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Effect of reduced water activity and reduced matric potential on the germination of xerophilic and non-xerophilic fungi

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ABSTRACT

Reduction in water activity (a_w) is used as a microbiological hurdle to prevent food spoilage. To minimize the levels of salt and sugar, which are commonly used to reduce a_w, the potential of food structure as a microbiological hurdle needs to be assessed. The concept of matric potential (Ψ_m) is used to measure the effect of food structure on water movement. This study reports the effect of reduced a_w and reduced Ψ_m on the germination of xerophilic fungi (represented by *Eurotium herbariorum*) and non-xerophilic fungi (represented by *Aspergillus niger*) on model glycerol agar media. Germination curves were plotted with the percentage of germinated spores against time. The germination time (t_G), which is defined as the time at which 50% of the total viable spores have germinated, was estimated using the Gompertz model. Total viable spores was defined as those spores that were able to germinate under the optimum a_w and Ψ_m conditions for each species, i.e. 0.95 a_w and 2.5% agar for *E. herbariorum* and 0.98 a_w and 2.5% agar for *A. niger*. As a_w decreased from 0.90 to 0.85 a_w, t_G increased significantly for both the xerophilic fungi and non-xerophilic species at equivalent matric potential values. When matric potential was reduced from −12 kPa (2.5% agar) to −38 kPa (12.5% agar), t_G of *A. niger* was significantly extended at 0.90 a_w; however, t_G remained the same for *A. niger* at 0.85 a_w, and for *E. herbariorum* at 0.80, 0.85 and 0.90 a_w. This study demonstrated that the germination time for non-xerophilic and xerophilic fungi was extended by reduced a_w, however the effect of reduced Ψ_m was limited.

1. Introduction

Fungal spoilage of food occurs when contaminated fungal spores germinate and grow to produce visible fungal colonies on food. Reduction in water activity (a_w) is used widely by microbiologists and the food industry to control fungal spoilage in food. Fungal growth is inhibited at a_w values below its optimum because water movement is restricted. The movement of water to food surfaces is affected by not only the solute but also the structure. Additives, sugar, salt and polyols, are commonly used to reduce a_w. Modification of food structure may be a useful means for meeting the increasing consumer demand for products with reduced additives.

The concept of matric potential (Ψ_m) has been used to measure the effect of food structure on water movement (Boddy and Wimpenny, 1992; Simatos and Karel, 1988). We reported that reduced Ψ_m decreased the growth rate of *Aspergillus niger* and the biomass density of *Eurotium herbariorum* in model gel systems (Huang et al., 2009). The effect of reduced Ψ_m on the germination of xerophilic and non-xerophilic fungi was studied previously (Huang et al., 2009), but the determination of the germination time, t_G, was not accurate enough. A more precise determination of t_G was used in this study using a predictive model. Germination of contaminating fungal species is the first step in food spoilage. The aim of this study was to determine if reduced a_w and reduced Ψ_m could inhibit the germination of both xerophilic and non-xerophilic fungi on glycerol agar media (GA).

2. Material and methods

2.1. Preparation of media

The growth medium, glycerol agar (GA media), used in this study was based on dichloran-glycerol agar (Oxoid CM729, Oxoid Australia Pty Ltd, Adelaide, South Australia), containing 0.5% peptone, 1% glucose, 0.1% KH_2PO_4, 0.05% $MgSO_4$ and 0.01% chloramphenicol, an antibacterial agent. Matric potential (Ψ_m) was modified by adding agar (LP0011, Oxoid Australia Pty Ltd), 2.5%, 5.0%, 7.5%, 10.0%, and 12.5% (w/w in water). The a_w of GA media was adjusted to 0.98 a_w 0.95 a_w, 0.90 a_w, 0.85 a_w and 0.80 a_w by the addition of glycerol (Selby Biolab, Australia). Only one agar concentration (2.5%) was tested at 0.98 a_w and 0.95 a_w, being the optimum a_w and Ψ_m conditions for *A. niger* and *E. herbariorum* respectively. All media were prepared in 100 ml Schott bottles, adjusted to pH 6.0 with 10 M KOH, and then sterilized by steaming for 4 h, with manual shaking at 45 min intervals. The media were then aseptically poured into each Petrislide

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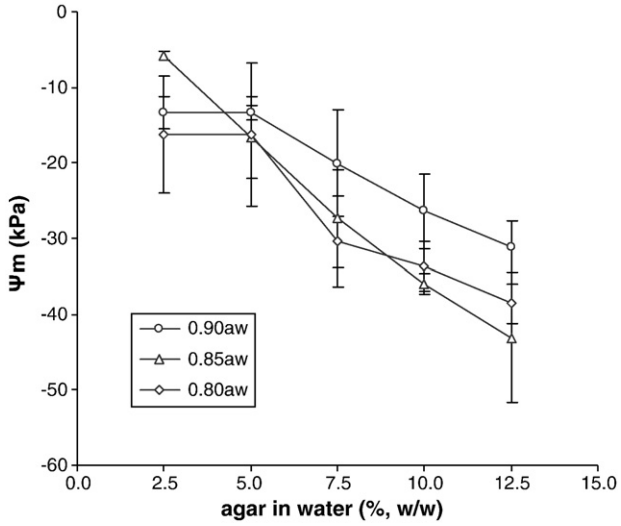

Fig. 1. Matric potential (Ψ_m, kPa) of glycerol agar media (GA) with various agar concentrations at 0.80 a_w to 0.90 a_w. Error bars denote the standard error of the mean of three replicates.

(Millipore, Australia), approximately 2 g per Petrislide. The media in Petrislides were allowed to set at ambient temperature, and then stored in plastic boxes with appropriate saturated salt solutions to control a_w (Robinson and Stokes, 1955).

2.2. Measurement of a_w and Ψ_m

The a_w of media in Petrislides was measured using an Aqualab CX-3 instrument (Decagon, Pullman, WA, USA), which has a stated accuracy of ± 0.003. Media were measured on the day of inoculation and at the time when visible growth was observed. The a_w of media during incubation in Petrislides was maintained at the target value (± 0.01) during incubation using appropriate saturated salt solutions (Robinson and Stokes, 1955).

The Ψ_m of agar-water gels and GA was measured by a filter paper technique as described previously (Huang et al., 2009).

2.3. Preparation of inocula

Two fungal isolates from the FRR culture collection of CSIRO Food and Nutritional Sciences, *E. herbariorum* (FRR 5354) and *A. niger* (FRR 5664), were used for this study. *E. herbariorum* was grown on Czapek

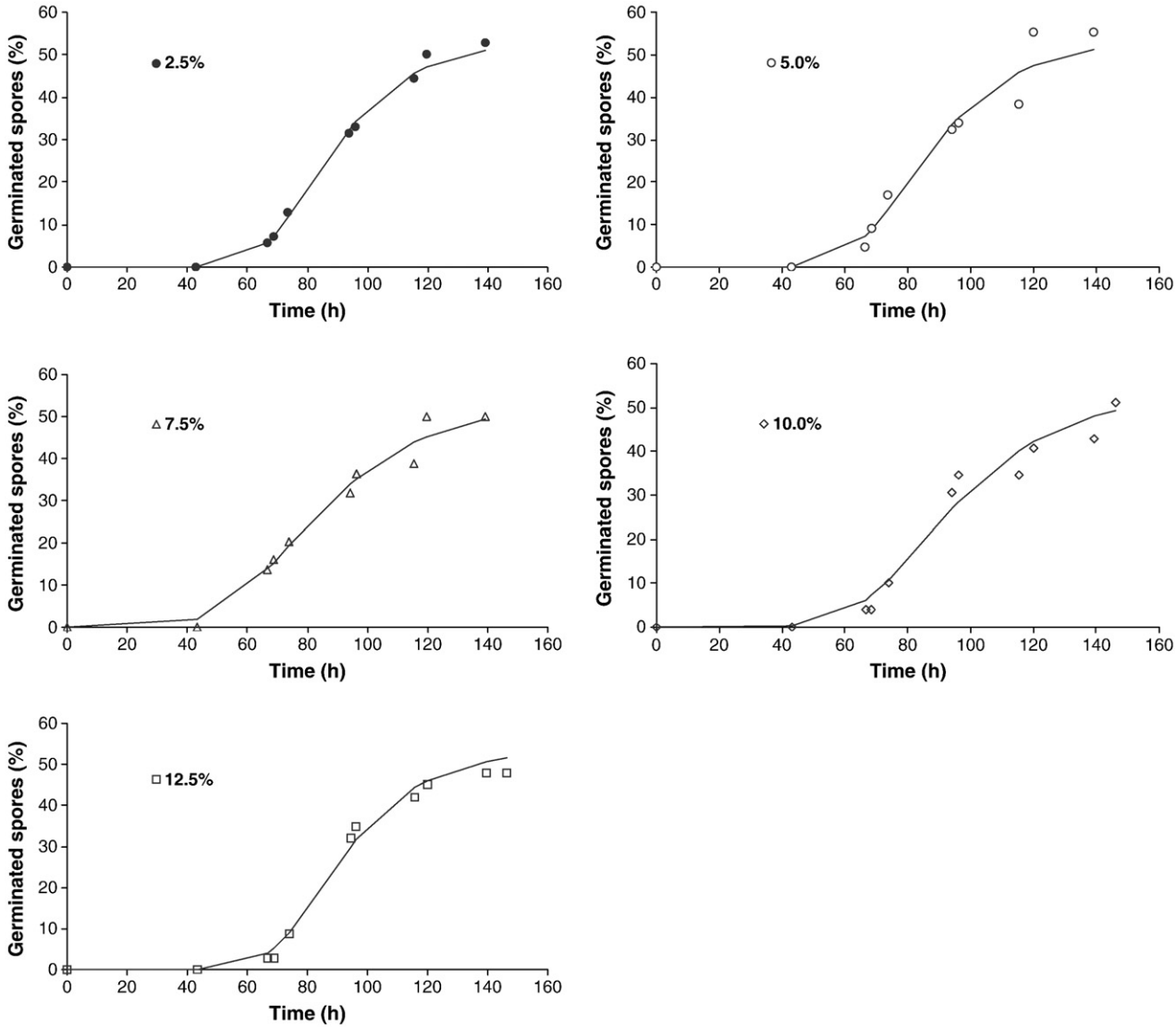

Fig. 2. Germination curves of *Eurotium herbariorum* on glycerol agar media (GA) at 0.85 a_w at 22 °C. Lines are fitted data using the Gompertz equation. Percentages (%) are agar concentration in water (w/w).

yeast extract 20% sucrose agar (CY20S) for 2–3 weeks at 25 °C and *A. niger* on Czapek yeast extract agar (CYA) for one week to allow adequate time for sporulation. CY20S and CYA were prepared according to Pitt and Hocking (1997). After incubation, colonies were wetted with sterile 0.05% Tween 80 solution and spores were collected by gently rubbing the surface of the colony with a Pasteur pipette. Ascospores of *E. herbariorum* were released from the asci by mixing with an equal weight of glass beads (<106 µm, Sigma G4649, Australia) and shaking twice in 'FastPrep FP120' shaker (Savant Instrument, USA) at speed setting of 5.5 for 35 s. The tubes were stored on ice for 2 min between the shakes. Conidia of *A. niger* were released by vortexing with 3 mm glass beads for 1 min. The spore/bead suspensions were filtered through sterile glass wool for the removal of hyphae and beads. The filtered spore suspensions were then diluted in sterile 50% glycerol to a final concentration of 6×10^4 cfu/ml, dispensed into sterile 1 ml Cryogenic vials (Nalgene, Australia) in aliquots of 200 µl, and stored at −80 °C. One aliquot of each isolate was used at each inoculation time.

2.4. Study of germination

The inoculum was prepared by making dilutions of thawed glycerol stock in 50% glycerol solutions to a final concentration of 2×10^4 cfu/ml. Each Petrislide was inoculated using a micropipette to dispense 5 µl of each inoculum. The inoculated media were left on the bench for 2 h to dry, then placed in polyethylene boxes with air-tight lids and incubated at 22 °C. The a_w of boxes was maintained by saturated salt solutions, KCr_2O_7 (0.980 a_w), KH_2PO_4 (0.953 a_w), $BaCl_2$ (0.902 a_w), $Sr(NO_3)_2$ (0.850 a_w), or $(NH_4)_2SO_4$ (0.800 a_w), in 250 ml containers. After 1 d incubation, a microscope field at 100× magnification was selected and the microscope stage Vernier scale values were recorded. Spores were considered to have germinated when the length of the germ-tube was equal to or greater than the longest axis of the swollen spore (Dantigny et al., 2006). Once initial germination was observed, total spores and germinated spores of each field were recorded at least 10 times at 22 °C until visible growth was observed or up to 90 d. Visible growth was determined as development of a colony 1–3 mm in diameter. At the end of a 90 d incubation, inocula without germination were transplanted onto CYA agar for *A. niger* or CY20S agar for *E. herbariorum* for confirmation of viability.

Experiment for each combination of organism/a_w/agar concentration was repeated once in all instances, but treated as individual samples for plotting the germination curve for the determination of the lag time and time to germination.

2.5. Estimation of the lag time (λ_{ge}) and the time to germination (t_G)

It is difficult to predict the sampling time to coincide with t_G. Dantigny et al. (2006) proposed standardize methods for assessing mould germination for the predictive modeling of fungal growth. We followed the widely accepted definition of the germination time (t_G), the time required for 50% of viable spores to germinate (Dantigny et al., 2006). The Gompertz and the logistic equations have been used to describe the kinetics of germination over time (Dantigny et al., 2007; Dantigny et al., 2006; Dantigny et al., 2003). Based on the method of Dantigny et al. (2007), the percentage of germinated spores was plotted against the time to produce a germination curve, which was fitted by the Gompertz equation and logistic function. We observed that t_G estimated by the Gompertz equation was similar to that by the logistic function (data not shown). The logistic function is symmetric with respect to the inflection point at t_G, but the Gompertz equation is not (Dantigny et al., 2006). Dantigny et al. (2007) reported that the Gompertz equation was more suitable for the germination curve of *Aspergillus carbonarius* under stressed conditions. Therefore, we preferred to use the Gompertz equation to calculate t_G for spores under water stress in this study.

The Gompertz equation was expressed as:

$$P_t = P_{max}\cdot\exp\cdot\left(-\exp\left[\left(\mu_m\cdot e/A\left(\lambda_{ge}-t\right)\right)+1\right]\right. \quad (1)$$

where t was the time; P_t was the percentage of germinated spores at time t; P_{max} (%) was the percentage of viable spores for each inoculum at the optimum conditions, 0.95 a_w and 2.5% agar for *E. herbariorum*

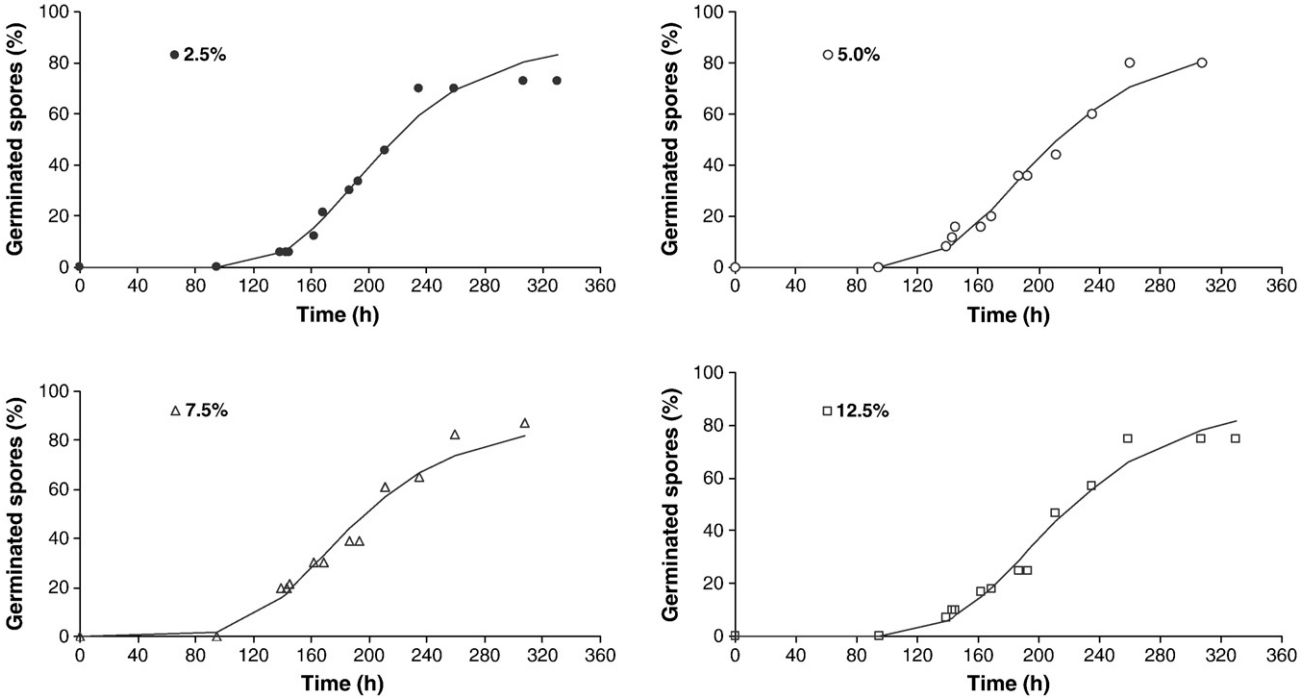

Fig. 3. Germination curves of *Aspergillus niger* on glycerol agar media (GA) at 0.85 a_w at 22 °C. Lines are fitted data using the Gompertz equation. Percentages (%) are agar concentration in water (w/w).

and 0.98 a_w and 2.5% agar for *A. niger*; μ_m (/h) was the slope term of the tangent line through the inflection point; and λ_{ge} (h), the lag time, was the time axis intercept of the tangent through the inflection point. Both μ_m and λ_{ge} were estimated to obtain the least squares fit.

Since t_G is the time (t) when $P = 50\%\ P_{max}$, further calculation is required to estimate t_G by rewriting Eq. (1) as:

$$t_G = \lambda_{ge} + [P_{max} / (e \cdot \mu_m)] \cdot [1 - \ln(-\ln(0.5))]. \quad (2)$$

2.6. Data analysis

Average absolute value of Ψ_m ($n = 3$), average estimated λ_{ge} and t_G ($n = 2$) of any two agar concentrations at equivalent a_w or any two a_w at equivalent agar concentration for each species were compared by single factor ANOVA using Excel 2003. P values of <0.05 were regarded as significantly different.

3. Results

3.1. Matric potential (Ψ_m) of GA

The matric potential (Ψ_m) significantly decreased with the increase in agar concentration at 0.85 to 0.90 a_w (Fig. 1). The matric potential was not significantly different amongst a_w values, 0.80, 0.85 and 0.90, for each single agar concentration ($P > 0.05$) (Fig. 1).

3.2. The lag time, the time to germination (t_G) and the time to visible growth

Both *E. herbariorum* and *A. niger* were studied at 0.80, 0.85 and 0.90 a_w. No germination was observed for *A. niger* at 0.80 a_w after 90 d incubation (data not shown).

Figs. 2 and 3 show examples of the germination data and curves fitted by the Gompertz equation. Less than 100% germination was observed for all conditions prior to visible growth. This was due to the outgrowth of early germinated spores to visible growth, which either masked or inhibited germination of underlying spores. The total viable spores of inocula, which were determined at 0.95 a_w and 2.5% agar for *E. herbariorum* and 0.98 a_w and 2.5% agar for *A. niger*, were 50.4% ± 0.8% ($n = 30$) and 85.0% ± 1.3% ($n = 10$) respectively.

The estimated lag time, t_G and the time to visible growth of *E. herbariorum* and *A. niger* are plotted against average value Ψ_m at each agar concentration in Figs. 4 and 5 respectively. We were unable to obtain the estimated lag time and t_G for *A. niger* on 10% agar at 0.85 a_w (average Ψ_m of −32 kPa) because the germination percentage was 13–30% (15–35% viable spores), which was below the 50% of viable spores required for the determination of t_G.

When a_w decreased at each Ψ_m value, the lag time, t_G and the time to visible growth were significantly extended: three times longer at 0.80 a_w than those at 0.90 a_w for *E. herbariorum*; three times longer at 0.85 a_w than those at 0.90 a_w for *A. niger*.

When Ψ_m reduced from −12 (2.5% agar) to −38 kPa (12.5% agar), there was a significant increase in the lag time, t_G, and time to visible growth for *A. niger* at 0.90 a_w; but no significant difference was observed for *A. niger* at 0.85 a_w, or for *E. herbariorum* at 0.80, 0.85 and 0.90 a_w.

4. Discussion

Water status in food is affected not only by solutes but also by the food structure. The term 'matric potential' (Ψ_m) describes water status as a result of interaction of water with matrices (Boddy and Wimpenny, 1992). We reported previously that Ψ_m decreased as agar concentration increased at 0.90 and 0.95 a_w (Huang et al., 2009). We observed a similar trend at 0.80, 0.85 and 0.90 a_w in this study.

The presence of liquid water or water vapor is essential for the germination of spores. The required concentration of water or its vapor in the environment of the spore to allow germination is specific to the particular fungal species (Gottlieb, 1978). The concept of a_w has been widely used to study fungal water relations. The minimal a_w for germination has been reported for many fungal species, e.g. for *A. niger*, 0.77 to 0.79 a_w (Gottlieb, 1978; Pitt, 1975); for *E. herbariorum*, 0.74 a_w (Pitt, 1975).

Reduced a_w causes water stress in fungal species, but water stress can also be manipulated by the physical microstructure of food. This study is an extension of our report on the inhibitory effect of physical microstructure on fungal growth (Huang et al., 2009). We have observed a significant inhibitory effect of reduced Ψ_m on the germination of *A. niger* at 0.90 a_w in this study; however, fungal germination, unlike fungal growth, was not affected by reduced Ψ_m under most of the tested reduced a_w conditions. This is not to say that food structure has no potential as a microbiological hurdle. The relationship of Ψ_m to a_w was given as Ψ_m (kPa) = 1000 ln(a_w)RT/W_A, where R

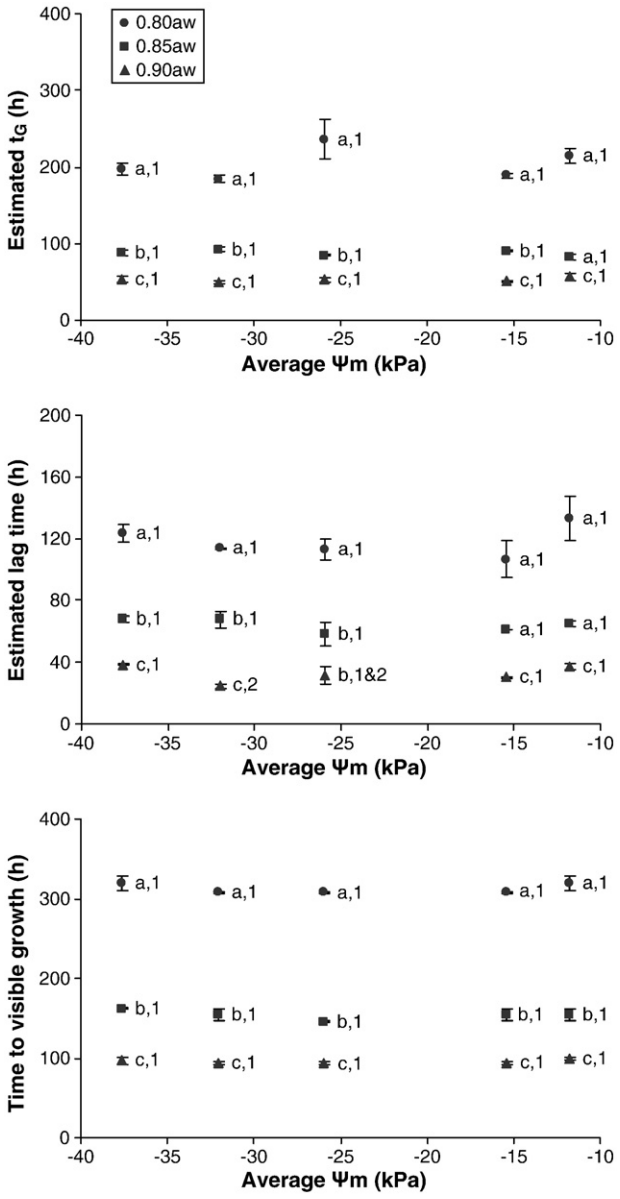

Fig. 4. Estimated time to germination (t_G) and lag time by the Gompertz equation, and the time to visible growth of *Eurotium herbariorum* on glycerol agar media (GA) with agar concentrations at 0.80 to 0.90 a_w at 22 °C. Error bars denote the standard error of the mean of duplicates. Different letters (a–c) indicate significant differences ($P < 0.05$) amongst a_w values for each Ψ_m. Different numbers (1–2) indicate significant differences amongst Ψ_m values at each a_w.

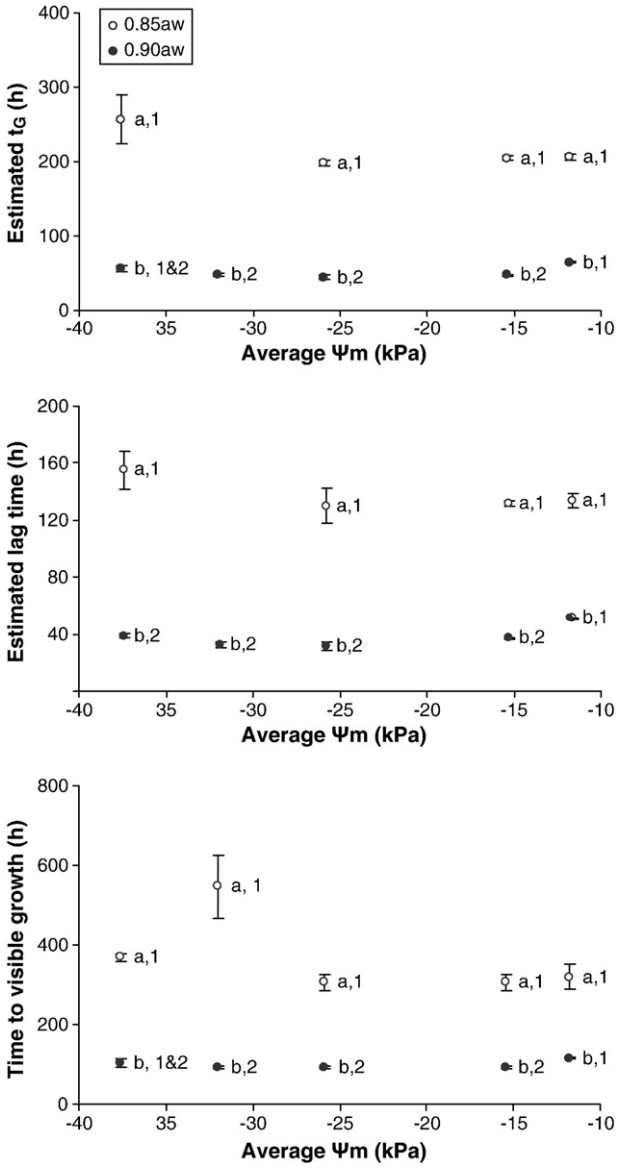

Fig. 5. Estimated time to germination (t_G) and lag time by the Gompertz equation, and the time to visible growth of *Aspergillus niger* on glycerol agar media (GA) with agar concentrations at 0.85 to 0.90 a_w at 22 °C. Error bars denote the standard error of the mean of duplicates. The estimated lag time and t_G for *A. niger* on 10% agar at 0.85 a_w were not obtained because the germination percentage was below the 50% required for t_G determination. Different letters (a–c) indicate significant differences ($P<0.05$) amongst a_w values for each Ψ_m. Different numbers (1–2) indicate significant differences amongst Ψ_m values at each a_w.

is the gas constant, 8.3143 J/(Kmol), T is the absolute temperature (K), and W_A is the molecular weight of water (18.016) (Lang, 1967). If we have a system with Ψ_m of −100 kPa, the a_w of the system will be 0.999, in other words, a Ψ_m of −100 kPa can only reduce water availability by the equivalent of 0.001 unit of a_w. Due to the technical difficulties in making gels with higher agar concentrations, the highest agar concentration we could apply in GA media was 12.5%, giving a Ψ_m of −38 kPa that is equivalent to a a_w reduction of less than 0.001 unit. Therefore, the effect of reduced Ψ_m of GA media cannot be compared with that of reduced a_w over the range tested here. Ψ_m and a_w are not independent factors, as change in Ψ_m can be measured as changes in a_w, when it was measured as the equilibrium relative humidity (%ERH). However, the ability to measure changes in Ψ_m in agar gels caused by changes in agar concentration (2.5 to 12.5%) is below the accuracy of Aqualab CX-3 (0.003 unit). We hypothesized that matric potential of food gel may affect the germination of spores, however that the maximum Ψ_m in the agar systems reported here was below the level, at which a biological effect is observed. Other researchers have observed significant effect of Ψ_m on germination of fungal spores in polyethylene glycol (PEG) systems (Ramirez, et al., 2004; Ritchie, et al., 2006). Further work will be required to investigate other methods of manipulating food structure to decrease Ψ_m to enable a significant effect of reduced Ψ_m on fungal germination.

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