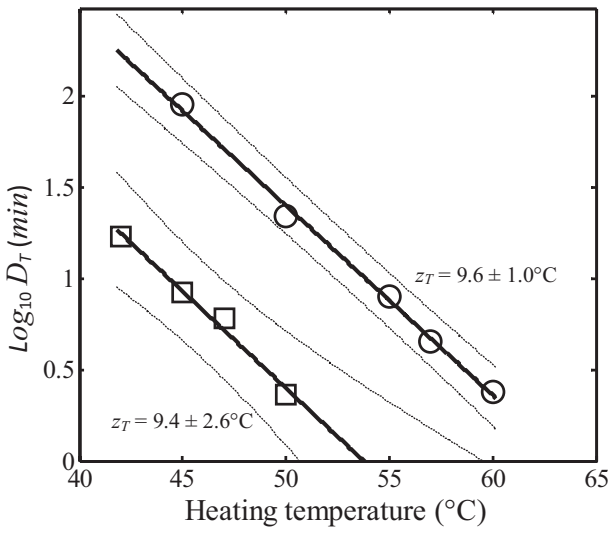

FIG. 3. DECIMAL REDUCTION TIME SPORES (LOG_{10} D_T) AS A FUNCTION OF HEATING TEMPERATURE (*T*)
Experimental data for *Aspergillus niger* (○) and *Candida apicola* (□) and predicted curves: solid line represents the fitting with $\log_{10} D_T = \log_{10} D_{T_{ref}} - (T - T_{ref})/z_T$ and dashed line the confidence interval ($P = 0.05$).

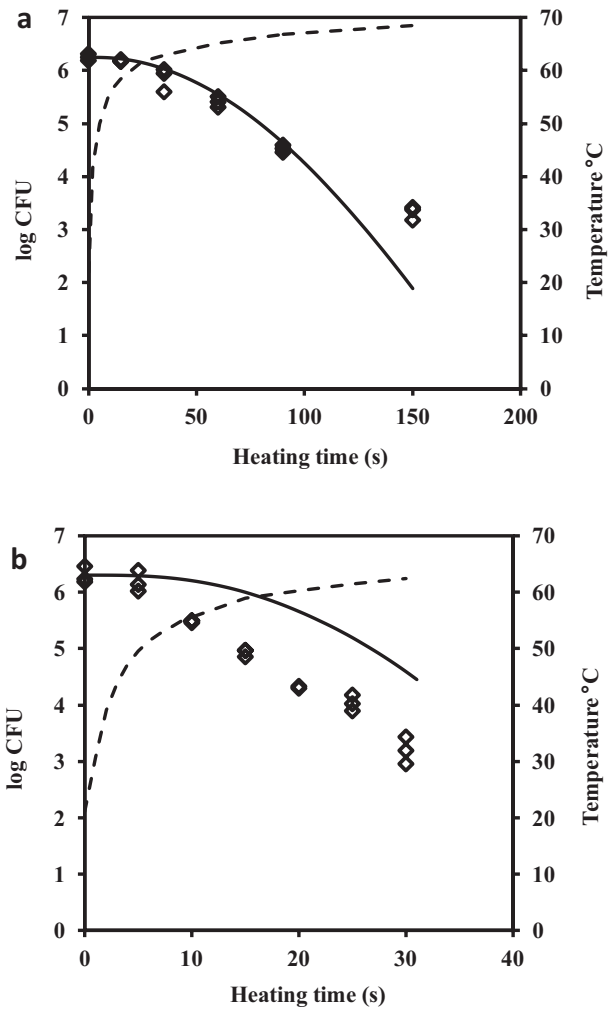

FIG. 4. EXPERIMENTAL (□) AND SIMULATED (—) SURVIVING POPULATIONS OF *ASPERGILLUS NIGER* (A) AND *CANDIDA APICOLA* (B) SPORES ON THE SURFACE OF DATE FRUIT DURING HEAT TREATMENT AT 70C WITH THE RECORDED SURFACE TIME–TEMPERATURE PROFILES (- - -)
Kinetic parameters for *A. niger* and *C. apicola* were 0.20 and 0.02 min, respectively, with T_{ref} = 70&deg*C*.

dry weight). A small load of *C. apicola* can rapidly alter the fresh date quality, hence the importance of eliminating this yeast on *Deglet-Nour* dates.

Unlike observations reported by other authors (King Jr *et al.* 1979; Fujikawa and Itoh 1996a; Sant'ana *et al.* 2009), the first-order kinetics visible in Fig. 2 draw a curve with neither shoulder nor tail. No resistant forms and no activation phenomenon were found in the strains studied here. This could be related to the heat sensitivity of the strains (wild strains) and/or to the isothermal conditions in which the heat treatments were carried out. The conidia from *A. niger* strain isolated from date surface exhibit higher heat resistance ($D_{60} = 2.4 \pm 0.3$ min) than strains isolated from other foods $D_{60} = 1.1$ min (Fujikawa and Itoh 1996b), acid foods $D_{60} = 0.45$ min (Shearer *et al.* 2002) or beer $D_{60} = 0.04$ min (Reveron *et al.* 2005). Moreover, these conidia were more resistant to temperature increase (z_T: 9.6 ± 1.0C) than that of strains isolated from food (z_T: 3.6–3.7C). Regarding the heat resistance of *C apicola*, we are not aware of any published data. The thermal sensitivities of *A. niger* and *C. apicola* spores are similar, with z_T values of 9.6 ± 1.0 and 9.4 ± 2.6C, respectively. These spores were therefore only weakly sensitive to temperature increases and both strains displayed the same behavior. On the contrary, according to our estimations of D_T values, the spores of both strains isolated in this study were heat sensitive, with *C. apicola* being more sensitive to the heat treatments than *A. niger*.

In studying the thermal inactivation of microorganisms on food surface, particularly on fruit surface, one needs to be able to maintain the initial microbial load adhering to the surface of the fruit during the thermal process in order to assess survival spores as compared with the initial load inoculated. Moreover, the temperature must be measured on the fruit surface during the heat treatment. These experimental conditions were obtained using the experimental setup pictured in Fig. 1. Comparing the survival of fungus and yeast spores obtained experimentally on date surfaces with those calculated using the estimated parameters z_T and $D_{T_{ref}}$ and the temperature–time profiles data (Fig. 4) validates not only both z_T and $D_{T_{ref}}$ parameters for *A. niger* and *C. apicola* spores but also the heat inactivation strategy of spore suspensions (isotonic aqueous solution), which could be used for inactivation fungal spores attached to the fruit surfaces in postharvest heat treatment processes.

Bigelow method is used to quantify the effect of non-isothermal heat treatments such as pasteurization processes. For pasteurization value calculations, the reference temperature is 70C for nonliquid foods with a z_T value of 10C. This is close to the z_T values obtained for *A. niger* and *C. apicola* isolated from *Deglet-Nour* date fruits. Regarding the date surface heat treatments at 70C, the pasteurization values derived from the temperature kinetics at the surface of date fruits and the spore counts of the surviving fungus and yeast validated the heat resistance parameters (D_T and z_T) determined for *A. niger* and *C. apicola* on laboratory media. These results show that the food matrix does not affect the heat resistance of *A. niger* and slightly decreases that of *C. apicola*. The calculation of target pasteurization values is thus greatly facilitated: e.g., in order to obtain a 9 $\log_{10}$ reduction of the *A. niger* conidia population on date surface, a pasteurization value of 2 min at 70C must be applied.

The modifications of the physicochemical properties of date during heat treatment mainly result in a nonenzymatic

browning (Belarbi 2001). For the *Deglet-Nour* date variety, the nonenzymatic browning caused by heat treatment is just located on the surface (Ben-Lalli *et al*. 2011). In addition, the advantage of using visual color was that it might be measured as an online quality parameter (Kara and Erçelebi 2013). It can be used as an indirect measure of other quality attributes such as flavor and contents of pigments because it is simpler, faster and correlates well with other physicochemical properties (Francis 1995; Pathare *et al*. 2013). To estimate thermal color degradation kinetics of date surface during a heat treatment, Ahmed and Ramaswamy (2005) represented thermal color degradation of date paste (with tristimulus Hunter color values $L \times a \times b$) as a first-order reaction kinetics. The rate constant at 70C was 0.002/min, and so for a heat treatment at 70C for 5 min, the color index was varied from 1.00 to 0.99. For a target reduction (12 $\log_{10}$) of the *A. niger* load (the worst-case scenario), a heat treatment at 70C for 2.4 min is sufficient, while the color index remains constant. In this case, no significant color variation was observed during heat treatment from 42 to 70C, where *A. niger* and *C. apicola* were inactivated. Indeed, the model validation process at 70C on the date surface depicts no color change observation. The farmer results are consistent with those reported by Reynes (1997) for thermal-treated dates (65C for 3 min) by microwave, showing no color change on the surface, texture or chemical composition. Nevertheless, Belarbi (2001) reported that treated dates with hot air (70C) for 1 h undergo little change in color of 5%. Noting that the *z* value varies from 17 to 45C for the overall sensory quality, texture and color changes in foods (Holdsworth and Simpson 2007). Consequently, a high-temperature/short-time (HTST) process is effective to provide the desired reduction of *A. niger* and *C. apicola* populations while avoiding negative quality impact on fresh *Deglet-Nour*.

Our findings could be used to design an effective postharvest heat treatment against the yeast and fungus spores responsible for quality deterioration of fresh dates, i.e., in-package pasteurization or heat treatment of bulk dates and aseptic packaging. The inactivation parameter values are useful to predict and optimize the pasteurization processes for both liquid (date-juice-based drinks) and solid (date fruit, plum, etc.) products. It is recommended to develop the HTST processes to eliminate fungi that cause spoilage of fresh dates while preserving its quality.

NOMENCLATURE

D_T decimal reduction time at temperature T (min)
$N^{(t)}$ viable spores at any time t (cfu/mL or cfu/g)
PV pasteurization value (min)
T temperature (C)
T_{ref} reference temperature (C)
z_T increase in temperature to achieve a 10-fold reduction of the D value (C)

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