

Molecular mobility in the cytoplasm of lettuce radicles correlates with longevity

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Abstract

Molecular mobility is hypothesised to be a key factor influencing storage stability of seeds because it may control the rate of deteriorative reactions responsible for reduced shelf life. The relationship between the longevity of lettuce seeds and the molecular mobility was investigated in the cytoplasm of lettuce radicles. Longevity of lettuce seeds was predicted using the viability equation of Ellis and Roberts, and the molecular mobility was determined by saturation transfer electron paramagnetic resonance spectroscopy measurements of rotational motion of a polar spin probe inserted into the cytoplasm. Increasing the temperature resulted in faster rotational motion and shorter longevity. There was a linear relationship between the logarithms of rotational motion and estimated seed longevity for temperatures above 5°C. Below 5°C, there was a deviation from linearity. Based on the hypothesis that the molecular mobility in the cytoplasm determines the rate of detrimental reactions, seed longevity at sub-zero temperatures was predicted by extrapolation of the rotational motion using the linear relationship. Predictions of longevity at sub-zero temperatures based on rotational motion indicated longer survival times than those based on the viability equation. A kinetic approach to ageing using molecular mobility measurements is expected to improve our understanding of seed storage stability and might eventually lead to realistic predictions of longevity at low temperatures.

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Introduction

Seed longevity can be determined by the environmental conditions during storage. In general, low temperatures and low moisture contents are beneficial to long storage life (Roberts, 1972). One of the hypotheses underlying the improved stability of germplasm at low temperatures and moisture contents is that the cytoplasm transforms into a glassy state under these conditions (Williams and Leopold, 1989; Leopold *et al*., 1994; Sun and Leopold, 1994; Leprince and Walters-Vertucci, 1995; Sun, 1997; Buitink *et al*., 1998b). The high viscosity of this glassy state has been suggested to be responsible for delayed ageing and increased longevity. The life span of biological materials such as seeds and pollen was increased significantly during storage under conditions in which the cytoplasm was brought in a glassy state (Sun and Leopold, 1994; Sun, 1997; Buitink *et al*., 1998b).

It has been known for a long time that stabilisation of macromolecules is greatly enhanced by the presence of aqueous glasses. The shelf life of food materials has been associated with the presence of a glassy state (Roos, 1995 for a review). In the pharmaceutical industry, it is currently recognised that the presence of an amorphous state has very important implications for storage of pharmaceutical products (Hancock and Zografi, 1997). Formation of the glassy state results in a dramatic increase in viscosity and concomitant decrease in rotational and translational motion in many glass-forming

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substances (Soesanto and Williams, 1981; Roozen and Hemminga, 1990; Roozen *et al.*, 1991; Blackburn *et al.*, 1996; Champion *et al.*, 1997; Hemminga and Van den Dries, 1998; Van den Dries *et al.*, 1998). It has been suggested that molecular mobility measurements could be used in the prediction of shelf lives of amorphous drugs, assuming a direct relation between the molecular mobility and the rate of degradation of the product (Hancock *et al.*, 1995). Therefore, characterisation of the molecular mobility in the cytoplasm of seeds of different plant species, and comparing this information with longevity, is expected to give an answer as to whether these two parameters are interrelated.

The determination of molecular mobility can be achieved using saturation transfer electron paramagnetic resonance (ST-EPR) spectroscopy (Hemminga and Van den Dries, 1998; Buitink *et al.*, 1999). By the insertion of a nitroxide spin probe in cells, one can determine the rotational correlation time (τ_R) of the spin probe in its local environment. The τ_R is defined as the time it takes for the spin probe to rotate one radian (~60°) around its axis (Knowles *et al.*, 1976). In other words, the shorter τ_R, the faster the rotational motion of the spin probe. In biological systems, the choice of spin probe to use must be carefully considered before performing measurements on seeds using EPR spectroscopy. A polar spin probe, such as 3-carboxy-proxyl (CP) will tend to reside in the cytoplasm, whereas an apolar spin probe, like TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy), will be present in the lipid fraction. Therefore, to measure the mobility of a spin probe in the cytoplasm, care must be taken that the spin probe is indeed present in the cytoplasm. Moreover, during drying, amphiphilic spin probes have the tendency to partition into lipids (Golovina *et al.*, 1998), so a spin probe that is not present in the lipids at high water content could be present in the lipids upon drying. Measurements of the rotational motion in the cytoplasm is particularly difficult in oily seeds, because even a very polar spin probe such as CP will partition partially into the lipids upon drying and heating. In this study, we demonstrate how to perform measurements of cytoplasmic molecular motion in seeds containing high quantities of lipids. To this end, we used the high field part of the ST-EPR spectrum, which is not influenced by the presence of the spin probe in lipids (Hyde and Dalton, 1979).

One way of estimating seed longevity is by the use of the viability equation from Ellis and Roberts (1980a). This empirical equation can give accurate predictions of longevity over a wide range of temperatures and moisture contents (Ellis and Roberts, 1980a,b; Kraak and Vos, 1987; Ellis *et al.*, 1989, 1990, 1992; Dickie *et al.*, 1990; Mwasha *et al.*, 1997; Hong *et al.*, 1998, 1999). However, it has been recognised that the viability equation has some limits to its application (Ellis *et al.*, 1989, 1990; Dickie *et al.*, 1990; Vertucci and Roos, 1990; Vertucci *et al.*, 1994). Below a certain critical moisture content, the equation fails to adequately predict the viability of seeds (Ellis *et al.*, 1989, 1990; Vertucci and Roos, 1990; Vertucci *et al.*, 1994). Likewise, the temperature range has a lower limit, due to the quadratic function of the temperature equation (Dickie *et al.*, 1990). Therefore, when calculating the predicted longevity, the temperature and moisture content limits of the equation should be considered.

Lettuce is one of the most studied oily seeds with respect to longevity. The viability of lettuce seeds has been determined under a wide range of storage conditions (Kraak and Vos, 1987; Dickie *et al.*, 1990). Constants for the viability equation have been determined from experimental storage conditions ranging from 5°C to 75°C for moisture contents below 18% (Kraak and Vos, 1987).

In this study, rotational motion was determined in radicles of lettuce seeds at one water content and different temperatures using ST-EPR. The viability equation was used to infer longevity data for lettuce seeds at different storage temperatures, following published viability constants (Dickie *et al.*, 1990). The calculated longevity was compared with the cytoplasmic mobility, determined under similar conditions. The relationship between rotational motion and inferred longevity is discussed in relation to low temperature storage.

Material and methods

Plant material and sample preparation

Highly viable (99.9% germination) lettuce (*Lactuca sativa* L.) seeds were acquired from Rijk Zwaan (De Lier, the Netherlands). Lettuce seeds were allowed to imbibe for 2 h at 15°C, after which the radicles (approx. 10–15) were excised and incubated in 3 ml of 1 mM 3-carboxy-proxyl (CP, Sigma) at 15°C. After 45 min, potassium ferricyanide was added to a final concentration of 200 mM, and the radicles were incubated for another 15 min. The ferricyanide was added to broaden the signal of CP outside of the cells to invisibility. Because ferricyanide cannot penetrate intact cells, the signal obtained is exclusively derived from the spin probe present in the cells. Subsequently, the lettuce radicles were dried at 20°C in dry air (3% RH) for 24 hours. After drying, radicles were stored over a saturated salt solution of $MgCl_2$ at 20°C (33% RH) for 7–14 days.

Ten to 15 radicles were sealed in a 2-mm diameter glass capillary for EPR measurements. To prevent water loss during the measurements, the capillaries

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were sealed at both sides. Temperature was controlled using a temperature controller with liquid nitrogen vapour as the coolant. Samples were rapidly cooled to –40°C and allowed to equilibrate for 30 min. Subsequently, scans were recorded from –40°C to 45°C in 5°C increments after equilibration for 5 min after each increment. After the measurements, the radicles were removed from the capillaries and water contents were determined. Water contents were analysed by weighing the samples before and after heating at 96°C for 36–48 h (0.057 g H_2O g^{-1} fresh weight (fw).

Saturation transfer electron paramagnetic resonance spectroscopy (ST-EPR)

At low water contents and temperatures, τ_R of CP in seed radicles becomes longer than 10^{-8} s, resulting in the appearance of a powder spectrum (Buitink *et al.*, 1998a; Fig. 1A top spectrum). ST-EPR spectroscopy enables measurements of rotational motion in the motional region from 10^{-7} s to 10^{-3} s (Hyde and Dalton, 1979). ST-EPR is based on the diffusion and recovery of saturation between different parts of the powder spectrum in competition with field modulation (Hemminga, 1983). For ST-EPR measurements, the second harmonic quadrature absorption signal was detected under the following conditions: field modulation amplitude 5 G, microwave power 100 mW, and field modulation frequency 50 kHz (Hemminga *et al.*, 1984). The phase was set with the self-null method (Thomas *et al.*, 1976).

ST-EPR spectra can be well characterised by independent line shape parameters, such as the line-height ratios L″/L or H″/H. The ratio H″/H is especially useful when the spectrum consists of two overlapping spectra: one of the spin probe present in the immobile fraction (i.e. glassy cytoplasm) and one of the spin probe in a mobile, liquid environment (Fig. 1B, spectrum recorded at 35°C). The typical three-line spectrum of a spin probe in a mobile environment does not influence the high field region (H″/H) of the solid spectrum. Therefore, the rotational motion of the spin probe in the immobile fraction (i.e. glassy cytoplasm) can be obtained by determining the ratio H″/H (see Fig. 1B). Using reference material with known viscosity, τ_R values are usually obtained in an empirical way. We used spectra of CP in anhydrous glycerol to construct a calibration curve (Hemminga and Van den Dries, 1998). Because the viscosity for anhydrous glycerol is known over a broad temperature range, τ_R of CP in glycerol can be obtained from the modified Stokes–Einstein equation (Roozen *et al.*, 1991),

$$\tau_R = (\eta V / k_b T)\, k + \tau_0, \qquad (1)$$

where τ_R is the rotational correlation time, η is the solvent viscosity, k_b is Boltzmann's constant, V the volume of the rotating molecule, T the absolute temperature, τ_0 is the zero viscosity rotational correlation time, and k is a dimensionless slip parameter (0.09 for CP). The slip parameter was assumed to be temperature-independent (Hemminga and Van den Dries, 1998). Anhydrous glycerol, containing 1 mM CP, was cooled to –150°C, and after equilibration for 30 min, spectra were recorded in 3°C increments. For each temperature the value of the line shape parameter H″/H was calculated. From the curves representing the line shape parameters of CP in glycerol against τ_R, the values of τ_R of CP in the lettuce radicles were obtained by interpolation of the corresponding line shape parameters.

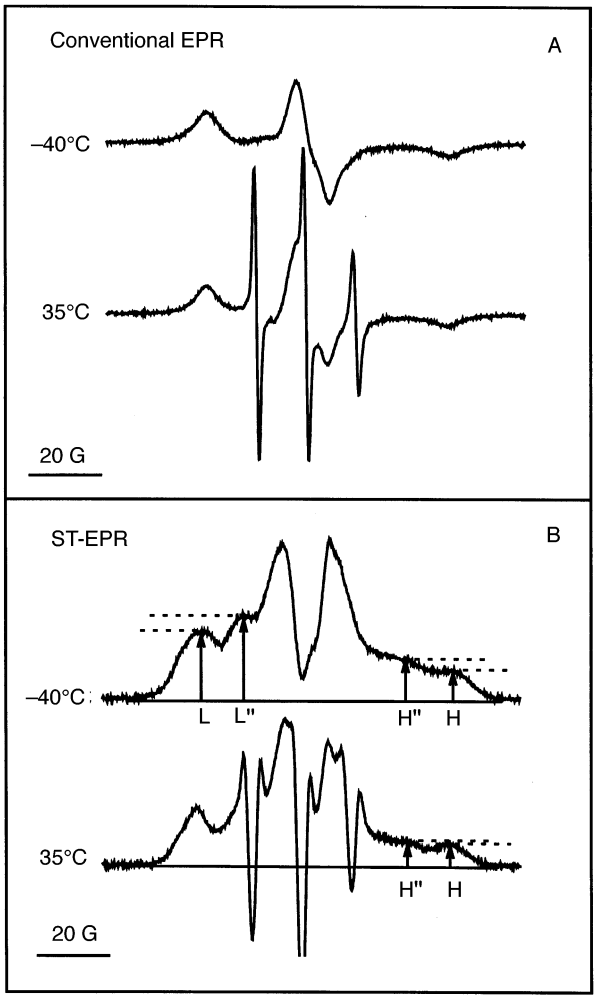


Figure 1. Conventional EPR (A) and saturation transfer EPR (B) spectra of CP in radicles of lettuce, recorded at two different temperatures. The lettuce radicles were equilibrated to 33% RH and had a moisture content of 0.057 g H_2O g^{-1} fw. The line height ratios L″/L and H″/H are indicated; H″/H was used to calculate the τ_R.

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Estimation of longevity of lettuce seeds

The longevity of lettuce seeds at different temperatures and 5.7% moisture on a fresh weight basis was estimated using the viability equation

$$\log \sigma = K_E - C_W \log m - C_H t - C_Q t^2 \qquad (2)$$

where m is seed moisture content, t is temperature (°C), and K_E, C_W, C_H and C_Q are seed viability constants for a certain species. The σ is the standard deviation of lifespans of individual seeds in a population under the storage conditions indicated (Ellis and Roberts, 1980a) and can be used as a measure of longevity, since it is the time in days during which percentage viability is reduced by one on the probit scale (Ellis and Roberts, 1980a; Dickie *et al.*, 1990). The constants used for lettuce were 6.985, 4.200, 0.0329, and 0.000478 for K_E, C_W, C_H and C_Q, respectively (Dickie *et al.*, 1990). The constants were derived from a fit of the equation to experimental viability data obtained under storage conditions of 5–75°C and 3.6–17.9% moisture on a fresh weight basis (Kraak and Vos, 1987).

Results

Using EPR spectroscopy, the rotational correlation time (τ_R) of a spin probe can be determined in its local environment. The longer the τ_R of the spin probe, the slower the rotational motion in the cytoplasmic surroundings. When the spin probe has a τ_R longer than 10^{-8} s, the conventional EPR spectrum takes the shape of a so-called powder spectrum (Fig. 1A, top spectrum). In this range of mobility, conventional EPR spectroscopy has become insensitive to rotational motion. However, line shape changes of ST-EPR spectra can be used to obtain information on rotational motion of the spin probe in the cytoplasmic surroundings in the range of 10^{-7} to 10^{-3} s (Fig. 1B, top spectrum). An often used line shape ratio is L″/L (Roozen and Hemminga, 1990; Roozen *et al.*, 1991; Hemminga and Van den Dries, 1998, Buitink *et al.*, 1999) (see indication of L″/L in Fig. 1B). One of the problems of applying spin probe EPR spectroscopy to oily seeds, such as lettuce, is that the spin probe partitions into lipids at elevated temperatures. The spin probe in liquid lipid bodies is characterised by a sharp three line conventional EPR spectrum (see Fig. 1A, bottom spectrum). Thus, the resulting spectrum consists of two overlapping spectra: that of the spin probe present in the immobile cytoplasmic surroundings, plus that in the fluid, mobile lipid bodies (compare Fig. 1A, top and bottom spectra). From Fig. 1B it is clear that the ratio L″/L cannot be determined when part of the spin probe is present in the lipids or any other mobile environment. However, the ratio H″/H is not influenced by the mobile spectrum (see Fig. 1B). Therefore, we calculated the t_R of the polar spin probe CP inserted in lettuce radicles using the H″/H ratio.

Figure 2 shows the dependence of τ_R values of CP on temperature for radicles of lettuce equilibrated to 33% RH. Increasing the temperature resulted in shorter τ_R values, indicating faster rotational motion. Around 0–10°C, the temperature dependence of the rotational motion of CP deviated from a straight line (see dashed line on the Arrhenius plot), indicating a change in activation energy of the rotational motion. Above these temperatures, rotational motion increased more rapidly with increasing temperature.

In order to compare cytoplasmic mobility with the longevity of lettuce seeds, the estimated longevity was calculated using the viability equation and constants (Dickie *et al.*, 1990). Figure 3 shows the dependence of longevity on temperature for lettuce seeds with a moisture content of 0.057 g H_2O g^{-1} fw. A decrease of temperature from 45°C to –20°C resulted in increased estimated longevity from about 24 to 19,000 days. Below a certain temperature, the relationship between temperature and longevity reversed (–36°C), resulting in a decrease of the longevity with a further decrease in temperature. This optimum in longevity as a function of temperature (Fig. 3) is the result of the quadratic term in the

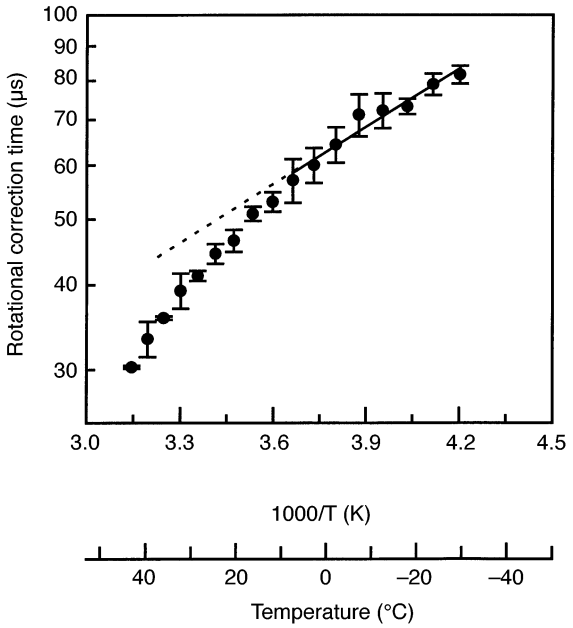


Figure 2. Arrhenius plot of the rotational correlation time (τ_R) of CP in lettuce radicles. The τ_R was calculated using the H″/H line height ratio. The lettuce radicles were equilibrated to 33% RH and had a moisture content of 0.057 g H_2O g^{-1} fw. Data are the average of three separate experiments, of which the standard errors are indicated.

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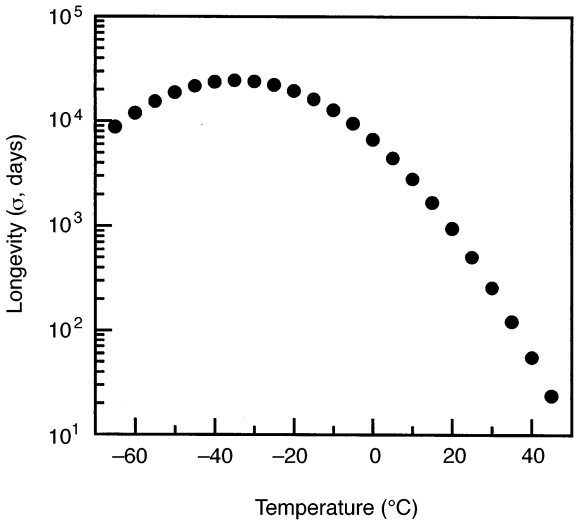


Figure 3. Estimated longevity (σ) of lettuce seeds, derived from the viability equation, in relation to temperature. Viability constants were derived from Dickie *et al.* (1990).

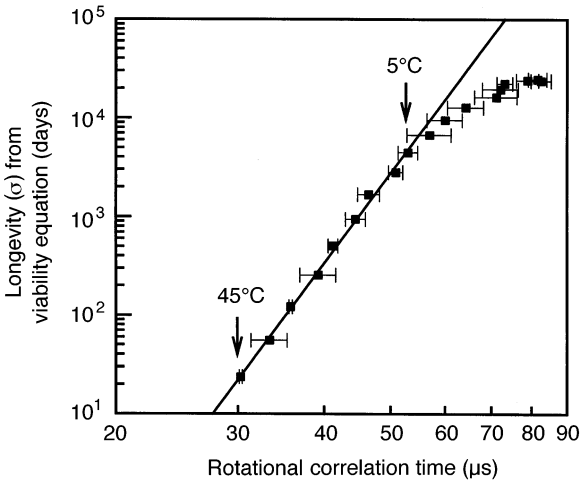


Figure 4. The relationship between rotational correlation time (τ_R) and estimated longevity of lettuce seeds, determined at different temperatures. The τ_R was determined using the H″/H ratio, obtained from ST-EPR spectra, whereas the estimated longevity was calculated using the viability equation with constants from Dickie *et al.* (1990). The τ_R and estimated longevity were determined every 5°C from –40°C to 45°C. The linear regression was drawn through data points in the temperature range from 45°C to 5°C (r^2 = 0.996). Constants for the linear regression {log $y = a + b \log x$} were a = –12.57, b = 9.42.

viability equation (eqn. 2) and is not based on experimental data (Dickie *et al.*, 1990).

To test the hypothesis that the molecular mobility in the cytoplasm influences the rate of deteriorative reactions associated with ageing, we plotted the rotational motion of CP in the cytoplasm of the lettuce radicles versus the estimated longevity using the viability equation (Fig. 4). The estimated longevity increased linearly with longer τ_R (slower rotational motion). This log–log relationship extended from 5 and 45°C (Fig. 4), corresponding to the same temperature range over which the viability constants for lettuce were determined based on experimental data (Kraak and Vos, 1987). At temperatures below 5°C, the relationship between rotational motion and estimated longevity deviated from the linear regression (Fig. 4), such that longevity was shorter than expected.

Assuming that the cytoplasmic molecular mobility is an accurate estimation of the rate of detrimental reactions, it is possible to predict longevity for temperatures below 5°C by linear extrapolation. Fig. 5 shows the predicted longevities as a function of temperature, both determined using the viability equation (closed symbols) and by extrapolation using linear regression between rotational motion and longevity (open symbols). Predictions of longevity at low temperatures, based on rotational motion, were greater than those based on the viability equation (Fig. 5). At –20°C, a temperature commonly used by gene banks to store seeds, the longevity predicted through rotational motion was approx. four-fold higher than that estimated by the viability equation. At –10°C, this difference in predicted longevity was about two-fold (Fig. 5).

Discussion

Molecular mobility in radicles of lettuce seeds

The rotational motion of the polar spin probe CP in the cytoplasm of lettuce radicles could be determined using the H″/H ratio of the ST-EPR spectrum. Above 0–10°C, the rotational motion of CP increased more rapidly with increase in temperature, apparent from the deviation from linearity in the Arrhenius plot. Around this temperature range, some change in the kinetics of rotational motion takes place. This change in ageing kinetics was not due to the melting of ice, because the water content (0.057 g H_2O g^{-1} fw) was far below the unfrozen water content of lettuce seeds (Roberts and Ellis, 1989). Alternatively, the change in kinetics might be due to the melting of neutral lipids. Although unlikely, the melting of oil bodies in the cells might have an impact on the mobility of CP in the cytoplasm. A possible explanation for the change in kinetics is that the break coincides with the melting of the glassy state (Sun, 1997). A steeper increase in rotational motion is expected when the glass has melted, and this would be manifest as an increase in

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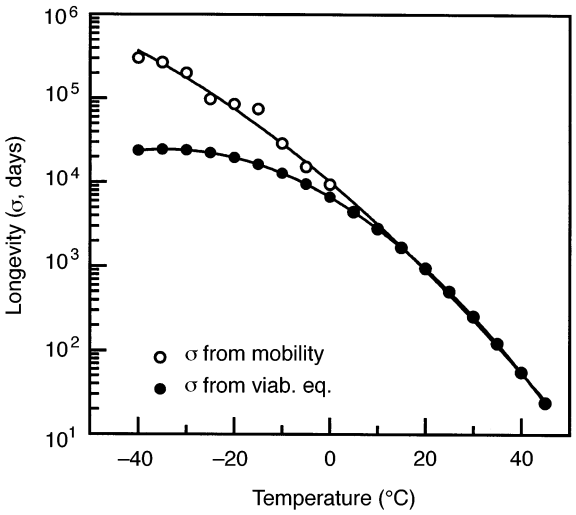


Figure 5. Comparison of the relationships between predicted longevity of lettuce seeds and temperature. Longevity was predicted either using the viability equation (closed symbols, derived from Fig. 3) or by extrapolation of rotational mobility data using the linear regression of Fig. 4 (open symbols).

activation energy, as found previously for various other glass forming substances (Roozen and Hemminga, 1990; Roozen *et al*., 1991; Blackburn *et al*., 1996; Van den Dries *et al*., 1998). An increase in activation energy upon melting of the glassy state has been found to be associated with altered ageing kinetics of *Typha* pollen and in the rotational motion of CP in pea axes (Buitink *et al*., 1998b, 1999). However, it was not possible to determine the glass transition temperature of lettuce seeds by differential scanning calorimetry, due to the large amounts of lipids obscuring any shift in baseline representing the glass transition. Moreover, whether a change in kinetics is due to the melting of the glassy state can only be certified by determining the temperature dependence of the rotational motion at different moisture contents. Because the glass transition temperature increases with decreasing moisture contents, the break would be expected to shift to higher temperatures in radicles with less water. Although further experiments are needed to establish the cause of the change in kinetics of motion, the change in kinetics is an important observation. If molecular mobility influences ageing rates, a change in the temperature dependence of the rotational motion implies that the kinetics of ageing is also expected to change at a certain temperature. In this regard, it is an interesting observation that in studies of the rate of viability loss in different seed species, the *Q*10 values varied sufficiently among temperature ranges to give a non-linear Arrhenius plot (Ellis and Roberts, 1980a; Dickie *et al*., 1990; Walters, 1998). This demonstrates that the kinetics of ageing in seeds deviates from Arrhenius behaviour, as found for the kinetics of rotational motion. However, that this deviation is due to the intracellular glass remains to be validated (Walters, 1998).

Molecular mobility correlates with longevity: use of the viability equation

The estimated longevity of lettuce seeds was calculated using the viability equation, in order to compare longevity with rotational motion in the cytoplasm at different temperatures. Although the lettuce cultivar used in this study was different from those used for the determination of the viability constants (Kraak and Vos, 1987), this will not influence the relation between temperature and longevity, because seed viability constants are generally applicable to all seed lots within one species (Ellis *et al*., 1982; Kraak and Vos, 1987). Because the viability constants for lettuce seeds were determined over a temperature range from 5 to 75°C (Kraak and Vos, 1987), the estimated longevity can be expected to be reasonably accurate within this temperature range. It is interesting to note that for the same temperature range for which experimental data were used to obtain the viability constants, the relationship between the logarithms of rotational motion and longevity was found to be linear (Fig. 4). This linearity suggests that molecular mobility influences the rate of ageing. Evidently, an increase in the cytoplasmic mobility results in faster reaction rates and eventually in faster cell death.

At low temperatures, it has been suggested that the viability equation fails to predict longevity accurately (Kraak and Vos, 1987; Dickie *et al*., 1990) due to the quadratic temperature term in the equation ($-C_Q t^2$). This is evident from the declining estimated longevity at temperatures below –36°C (Fig. 3). Therefore, the deviation of the linearity between estimated longevity and rotational motion at temperatures below 5°C might stem from inaccurate predictions from the viability equation.

If the predictions of longevity based on the extrapolation of rotation mobility data on the linear regression between rotational motion and estimated longevity (Fig. 4) are accurate, longevity at sub-zero temperatures should be higher than estimated by the equation. Previously, Dickie *et al*. (1990) found a higher estimate of longevity of seeds using a slightly modified viability equation, correcting for the quadratic temperature term using an exponential function. However, these authors stated that, for the time being, it is best to use the most conservative estimate of longevity, which is obtained using the viability equation. A kinetic approach to ageing using molecular mobility measurements could improve our understanding of seed storage stability and might

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eventually lead to improved predictions of longevity at low temperatures.

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