

Understanding and Predicting Optimal Storage Conditions and Longevity:

a biophysical approach



Julia Buitink

Institut National d'Horticulture, UMR Molecular Seed Physiology (INH/INRA/Université d'Angers),
16 bd Lavoisier, 49045 Angers, France

Folkert A. Hoekstra

Laboratory of Plant Physiology, Wageningen University, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands

Summary

Longevity of dry seeds has been attributed to the presence of an intracellular glassy state. It is assumed that the high viscosity of the glass decreases molecular mobility and impedes diffusion within the cytoplasm, thus slowing deleterious chemical reactions and changes in structure and chemical composition during ageing. To obtain a more profound insight in the role of glasses in longevity, we characterised the molecular mobility in the cytoplasm as a function of water content (wc) and temperature, both above and below the glass transition. Using electron paramagnetic resonance spectroscopy, we measured the rotational motion of a spin probe that was incorporated in the cytoplasm. Rotational motion in the cytoplasm of seed and pollen of various plant species was found to change as a function of moisture content and temperature in a manner similar to ageing rates or longevity, suggesting that detrimental ageing rates might be associated with molecular mobility in the cytoplasm. A linear relationship was established between the logarithms of rotational motion and ageing rates. This linearity enabled us to predict vigour loss or longevity by measuring the rotational motion at low temperatures at which experimental determination is practically impossible. Predictions on the basis of molecular mobility support the contention of the existence of an optimum water content for storage that shifts to higher values with decreasing storage temperatures. Furthermore, the predictions suggest that longevity at sub-zero temperatures and elevated water contents is higher than estimated by the seed viability equation.

Introduction

An important goal of germplasm facilities is the long-term preservation of viability. Most seeds and pollens are particularly suitable for this purpose, because they are able to tolerate severe desiccation. Determination of optimum storage conditions is needed to ensure a prolonged lifespan. Nevertheless, in spite of the long lifespans that are achievable by drying to low water contents, these desiccation-tolerant systems eventually lose viability. It is therefore important to find effective tools to predict longevity under given storage conditions and to minimise damage to the germplasm.

Over the past decade, it has been demonstrated that the increased longevity upon drying is related to an increased intracellular viscosity (Leopold *et al.*, 1994; Sun, 1997; Buitink *et al.*, 1998a, 2000c). Considering that the rate of a chemical reaction is related to the viscosity of the reaction mixture (Hancock *et al.*, 1995), it was hypothesised that a dehydration-induced increase in cytoplasmic viscosity leads to a decrease in the rate of detrimental reactions and, consequently, to an increase in longevity.

When the water content falls below a certain value (approximately 10%, or 0.1 g H₂O g dw⁻¹, at room temperature), the cytoplasm becomes so viscous that it

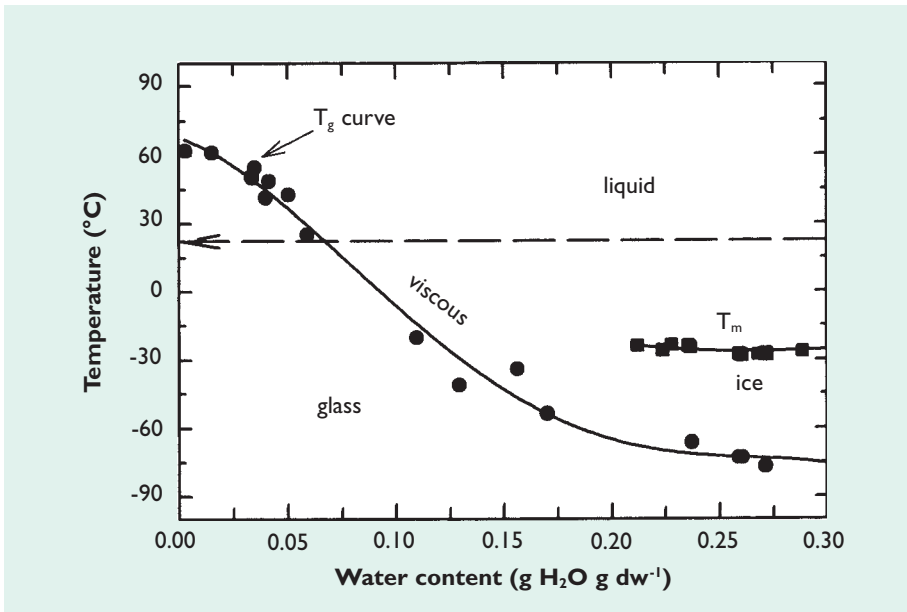


Figure 37.1 State/phase diagrams for pollen of *T. latifolia*, describing the interaction between water content and temperature on the glass transition temperature (T_g) and melting events (T_m). Onset temperature for T_m and T_g were determined from DSC thermograms (Buitink *et al.*, 1998b, with permission of Blackwell Publishing). Samples were scanned at $10^\circ\text{C}/\text{min}$ from -120 to 100°C . The fit for the glass transition was a fourth order polynomial function. Also indicated are the physical states of water: glass, liquid, viscous and ice.

transforms into a so-called glass. A glass is a thermodynamically unstable solid state with an extremely high viscosity. Low tissue water contents and low temperatures promote its formation (Franks *et al.*, 1991). A state diagram (T_g curve) as shown in Figure 37.1 for *Typha latifolia* L. pollen (Buitink *et al.*, 1996) describes the relation between glass transition temperature (T_g) and tissue water content. The lower the water content of the tissues, the higher is the temperature at which the glass is formed. For example, when the pollen is stored at $0.1 \text{ g H}_2\text{O g dw}^{-1}$ and -20°C , its cytoplasm will be in a glassy state, whereas it will be in a liquid phase at $0.15 \text{ g H}_2\text{O g dw}^{-1}$ and 20°C . The combinations of water content and temperature below the T_g curve (cytoplasm in the glassy state) can be considered as optimal storage conditions. Also indicated in Figure 37.1 are the physical states of water in the cytoplasm (ice, liquid, viscous, glass). The melting temperature (T_m) of the water in the pollen can aid in predicting when the cytoplasm will be transformed into ice (at a cooling rate of $10^\circ\text{C min}^{-1}$). Care has to be taken that the way to reach the glassy state does not first allow ice crystals to be formed. Therefore, tissues that are desiccation-tolerant should first be dried to water contents below the unfrozen

water content (where T_m becomes undetectable), and then the temperature should be decreased. In this way, the state-phase diagram can be used as a first approximation to determine a drying protocol and storage conditions for optimal longevity (Sacandé *et al.*, 2000).

Nevertheless, even when seeds or pollens are stored under conditions that allow the cytoplasm to be in the glassy state, they still age, with their ageing rates being dependent on the temperature and water content of the tissues. For example, ageing rates will be higher at elevated temperatures or will increase when tissues are dried to very low water contents (Roberts, 1972; Vertucci *et al.*, 1994; Buitink *et al.*, 1998b). To better understand the rate of ageing under various conditions, we hypothesised that the molecular mobility in the cytoplasm might be involved. This led us to investigate the possibility to determine molecular mobility in seeds and to compare these results with their longevity in relation to water content and temperature of storage.

How to Measure Molecular Mobility:

Determination of the Rotational Mobility of Guest Molecules in the Cytoplasm using Electron Paramagnetic Resonance Spectroscopy

In order to measure a parameter like molecular mobility in cells it is necessary to resort to biophysical techniques. Electron paramagnetic resonance spectroscopy (EPR) has proven to be an excellent technique to study molecular mobility *in vivo* in biological samples (for more details see Hemminga and van den Dries, 1998; Buitink *et al.*, 1999). After incorporation of spin probe molecules into the tissues and removal of the extracellular signals by the addition of potassium ferricyanide, this technique can provide a spectrum of the spin probe from the cytoplasm. From the line shapes of spectra, a measure of molecular mobility can be derived (see Buitink *et al.*, 1999). To be more precise, the final result is an estimation of the rotational correlation time (τ_R) – a measure that is equivalent to the time it takes for the spin probe to rotate around its axis. Thus, the shorter τ_R , the faster the spin probe molecule rotates (for example, 10^{-11} s implies a faster rotation than 10^{-3} s). There are two applications of the same technique: Continuous Wave (CW) EPR can detect changes in τ_R of spin probes ranging from 10^{-12} to 10^{-8} s, and Saturation Transfer EPR (ST-EPR) can detect τ_R in the order of 10^{-7} to 10^3 s. Both applications have been successfully used to characterise the rotational motion of spin probes in polymers, food systems, and biological tissues such as pollens and seeds (Buitink *et al.*, 1998a; Hemminga and van den Dries, 1998; Leprince and Hoekstra, 1998). This rotational mobility can be taken as a measure for the intracellular viscosity of the environment in which the spin probe resides.

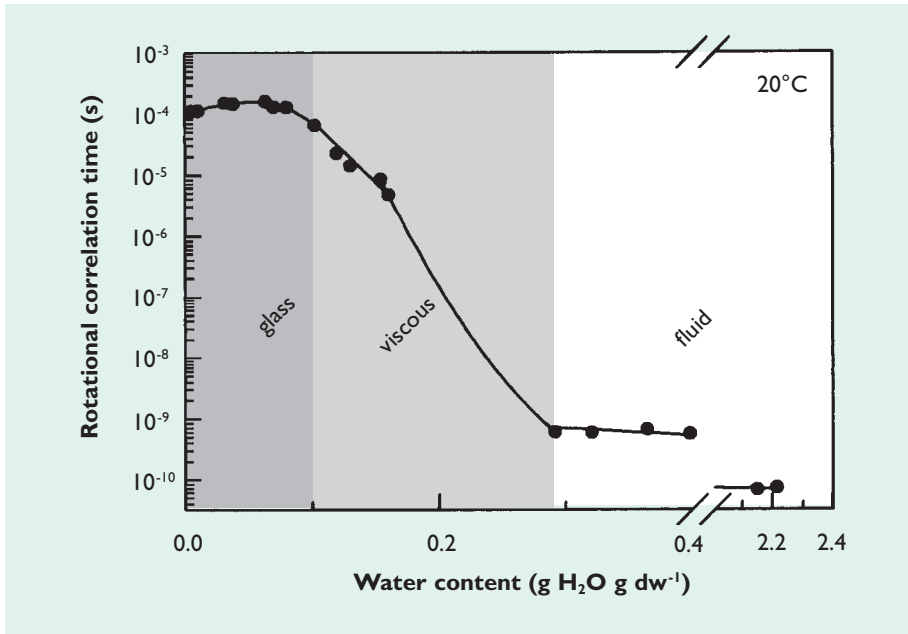


Figure 37.2 Decrease in molecular mobility of the spin probe, 3-carboxy-proxyl (CP), in the cytoplasm of pea embryonic axes during drying at 20°C. Molecular mobility is expressed as the rotational correlation time (τ_R). A long τ_R indicates a slow mobility, whereas a short τ_R indicates a fast mobility. τ_R was measured by (saturation transfer-) electron paramagnetic resonance spectroscopy, as described in Buitink *et al.* (1999).

Using 3-carboxy-proxyl, a polar nitroxide spin probe with the size of approximately a glucose molecule, the rotational mobility in pea (*Pisum sativum* L.) axes was determined during drying at room temperature, using both CW-EPR and ST-EPR (Figure 37.2; drying according to the dashed arrow in Figure 37.1). The spin probe was incorporated into the cells by allowing the pea seeds to imbibe for 16 h at 15°C, after which the axes were incubated for 1 h in a solution of 1 mM 3-carboxy-proxyl (CP). Potassium ferricyanide (200 mM) was added to broaden the signal of CP outside of the cells. Because ferricyanide cannot penetrate intact cells, the signal obtained is exclusively derived from the cytoplasm. Subsequently, the pea axes were taken at intervals during drying. After determining their fresh weight, they were enclosed in a glass capillary tube for spectra recording in an EPR spectrometer (for further details, see Buitink *et al.*, 1999).

During the initial drying, the τ_R of the spin probe in the cytoplasm increased slightly, from 7×10^{-11} s in the hydrated axes to 7×10^{-10} s at $0.3 \text{ g H}_2\text{O g dw}^{-1}$, indicating a slight decrease in molecular mobility (liquid region in Figure 37.2).

Then, around the water content corresponding to the appearance of “unfrozen water” (see Figure 37.1, where T_m becomes undetectable during drying) (Pammenter *et al.*, 1993; Buitink *et al.*, 1996), τ_R decreased rapidly over 5 orders of magnitude (viscous region). Finally, at around 0.10 g H₂O g dw⁻¹ at room temperature, the cytoplasm turned into a glass (glass region in Figure 37.2). Although glass formation in seeds drastically decreases molecular mobility, the molecules in a glass are not completely restricted in their movement. In time, diffusion will be possible, albeit at a rate which is considerably slower than that in hydrated cytoplasm.

Cytoplasmic Mobility and Ageing Rates are Linked

To determine how cytoplasmic molecular mobility is related to ageing rate, we studied the dependence of both parameters on temperature. Increasing the temperature of storage resulted in an increase in ageing rates, as exemplified in Figure 37.3 for pea seeds at 0.07 g H₂O g dw⁻¹ (closed symbols). These ageing rates were expressed as the slope of the rate of vigour loss (% germination multiplied by radicle length) derived from Vertucci *et al.* (1994). The τ_R values in the embryonic axes of pea seeds containing 0.07 g H₂O g dw⁻¹, as determined with CP using ST-EPR, became shorter with increasing temperature, indicating faster molecular mobility at increasing temperatures (Figure 37.3, open symbols). A replicate of the τ_R measurement is shown to demonstrate the high reproducibility of the ST-EPR measurements. Figure 37.3 shows that both molecular mobility and ageing rates changed in a similar manner upon an increase in temperature.

To ascertain the relationship between molecular mobility (τ_R) and ageing rate, these two parameters determined in pea seeds for several temperatures were plotted against each other (Figure 37.4). Each symbol type shown represents the τ_R and rate of vigour loss determined at temperatures ranging from 65 to 15°C for the same water content (vigour data derived from Vertucci *et al.*, 1994, see Buitink *et al.*, 2000c). For four different water contents, the relationship between rate of seed vigour loss and axis molecular mobility was found to be linear on a logarithmic scale. Likewise, the relationship between both parameters determined for one temperature but at different water contents was also linear (data not shown, see Buitink *et al.*, 2000c). Similar linear relationships were also observed for seeds of other species, such as *Impatiens walleriana* Hook.f., *Capsicum annum* L. and *Lactuca sativa* L. (Buitink *et al.*, 2000a, b), as well as for *T. latifolia* pollen (Buitink *et al.*, 2000d). The interdependence of both parameters appears to confirm the hypothesis that ageing rates are related to the mobility of molecules in the cytoplasm.

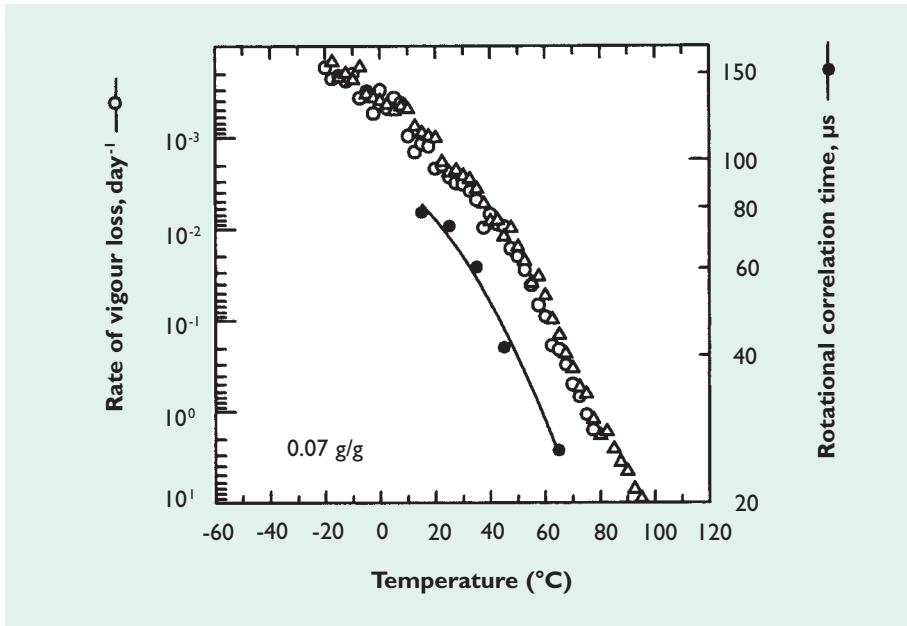


Figure 37.3 Dependence of ageing rate (expressed as rate of vigour loss, day⁻¹) of pea seeds (closed symbols) and τ_R (open symbols) of pea embryonic axes at a water content of 0.07 g H₂O g dw⁻¹ on temperature. Two separate measurements of the τ_R of CP in pea axes at 0.07 g/g are shown to indicate the high reproducibility of the ST-EPR measurements. Rates of vigour loss were taken from Vertucci *et al.* (1994). The figure is partly derived from Buitink *et al.* (2000c).

To test the general validity of the linear relationship between ageing rate and molecular mobility, we estimated longevity using the viability equation from Ellis and Roberts (1980). This equation has been demonstrated to adequately describe longevity of seeds and pollen. The loss of viability over time can be described by the equation $\log \sigma = K_E - C_W \log m - C_H t - C_Q t^2$, where σ (in days) is the standard deviation of the distribution of deaths in time, K_E , C_W , C_H , and C_Q are experimentally determined constants that are specific for each species, t is temperature and m is water content on a % fresh mass basis (Ellis and Roberts, 1980). The viability constants for most species with water contents below 17% (fresh weight basis), which have been published so far, have been experimentally determined from storage data between 30 and 90°C. In the present study, the viability constants for pea seeds were obtained from Ellis *et al.*, (1989) and used to calculate σ for a range of temperatures (t) at the four different water contents (m), at which the respective τ_R values of pea axes were

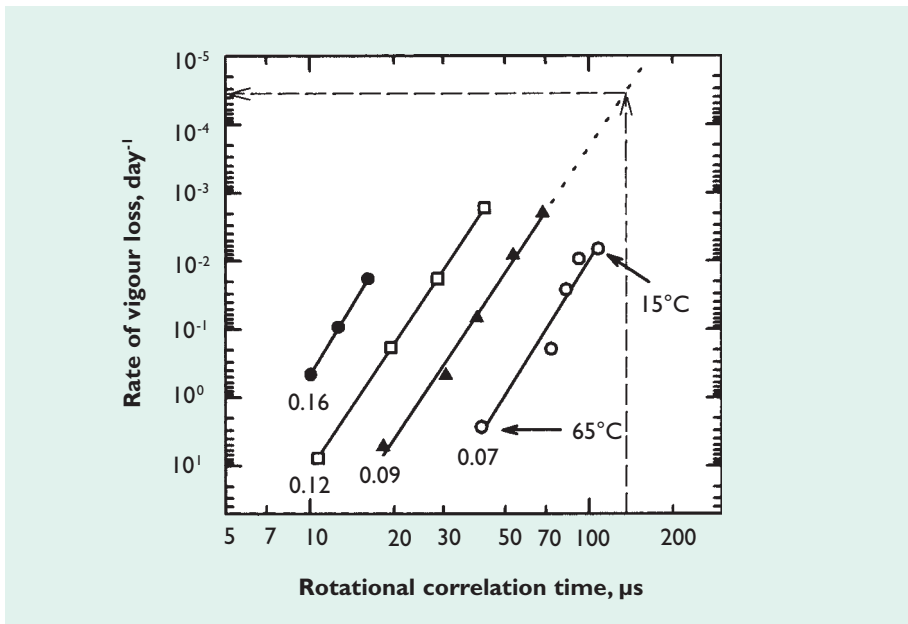


Figure 37.4 Relationship between ageing rate (expressed as rate of vigour loss, day⁻¹) of pea seeds and τ_R of pea axes. Ageing rates were determined at different temperatures (15, 25, 35, 45 and 65°C) and water contents (indicated values in g/g). Data were plotted on a double logarithmic scale $\{\log y = a + b \log x\}$. Water contents, constants and correlation coefficients of the linear regressions are: 0.16 g/g ($a = 6.53$, $b = -6.86$, $R^2 = 0.9995$); 0.12 g/g ($a = 7.21$, $b = -6.15$, $R^2 = 0.99993$); 0.09 g/g ($a = 8.53$, $b = -6.10$, $R^2 = 0.991$); 0.07 g/g ($a = 11.12$, $b = -6.57$, $R^2 = 0.946$). Ageing rates of pea seeds were obtained by interpolation of previously determined data (Vertucci *et al.*, 1994). The figure is partly derived from Buitink *et al.* (2000c).

also obtained (0.16, 0.12, 0.09, 0.07 g H₂O g dw⁻¹). The value of σ was calculated for every 2.5°C between 35 and 65°C. For water contents of 0.12 and 0.16 g H₂O g dw⁻¹, the highest temperature for which σ was calculated was 55 and 45°C, respectively, because τ_R in pea axes at these high water contents could not be determined at higher temperatures due to partitioning of the spin probe into the lipid phase (for more details see Buitink *et al.*, 1998a). The relationship between the logarithms of σ and rotational motion of CP in the cytoplasm of pea axes was linear (Figure 37.5). When the σ values were calculated for temperatures lower than 35°C, the relationship between σ and τ_R started to deviate from a straight line below about 10–15°C (Figure 37.5, open symbols), indicating that below these temperatures the predictions from the viability equation were not correlated with the cytoplasmic molecular mobility.

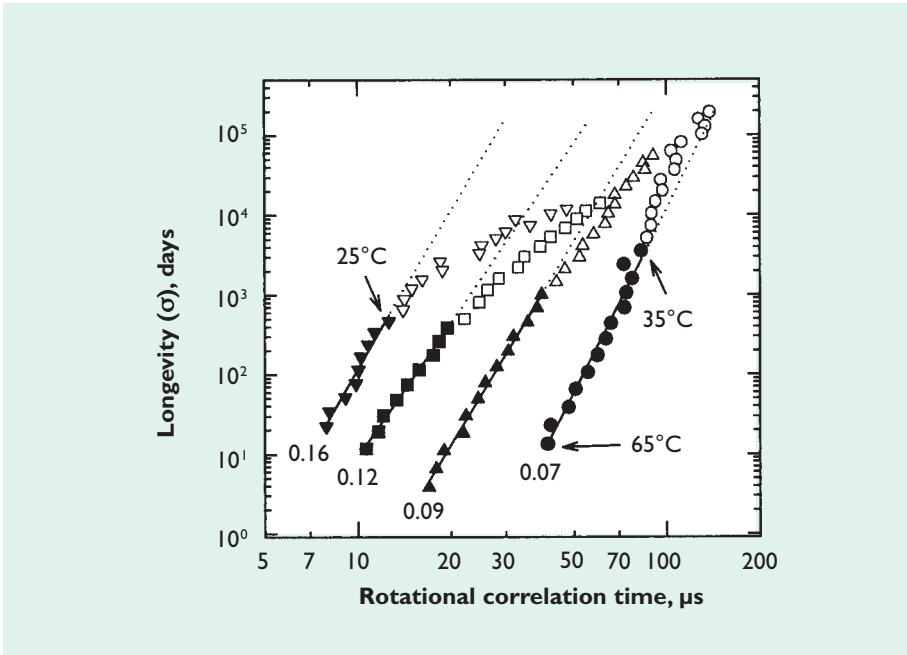


Figure 37.5 Relationship between the longevity (σ) of pea seeds and τ_R of pea axes. The σ (the time to lose one probit of viability) was calculated for every 2.5°C at different water contents using the viability equation (Ellis and Roberts, 1980), with experimental constants obtained from (Ellis *et al.*, 1989). Data were plotted on a double logarithmic scale. Water contents and temperature range for which σ was determined, together with constants and correlation coefficients are respectively: downward triangles, 0.16 g/g, 25 to 45°C ($a = -4.73$, $b = 6.79$, $R^2 = 0.964$); squares, 0.12 g/g, 35 to 55°C ($a = -4.58$, $b = 5.546$, $R^2 = 0.992$); upward triangles, 0.09 g/g, 35 to 65°C ($a = -7.14$, $b = 6.355$, $R^2 = 0.994$); circles, 0.07 g/g, 35 to 65°C ($a = -11.2$, $b = 7.64$; $R^2 = 0.966$). Open symbols represent the σ calculated for temperatures below 30°C. The figure is partly derived from Buitink *et al.* (2000c).

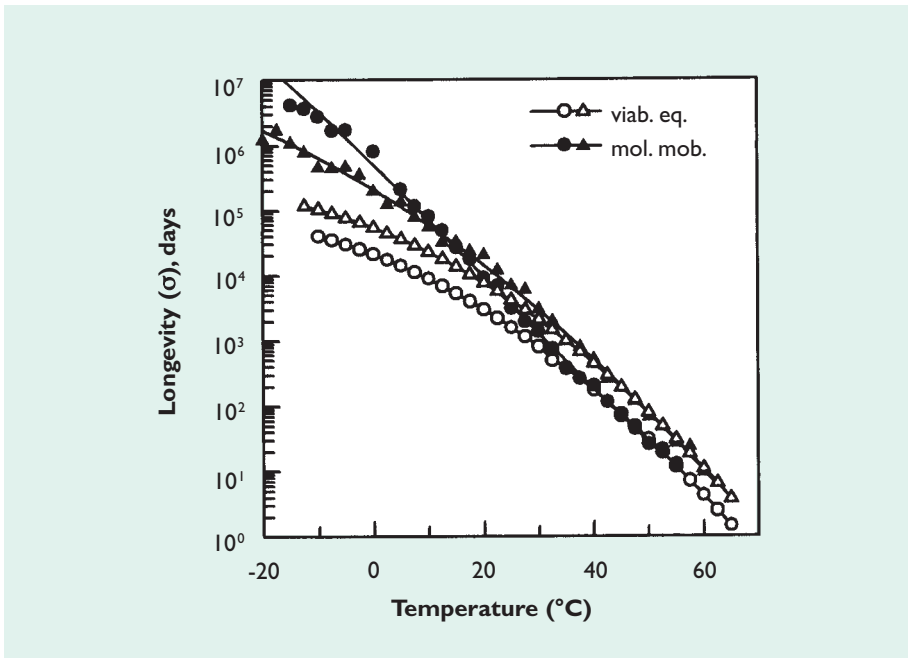


Figure 37.6 The effect of temperature on the estimated longevity of pea seeds, either by the viability equation (open symbols) or using the extrapolation of the linear regression of the relationship between molecular mobility and longevity from Figure 37.5 (closed symbols). The longevities were determined at two water contents, 0.09 (triangle) and 0.12 (circle) g H_2O g dw^{-1} .

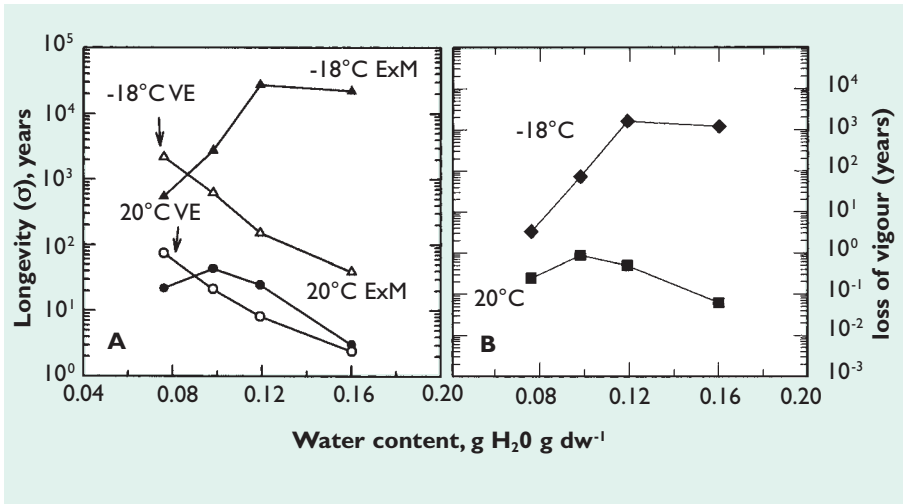


Figure 37.7 Relationship between rate of vigour loss or longevity of pea seeds and water content determined for different temperatures. (A) Estimation of the longevity (σ) calculated using the viability equation (open symbols) and estimation of the longevity (σ), extrapolated from the linear regressions in Figure 37.5 (closed symbols). (B) Estimation of the loss of vigour, extrapolated from Figure 37.4. VE stands for viability equation and ExM stands for extrapolation of mobility. The figure is partly derived from Buitink *et al.* (2000c).

Predictions of Longevity:

Molecular Mobility and the Viability Equation

The established linear relationship between the logarithms of rotational motion and longevity or ageing rates enabled us to extrapolate ageing rates or longevity to sub-zero temperatures by simply measuring the τ_{RS} at the required temperatures, assuming that the relationship between ageing rate and rotational motion remains linear at low temperatures (see dashed lines in Figures. 37.4 and 37.5). Thus, we estimated the longevity at temperatures as low as -20°C using the molecular mobility extrapolation and compared these estimates with the predictions based on the viability equation (Figure 37.6). The estimations of longevity derived from the viability equation increased with a decrease in temperature in a manner similar for both water contents (open symbols). The estimates based on the extrapolation of the molecular mobility also showed an increase in longevity with decreasing temperature, but especially at temperatures <20°C this increase was much stronger than that

determined by the viability equation. Furthermore, the increase in the longevity of pea seeds with decreasing temperature was stronger at $0.12 \text{ g H}_2\text{O g dw}^{-1}$ (circles) than at $0.09 \text{ g H}_2\text{O g dw}^{-1}$ (triangles). This resulted in a crossover of the two curves at around 10°C , implying that the longevity for any given temperature below 10°C will be higher for seeds stored at $0.12 \text{ g H}_2\text{O g dw}^{-1}$ than for seeds stored at $0.09 \text{ g H}_2\text{O g dw}^{-1}$. Likewise, the curve of longevity predicted using the mobility model for pea seeds stored at $0.07 \text{ g H}_2\text{O g dw}^{-1}$ crosses the curve of seeds stored at $0.09 \text{ g H}_2\text{O g dw}^{-1}$ at a temperature of 20°C (data not shown), indicating that the longevity for any given temperature below 20°C could be higher for seeds stored at $0.09 \text{ g H}_2\text{O g dw}^{-1}$ than for seeds with $0.07 \text{ g H}_2\text{O g dw}^{-1}$.

The final results of these calculations are plotted in Figure 37.7, showing the predicted longevity (σ) of pea seeds stored at different water contents at 20°C and -18°C , estimated using the viability equation (open symbols) and using the molecular mobility model (closed symbols, from Figure 37.5). Also shown are the experimental data of the rate of vigour loss derived from Vertucci *et al.* (1994) at 20°C together with the predictions made for -18°C using the linear regressions of Figure 37.4. The estimated longevity derived from the viability equation increased with decreasing water content for both temperatures (Figure 37.7A, open symbols). The predictions of longevity based on the extrapolations of the linear regressions in Figure 37.5 (closed symbols) showed an entirely different pattern upon a decrease in water content. At 20°C , a lowering of the water content from 0.16 to $0.09 \text{ g H}_2\text{O g dw}^{-1}$ increased longevity. However, a further decrease of the water content resulted in a subsequent decrease in longevity. At -18°C , the water content at which the estimated longevity was highest occurred around $0.12 \text{ g H}_2\text{O g dw}^{-1}$, and further reduction of the water content resulted in a decrease in longevity. The loss of vigour, either calculated from experimental data (20°C) or derived from predictions using the molecular mobility model showed a similar pattern, with optimum storage conditions around $0.09 \text{ g H}_2\text{O g dw}^{-1}$ at 20°C and $0.12 \text{ g H}_2\text{O g dw}^{-1}$ at -18°C . To validate the predictions and to demonstrate that the predictions of vigour loss are comparable to the loss of percentage germination, we plotted the linear regression in Figure 37.4 against the linear regression in Figure 37.5 for each water content. The regression coefficients of these plots were close to unity for all water contents: -0.97 for $0.07 \text{ g H}_2\text{O g dw}^{-1}$, -1.00 for $0.09 \text{ g H}_2\text{O g dw}^{-1}$, -1.10 for $0.12 \text{ g H}_2\text{O g dw}^{-1}$, and -1.00 for $0.16 \text{ g H}_2\text{O g dw}^{-1}$ (data not shown). Moreover, over a range of 30°C (between 5 and 35°C), one data set (loss of vigour) was based directly on experimental data, while the other was based on the extrapolation of the relationship between mobility and σ derived from the viability equation. This implies that rate of vigour loss can be considered as a measure of longevity. Furthermore, these results reinforce the hypothesis that cytoplasmic molecular mobility can indeed be used to describe and predict longevity.

Conclusion

Our predictions on the basis of molecular mobility support the contention of the existence of an optimum water content for storage that shifts to higher values with decreasing storage temperatures (Vertucci and Roos, 1993; Vertucci *et al.*, 1994; Buitink *et al.*, 1998b). Furthermore, if the predictions of longevity based on the molecular mobility model are correct, then longevity at sub-zero temperatures is higher than estimated by the viability equation at elevated water contents. Previously, Dickie *et al.* (1990) found a notably higher estimate of longevity of seeds using a slightly modified viability equation, correcting for the quadratic temperature term using an exponential function. Currently, protocols recommend seed storage at 5% moisture and -18°C. The predictions of longevity based on the molecular mobility model suggest that longevity at -18°C could be increased considerably by storing non-oily seeds at a water content higher than 7%. We suggest that future studies should be focussed on the dangers of over-drying germplasm for storage.

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