

Biological Glasses

**Nature's Way to
Preserve Life**

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CENTRALE LANDBOUWCATALOGUS



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Nature's Way to Preserve Life

Julia Buitink

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Propositions

1. The properties of intracellular glasses cannot be generalised from studies of simple solutions and must be independently studied – R.J. Williams et al. (1993) Japan. J. Freezing and Drying, 39:3-12; this thesis.
2. The statement “a good glass former” should not be based on the glass transition temperature only, but it should also take into account the molecular mobility – this thesis.
3. The role of oligosaccharides in the survival of seeds cannot be explained by the current hypotheses – this thesis.
4. The crossover temperature T_c , below which dynamic transitions of molecules are hindered, is important for the suppression of functions of biological molecules. Therefore, the high T_c in biological systems could play an important role in the preservation of life – A.P. Sokolov et al. (1999) J. Chem. Phys.; this thesis.
5. The most important “ingredient” of any theory of glass transition is the molecular mobility – J. Perez (1994) J Food Eng 22:89-114.
6. The removal of the evolution theory from school science and the imposition of creationism as “science” in Kentucky (USA) is the result of the creation of a false choice between creationism and atheism, which the creationists equate with orthodox science.
7. The absence of evidence is not evidence of absence – R. Dixon, 1981.
8. Whenever the methods of observation are such that a complicated mathematical treatment is required to establish a relationship between the data and the physical nature of the system studied, the methods of observation are inadequate and must be changed, whenever possible – A. Abragam, The principles of nuclear magnetism, 1961.
9. Successful research starts with asking the right question.
10. Scientific researchers who try to cross disciplinary boundaries are faced with the dilemma, that though multidisciplinary approaches are often recommended and praised, professional evaluation of scientific performance is, almost without exception, carried out in monodisciplinary mode – V. Curtis, 1998.

Propositions belonging to the PhD thesis entitled “Biological glasses: nature's way to preserve life”.

Wageningen, 28th June 2000

Julia Buitink

Contents

Voorwoord	1
Abbreviations	2
General Introduction -----	3
Section I - Intracellular Glasses -----	19
1. Calorimetric properties of dehydrating pollen: analysis of a desiccation-tolerant and an -intolerant species	21
2. Storage behaviour of <i>Typha latifolia</i> pollen at low water contents: Interpretation on the basis of water activity and glass concepts	33
Section II - Molecular Mobility -----	47
3. Influence of water content and temperature on molecular mobility and intracellular glasses in seed and pollen.....	49
4. Pulsed EPR spin-probe study of intracellular glasses in seed and pollen	67
5. Characterization of molecular mobility in seed tissues: an EPR spin probe study	77
Section III - Ageing Kinetics and Molecular Mobility -----	91
6. The effects of moisture and temperature on the ageing kinetics of pollen: interpretation in terms of cytoplasmic mobility	93
7. Molecular mobility in the cytoplasm of lettuce radicles correlates with longevity	105
8. Molecular mobility in the cytoplasm: an approach to describe and predict lifespan of dry germplasm	115
Section IV - Composition and Properties of Intracellular Glasses -----	129
9. A critical assessment of the role of oligosaccharides in intracellular glass stability.....	131
10. Is there a role for oligosaccharides in seed longevity? An assessment of intracellular glass stability.....	137
11. High critical temperature above T_g may contribute to the stability of biological systems	151
General Discussion -----	169
References.....	182
Summary	193
Samenvatting	197
List of publications.....	200
Curriculum Vitae	202

Voorwoord

Voor u ligt het proefschrift waar ik de afgelopen vier jaar aan heb gewerkt. Het waren vier fantastische jaren: vol verbazing, spanning, lol, vrijheid, een beetje terleurstelling en frustratie, en ook met schitterende momenten. Dit boekje had niet tot stand kunnen komen zonder de vele mensen die ik hieronder graag wil bedanken. Ten eerste, Folkert, jij bent degene geweest die mij geïntroduceerd heeft in de wonderen van de wetenschap. Het enthousiasme dat je me hebt bijgebracht voor de wetenschap, samen met het inzicht in onderzoek en het communiceren daarvan is van vitaal belang geweest voor het tot stand komen van dit boekje. I also would like to express my gratitude to Prof. Sergei Dzuba, with whom I collaborated during my stay at the Institute of Chemical Kinetics and Combustion in Novosibirsk, Russia. Sergei, thank you for your enthusiastic introduction into echo-detected EPR spectroscopy. I thoroughly enjoyed my stay in Novosibirsk, and the interaction I had with you and your colleagues. Mark, bedankt voor je gezelschap (is de koffie al klaar!?) en je computerprogramma's die het mogelijk maakten al de data snel uit te werken. Linus, de laatste loodjes waren een stuk makkelijker door jouw inzet, bedankt.

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Abbreviations

BET	Brunauer, Emmett and Teller
CP	3-carboxy-2,2,5,5-tetramethylpyrrolidine-1-oxyl; 3-carboxy-proxyl
C_p	heat capacity
DMTA	dynamic mechanical thermal analysis
DSC	differential scanning calorimetry
dw	dry weight
E_a	activation energy
ED EPR	echo-detected electron paramagnetic resonance
EPR	electron paramagnetic resonance
FTIR	fourier transform infrared
fw	fresh weight
G	Gauss
η	viscosity
ΔH	enthalpy
k	rate constant
M_2	second moment
M_w	molecular weight
M_n	average molecular weight
NMR	nuclear magnetic resonance
ν_{OH}	band position of OH-stretch
PolyLys	poly-L-lysine
R	ideal gas constant
RH	relative humidity
SD	standard deviation
Stach	stachyose
ST-EPR	saturation transfer electron paramagnetic resonance
Suc	sucrose
T_c	critical temperature
TEMPO	2,2,6,6-tetramethyl-1-piperidinyloxy
TEMPOL	4-hydroxy-2,2,6,6-tetramethyl-1-piperidinyloxy
TEMPONE	4-oxo-2,2,6,6-tetramethyl-1-piperidinyloxy
T_g	glass-to-liquid transition temperature
τ_R	rotational correlation time
TSDC	thermally stimulated depolarisation current
P_{50}	mean viability period
wc	water content, $g H_2O g dw^{-1}$
WLF	Williams, Landel and Ferry

General Introduction

"The major biological reason for the existence of anhydrobiosis is a device serving to conserve reserves and stabilize the organisms under adverse conditions until adequate water becomes available."

James S. Clegg (1986)

Storage stability of germplasm

Desiccation-tolerant organisms (anhydrobiotes), such as seed and pollen, are capable of surviving the removal of the major part of their cellular water. Apart from the ability to survive dehydration and rehydration, these tissues also have to be able to survive for extended periods of time in the dry state. The lifespan of seeds can be remarkably long, ranging from decades (Priestley et al., 1985) to century's (Kivilaan and Bandurski, 1981; Steiner and Ruckenbauer, 1995) and even millennia (Shen-Miller et al., 1995). The most remarkable discovery was that on ancient seeds of Sacred Lotus from China; radiocarbon dating showed an age of these seeds of 1288 ± 271 years while still being capable to germinate (Shen-Miller et al., 1995). Apparently, nature has endowed seeds with mechanisms to survive the dry state for extended periods of time. In this thesis we will investigate the physical properties of dry anhydrobiotes to obtain a better understanding of desiccation tolerance and lifespan.

The processes leading to cell death in seeds have received considerable attention (Priestley, 1986; Smith and Berjak, 1995; Walters, 1998; Pammenter and Berjak, 1999 for reviews). As seeds age, membranes become leaky, enzymes lose catalytic activity and chromosomes accumulate mutations. It also has been proposed that food reserves are depleted as the seed deteriorates, and there is growing evidence pointing to the toxicity of by-products of catabolic reactions (Yaklich, 1985; Wettlaufer and Leopold, 1991; Bernal-Lugo and Leopold, 1992, 1995; Baker and Bradford, 1994; Zhang et al., 1994; Sun and Leopold, 1995). The rates at which all these detrimental reactions take place most likely determine the rate of ageing, and thus the lifespan of seed and pollen.

Apart from these intrinsic properties, the lifespan is influenced by the conditions under which the dry germplasm is stored. Temperature and water content are important factors playing a role in storage stability. It is common knowledge that low water contents and low temperatures are beneficial to the longevity of germplasm (Roberts, 1972; Justice and Bass, 1978; Priestley, 1986). Several models have been developed to describe the effects of these two parameters on viability. Early models, such as Harrington's Thumb Rules, propose that the time for 50% of the seeds to die (P_{50}) doubled for every 5°C reduction in temperature or 1% reduction in moisture level (Justice and Bass, 1978). The empirical viability equation of Roberts and Ellis was developed to account for differences in seed species and seed lots and to provide a more absolute prediction of lifespan (Roberts, 1972; Ellis and Roberts, 1980a; Ellis, 1991). This equation relates the seed storage conditions (water content and temperature) to the viability lost within the population during storage. This model has proven its validity in a number of studies (Ellis and Roberts, 1980a,b; Kraak and Vos, 1987; Ellis et al., 1989, 1990b, 1992; Dickie et al., 1990; Mwashia et al., 1997; Hong et al., 1998, 1999). Nonetheless, it has been recognised that there are some limits to its application with respect to low temperature and low and high water contents (Ellis et al., 1989, 1990b,

1991, 1995; Dickie et al., 1990; Vertucci and Roos, 1990, 1993a; Vertucci et al., 1994b; Walters and Engels, 1998). Although these empirical models are useful in estimating the effects of water content and temperature on longevity, they do not provide an understanding what the underlying causes are for these observations.

A more theoretical approach to understand longevity was based on the hypothesis that seed ageing reactions are controlled by the thermodynamic status of water in the seeds (Priestley, 1986; Leopold and Vertucci, 1989; Vertucci and Roos, 1990, 1993b). Vertucci and co-workers used water sorption isotherms to determine the thermodynamic states of water in seeds and pollen (Vertucci and Roos, 1990, 1993b). This approach resulted in the discovery that the optimum water contents of storage shifted to higher values with lower temperatures (Vertucci et al., 1994b).

While water-activity and water-binding models help to understand the nature of chemical reactions in relation to water content, they do not address how the kinetics of these reactions are affected by water content. Over the past years, a new approach has been introduced to understand the effects of water content and temperature on longevity of germplasm, based on the formation of glasses. Glasses are semi-equilibrium solid liquids with an extremely high viscosity (Franks et al., 1991). Low temperatures and low water contents drive the viscosity to such high values that the cytoplasm will form a glassy state. The high viscosity is thought to be responsible for the decreased ageing rates observed at these low water contents and temperatures. The formation and properties of glasses in sugar and polymer systems and biological tissues will be discussed in the following sections.

Properties of glasses

Solids can exist in a crystalline or amorphous form. Crystalline materials have defined structures, stoichiometric compositions and melting points. They are characterized by their chemical, thermal, electrical, optical and mechanical properties. By contrast, amorphous materials have no defined structure. The three-dimensional long-range order that normally exists in a crystalline material does not exist in the amorphous state, and the position of molecules relative to one another is more random, comparable with that in the liquid state (Atkins, 1982; Fig. 0.1). Glasses are not an equilibrium phase, implying that eventually they will move to equilibrium, i.e. they will crystallise. Due to the high viscosity of the glassy state, this transformation to equilibrium occurs at such slow rate that a glass is referred to as "semi"-equilibrium state. The transition from equilibrium to equilibrium phase (for instance crystal to liquid) is a first order transition, a *phase* transition. The glass-to-liquid or glass-to-crystal transition is a second order transition, a transition from a non-equilibrium state to an equilibrium phase. This transition is referred to as a *state* transition.

Glasses are created by rapidly cooling a liquid so that it remains in the liquid state well below the normal freezing point. In principle, a liquid should freeze (crystallise) when cooled to a temperature below its freezing point.

However, if the rate of cooling is high relative to the rate of crystallisation, then the liquid state can persist well below the normal freezing point. As cooling continues, there is a rise in the rate of increase of the viscosity of the supercooled liquid per unit drop in temperature. The initially mobile fluid turns into a syrup, then into a viscoelastic "rubber" (in case of large molecules) and

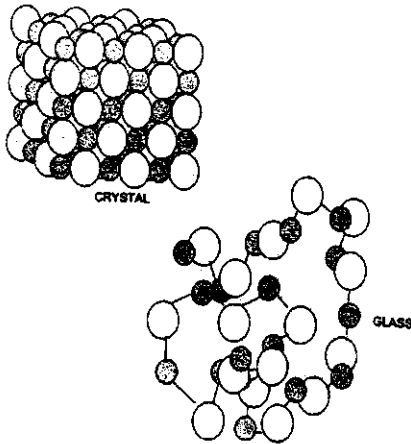


Figure 0.1. Schematic diagram of crystalline and glassy solids. Crystalline structure is regular, whereas there is no particular orientation to the molecules in the glass (adapted from Walters, 1998).

finally into a brittle glass. A glass is therefore a supercooled liquid with an extremely high viscosity - typically in the order of 10^{14} Pa s (Franks et al., 1991). Franks et al. (1991) gave the following elegant example: "The physical significance of such a high viscosity is more easily appreciated if it is converted to a flow rate. The flow rate of a typical liquid is in the order of 10 m/s, compared to 10^{-14} m/s in the glassy state. In other words, a glass is a liquid that flows about 30 μm in a century".

Glass formation can be viewed as an intrinsic property of all liquids, including water and aqueous solutions, given only that the formation of the crystalline phase is substantially avoided during, for instance, cooling or drying. Examples of well-known and thoroughly studied molecules that can undergo glass transitions can be found in the food and polymer science, and include sugars, proteins, starch, complex food systems, and a wide range of polymers (Sperling, 1986; Levine and Slade, 1988; Slade and Levine, 1991a,b, 1994; Roos, 1995).

The glass-to-liquid transition

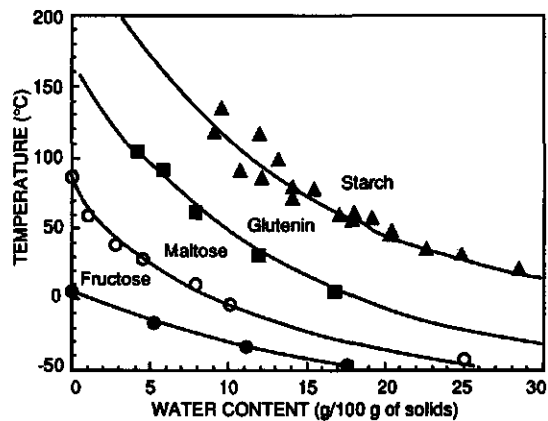
The glass-to-liquid transition occurs over a small range of temperatures (Franks et al., 1991), and can be identified experimentally by the use of various techniques, such as differential scanning calorimetry (DSC), thermally stimulated depolarisation current (TSDC) method, electron paramagnetic resonance (EPR) spectroscopy, nuclear magnetic resonance (NMR) spectroscopy, and dynamic mechanical thermal analysis (DMTA) (Williams, 1994; Roos, 1995 for reviews). The most popular technique to detect glass transition temperatures (T_g), however, is DSC that detects the transition from

glassy to liquid by a discontinuity in specific heat during constant heating of a glassy material (Franks et al., 1991).

The magnitude of the T_g is dependent on the composition of the amorphous state. For pure synthetic polymers, in the absence of a diluent, T_g is known to vary with M_w in a characteristic and theoretically predicted fashion (Ferry, 1980; Slade and Levine, 1991b). For a homologous series of amorphous linear polymers, T_g increases with increasing number-average M_w (M_n) until the entanglement region is reached ($M_n > 3000$). This increase in T_g with M_n is due to decreased free volume (increasing local viscosity) contributed by side ends of the polymers (Ferry, 1980; Sperling, 1986). The relationship between the T_g and solute M_w has been established from DSC measurements of over 150 different food carbohydrates (Levine and Slade, 1988; Slade and Levine 1991a,b).

The temperature of the glass transition is not only dependent on the composition of the solute, but also on the presence of plasticizers. In the polymer field, the classical definition of a plasticizer is 'a material incorporated in a polymer to increase the polymer's workability, flexibility or extensibility' (Sears and Darby, 1982). Characteristically, the T_g of an undiluted polymer is much higher than that of a typical low M_w glass forming diluent. As the diluent concentration of a solution increases, T_g decreases monotonically, because the average M_w of the homogeneous polymer-plasticizer mixture decreases, and its free volume increases (Ferry, 1980). It has been stated that water is the most ubiquitous plasticizer in the world (Sears and Darby, 1982). Plasticization, on a molecular level, leads to increased intermolecular space or free volume, decreased local viscosity, and concomitant increased mobility (Ferry, 1980).

Figure 0.2. State diagram representing the effect of water on the glass transition temperature of several food-related materials (adapted from Roos, 1995).



The effect of plasticization of water on the T_g is usually depicted in a state diagram (Fig. 0.2). Increasing the water content of a sugar-water mixture results in a decrease in T_g (Ferry, 1980). Figure 0.2 shows that a higher M_w compound exhibits a higher T_g over the entire range of water contents.

Glasses, stability and molecular mobility

It has been known for a long time that stabilisation of many 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 this respect, the extensive work of Levine and Slade, who applied the glass concept from polymer science to food systems, is of particular importance (Levine and Slade, 1988; Slade and Levine, 1991a,b, 1994). In the pharmaceutical industry, it is currently recognised that the presence of an amorphous phase has very important implications for storage of pharmaceutical dosage forms (Hancock and Zografi, 1997). The high stability of the glassy state is related to the high viscosity and concomitant low molecular mobility. Upon formation of the glassy state, viscosity increases steeply over several orders of magnitude. It is this high viscosity that is thought to confer stability by slowing down detrimental reactions and by preventing crystallisation and conformational changes. In general, high M_w sugars will exert a higher stability because of their higher T_g . Under similar conditions of temperature and water content, low M_w sugars might be above their T_g , while high M_w sugars are still in a glassy state (below T_g) (see Fig. 0.2).

The temperature dependence of reaction rates or mobility below T_g and about 100°C above T_g can be described by the Arrhenius equation (Levine and Slade, 1988; Karmas et al., 1992; Nelson and Labuza, 1994),

$$k = k_0 \exp(-E_a/RT) \quad (0.1)$$

where k is the rate constant at temperature T , k_0 is a pre-exponential factor, R is the ideal gas constant and E_a is the activation energy.

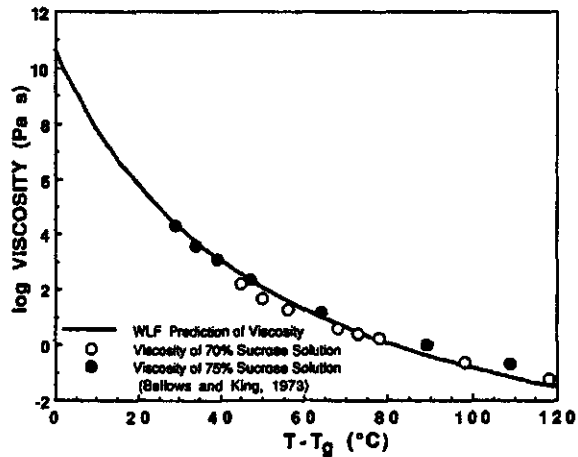
For the first 100°C above T_g , the temperature dependence of viscosity, translational or rotational relaxation times of model glasses such as sugar-systems and polymers cannot be described by an Arrhenius-like relationship. Several models have been developed which describe the kinetics of viscosity or molecular mobility for glass-forming substances above T_g (Perez, 1994). The Williams-Landel-Ferry (WLF) equation (eqn 0.2) is an often used model in food-polymer science to predict the effect of increasing temperature on relative relaxation times above T_g (Williams et al., 1955; Ferry, 1980; Soesanto and Williams, 1981; Chan et al., 1986; Roos and Karel., 1991; Steffen et al., 1992; Champion et al., 1997),

$$\log a_T = -C_1(T-T_{ref})/(C_2+(T-T_{ref})) \quad (0.2)$$

where C_1 and C_2 are system-dependent coefficients (Ferry, 1980), and a_T is defined as the ratio of the relaxation phenomenon at T to the relaxation at the reference temperature T_{ref} . This empirical equation was originally derived from the free volume interpretation of the glass transition. Average values for the WLF coefficients ($C_1=17.44$ and $C_2=51.6$) were calculated by Williams et al.

(1955) using the available values for many synthetic polymers. The universal constants have been shown to also apply to molten glucose (Williams et al., 1955), amorphous glucose-water systems (Chan et al., 1986), amorphous sucrose and lactose powders at low moisture (Roos and Karel, 1991), and concentrated solutions of mixed sugars (Soesanto and Williams, 1981). However, several problems are associated with the use of the average coefficients in the WLF equation (Peleg, 1992), and other values have been used to obtain a better fit (Ferry, 1980; Slade and Levine, 1991a; Champion et al., 1997). The WLF equation describes that melting of the glass results in a dramatic decrease in viscosity and increase in translational and rotational motion, as illustrated by the solid line in Fig. 0.3 (Soesanto and Williams, 1981; Roozen et al., 1991; Steffen et al., 1992; Blackburn et al., 1996; Deppe et al., 1996; Champion et al., 1997; Hemminga and Van den Dries, 1998; Van den Dries et al., 1998a). A consequence of the abrupt increase in molecular mobility in sugar glasses upon increasing the temperature above T_g is that the

Figure 0.3. Viscosity of 70 and 75% sucrose solutions and the WLF prediction of viscosity above the T_g (adapted from Roos and Karel, 1991).



systems exhibit a collapse at a certain temperature above T_g , observed as a macroscopically visible change in physical properties. The collapse is generally attributed to a reduction in viscosity upon increasing the temperature above T_g such that flow on a practical time scale is observed (for review see Roos, 1995). Using the WLF equation, the calculated viscosity at which the collapse is observed is generally around 10^8 Pa s (Slade and Levine, 1991a). For various mono- and disaccharides, the temperature of structural collapse and glass softening occurs 10-17°C above T_g (Slade and Levine, 1991a; Roos, 1995; Sun, 1997a). This collapse phenomenon results in a dramatic loss of stability of the matrixes, such as loss and oxidation of encapsulated lipids and flavours, loss of enzymatic activity, nonenzymatic browning, stickiness and caking or structural collapse (for review see Slade and Levine, 1991a; Roos, 1995). The sharp increase in mobility above T_g also leads to the crystallisation of pure

amorphous sugars. Indeed, the crystallisation of sucrose was found to follow WLF behaviour (Roos and Karel, 1991; Fig. 0.3).

Since the late 1980s, also seed biologists have embraced the concept of glass formation. What has been discovered in the past decade concerning glasses in biological systems is discussed in the next section.

Glass formation in biological systems

In general, desiccation-tolerant biological systems have the ability to form glasses. Glass formation in seeds should be viewed as a natural consequence of drying (Walters, 1998). The complex mixture of molecules present in the cytoplasm of the cells is likely to prevent crystallisation, thus making glass formation inevitable. Some well-known examples of tissues that form glasses are seeds (Williams and Leopold, 1989; Leopold et al., 1994, Leprince and Walters-Vertucci, 1995), pollen (Buitink et al., 1996), prokaryotes (Potts, 1994), and the so-called resurrection plants (Wolkers et al., 1998b). These tissues are capable of resisting extreme dehydration and are able to completely resume metabolic activity after the addition of water. All these tissues show a large accumulation of soluble sugars, predominantly sucrose, trehalose, and, in the case of seeds, oligosaccharides like raffinose and stachyose (Amuti and Pollard, 1977; Hoekstra et al., 1992; Ghasempour et al., 1998). It has been suggested that these sugars play an important role in the glass formation in biological tissues because of their reputation as being excellent glass-formers (Hirsh, 1987; Koster, 1991).

Just over a decade ago, the hypothesis that the cytoplasm of seeds could form glasses was put forward. Leopold and Vertucci (1986) suggested that in desiccation-tolerant systems, a mixture of sugars might serve to defer crystallisation, thus maintaining the effectiveness of sucrose in replacing water at the hydrophilic sites of the cellular membranes. At the same time, Burke (1986) suggested that in anhydrous organisms, glasses could be formed from cell solutes like sugars that are known to provide protection from denaturation of large molecules and formation of molecular aggregates. Also, glasses might fill spaces in a tissue, and during dehydration may serve to stop excessive increases in tissue collapse, solute concentration, and pH alteration. Furthermore, glasses are exceedingly viscous and should stop all chemical reactions that require molecular diffusion. In so doing, they may assure stability over time (Burke, 1986).

Evidence for the occurrence of aqueous glasses in organisms came from Hirsh et al. (1985), who found that at -28°C , ice crystals were absent from plants that were resistant to freezing, as derived from the direct observation of second-order transitions in the DSC. Subsequently, Leopold and co-workers focussed on the presence and significance of glasses in seed tissue. Williams and Leopold (1989) demonstrated the occurrence of a second order transition due to glass transitions in corn embryos using DSC, and it was concluded that these tissues were in a glassy state at room temperature below 12% moisture. It has been suggested that the solutes contributing to the glassy state could be

sugars, based on a study on sugars and desiccation tolerance (Koster and Leopold, 1988). Thereafter, many reports emerged on the detection of glass transitions in seed tissues with the use of different techniques, such as DSC (Vertucci, 1990; Leprince and Walters-Vertucci, 1995), EPR (Bruni and Leopold, 1991), TSDC (Bruni and Leopold, 1992; Konsta et al., 1996; Pissis et al., 1996), and mechanical analysis (Williams, 1994; Maki et al., 1994). Seed species investigated were pea, maize, soybean, wheat and bean. In subsequent papers, the emphasis shifted to the composition and function of the glasses in seeds.

Composition of intracellular glasses

Several authors have investigated the composition of intracellular glasses and especially the role of soluble sugars in the glass formation in dry anhydrobiotes. Mixtures of soluble sugars similar to those found in desiccation-tolerant maize embryonic axes (85% (w/w) sucrose, 15% (w/w) raffinose) are capable of forming glasses at temperatures above 0°C, whereas sugar mixtures similar to those found in desiccation-sensitive axes (75% (w/w) glucose and 25% (w/w) sucrose) only form glasses at subzero temperatures (Koster, 1991). Based on these results, it has been suggested that the composition of soluble sugars is important for glass formation in desiccation-tolerant systems. Bernal-Lugo and Leopold (1995) demonstrated that during accelerated ageing of maize seeds, raffinose contents decreased, concomitant with a decrease in the magnitude of the glass signal, measured by TSDC. It has been suggested that in maize seeds storage stability is a reflection of the mixture of sucrose with raffinose rather than of the total soluble sugars. Williams and Leopold (1995) showed that after 50h of imbibition, the T_g of pea embryonic axes was remarkably lower than that of axes imbibed for 14h, accompanying the loss of oligosaccharides and replacement by monosaccharides. It was suggested that the presence of galactosides and sucrose may contribute to the stability of pea seeds to drying, achieved by maintaining a vitreous state at high temperature.

However, some papers question the role of sugars in intracellular glass formation. For instance, Sun and Leopold (1993) found that during accelerated ageing of soybean seeds the magnitude of the glassy signal and T_g , both measured by TSDC, decreased. Yet, no differences were observed in sucrose, raffinose and stachyose contents during accelerated ageing. Despite different sugar compositions in soybean axes as compared to oak cotyledons, their T_g state diagrams were similar (Sun et al., 1994). Also, maturation of soybean axes, which resulted in accumulation of oligosaccharides, did not change the state diagram (Sun et al., 1994). Another indication that sugars alone are not sufficient to explain the formation of the vitreous state in seeds came from Sun and Leopold (1997), who showed that the state diagram of corn embryos was different from that of a representative carbohydrate-mix. An extensive calorimetric study on the glass transition in bean axes revealed the complexity of intracellular glasses (Leprince and Walters-Vertucci, 1995). Correspondence

of DSC data from bean to a model that predicts the effects of glass components on the T_g suggested that intracellular glasses could be composed of a highly complex oligomeric sugar matrix, such as, for instance, malto-dextrin. It is clear from these data that in addition to the soluble sugars, other seed constituents could contribute to the glassy state, including glycosides, larger carbohydrates, and even proteins (Leopold et al., 1994).

Function of intracellular glasses

Intracellular glasses were originally suggested to play a role in both desiccation tolerance and storage stability. Soluble sugars similar to those found in desiccation-tolerant seeds are capable of forming glasses at temperatures higher than zero, whereas sugar mixtures similar to those found in axes that do not tolerate desiccation only form glasses at subzero temperatures (Koster, 1991). However, Sun et al. (1994) showed that during maturation, soybean embryonic axes at desiccation-intolerant stages were able to form glasses upon drying. Furthermore, the T_g values of desiccation-sensitive red oak cotyledons as a function of water content were found to be indistinguishable from that of mature desiccation-tolerant soybean axes (Sun et al., 1994). It is important to realise that the water content at which glass formation occurs during drying at room temperature in seeds (~10-15% moisture) is much lower than the critical water content most desiccation-sensitive species exhibit. Apparently, dehydration-induced damage in these seeds occurs at water contents far above that at which protection of the glassy state can be effective.

The major function of intracellular glasses in dry seeds may be its contribution to the stability of the seed components during storage. Sun and Leopold (1993) found that during accelerated ageing of soybean seeds, the magnitude of the glassy signal as well as the T_g (TSDC) gradually decreased, and that the embryonic axes eventually lost their ability to enter the glassy state. It has to be noted, however, that the ageing conditions brought the tissues above their T_g , and that the glassy signal decreased prior to the loss of germination capacity. It was nevertheless proposed that the loss of the glassy state during accelerated ageing leads to an enhanced rate of subsequent deteriorative reactions in seeds and accelerates the loss of viability (Sun and Leopold, 1993; Bernal-Lugo and Leopold, 1995, Leprince and Walters-Vertucci, 1995). The explanation for the loss of the glass transition was presumably due to the crystallisation of some liquid components (Sun and Leopold, 1993). However, no sugar crystallisation could be detected in tissues that had lost their glassy state (Sun and Leopold, 1993).

It is assumed that the high viscosity of intracellular glasses decreases molecular mobility and impedes diffusion within the cytoplasm, thus slowing down deleterious reactions and changes in structure and chemical composition during ageing. Some evidence for the role of glasses in ageing kinetics comes from Sun and Leopold (1994), who found that seed deterioration appeared to be accelerated when seeds were not in the glassy state, as estimated through the viability equation of Ellis and Roberts (1980a). Additional evidence that

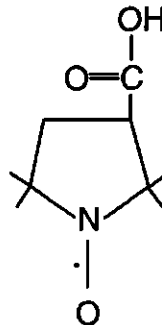
glasses are involved in storage behaviour came from a study on the changes in stachyose content during storage at 5°C (Sun and Leopold, 1995), indicating sugar hydrolysis during storage. For 29 soybean seed lots, stachyose hydrolysis was apparently inhibited below water contents of 0.12 g H₂O g dw⁻¹, and those tissues were expected to be in the glassy state. At water contents above 0.12 g H₂O g dw⁻¹, the decline of stachyose indicated sugar hydrolysis, and this may presumably have stimulated the Maillard reaction in the seeds (Sun and Leopold, 1995).

Despite numerous assumptions that molecular mobility might play an important role in storage stability (Sun and Leopold, 1993, 1994, 1995; Leopold et al., 1994; Sun, 1997b), not much is known about the translational, rotational or vibrational motion of molecules in the cytoplasm of seed and pollen. In the next section, a spectroscopic method is discussed that can be used to elucidate the importance of molecular mobility in storage stability.

Measurements of mobility: Electron Paramagnetic Resonance Spectroscopy

A technique to study rotational motion is electron paramagnetic (or spin) resonance (EPR) spectroscopy, which measures the rotational correlation time

Figure 0.4. Structure of the nitroxide spin probe 3-carboxy-proxyl (CP).



(τ_R) of spin probes dissolved in samples. For random tumbling, τ_R roughly corresponds to the average time for a molecule to progress one radian (Knowles et al., 1976). The rotational motion can be detected using the line shape of the spectrum of a nitroxide spin probe.

The choice of which spin probe to use is an important consideration before performing the measurements on the EPR machine. Depending on its polarity, the spin probe will be present in the cytoplasm or lipid fraction. To determine the molecular mobility in the cytoplasm of tissues, a very polar spin probe, 3-carboxy-proxyl (Fig. 0.4) can be used.

Figure 0.5 shows the various motional regions and characteristic EPR and ST-EPR spectra for isotropic motion, together with the wide range of rotational motions that can be covered. Continuous Wave (CW) EPR can detect changes in τ_R of spin probes ranging from 10⁻¹² to 10⁻⁸ s, and has been applied previously to biological systems (Bruni and Leopold, 1991; Buitink et al., 1998b; Leprince and

Hoekstra, 1998). In this motional range, the EPR spectrum of nitroxides consists of three lines (see Fig. 0.5), and τ_r can be calculated according to Knowles et al. (1976).

For rotational correlation times $> 10^{-7}$ s, conventional EPR spectroscopy yields the rigid-limit powder line shape (Fig. 0.5) that is insensitive to the rate of

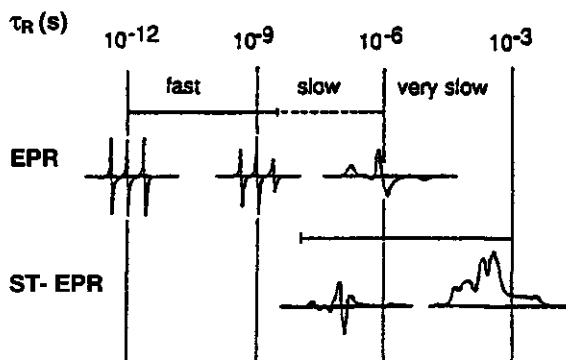


Figure 0.5. Survey of the various motional regions and the corresponding characteristic EPR and saturation EPR spectra for isotropic motion of a nitroxide spin label with rotational correlation time τ_r .

molecular motion. It is, however, possible to obtain line shapes which are sensitive to τ_r in this region by carrying out EPR under saturation conditions. This non-linear EPR technique is usually referred to as saturation transfer (ST) EPR spectroscopy (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 a review). ST-EPR spectra can be well characterized by independent line shape parameters (Hemminga, 1983). Using reference material with known viscosity, τ_r values are usually obtained in an empirical way (Hemminga and van den Dries, 1998).

ST-EPR spectroscopy has been successfully applied to determine the τ_r of spin probes in sugar glasses (Roozen and Hemminga, 1990; Roozen et al., 1991, Hemminga and Van den Dries, 1998; Van den Dries et al., 1998a). Likewise, information can be derived from the molecular mobility in biological glasses. After the introduction of a polar nitroxide spin probe into biological tissues, the τ_r of the spin probe in the cytoplasm can be estimated through the analysis of the line shape parameters of the nitroxide spectra.

Aim and outline of the thesis

Desiccation-tolerant tissues are capable of forming a glassy state under conditions of low temperatures or low water contents. The viscosity and stability of the glassy state are determined not only by the temperature and water content but also by the composition of the glass. It is to be expected that a compositional change of the glassy cytoplasm might affect the viscosity and thereby change the ageing rates of the anhydrobiotes, resulting in a modified longevity. Some treatments, such as osmo-priming of seeds, are known to

change the sugar composition of the cytoplasm (Hoekstra et al., 1994), and lead to a reduction in longevity (Tarquis and Bradford, 1992; Saracco et al., 1995). Considering the reputation of sugars as being excellent glass formers, it was hypothesised that the change in sugar composition in seeds due to priming might lead to a decrease in the intracellular viscosity and thereby to an increase in ageing rates.

To answer the question as to whether the shorter longevity of primed seeds is related to changes in the intracellular glass properties, we decided to first characterize the formation of intracellular glasses (section I) and their physical properties, with emphasis on molecular mobility as a measure of viscosity (section II). Subsequently, by manipulation of the water content and temperature, we changed the cytoplasmic molecular mobility and the longevity of seed and pollen, to confirm the hypothesised link between molecular mobility and rate of ageing (section III). Finally, we investigated what the impact of compositional changes in soluble sugars was on the properties of the intracellular glass, using priming treatments to manipulate sugar contents and longevity (section IV).

In **Section I**, the role of intracellular glasses in storage behaviour was ascertained, using pollen as a model system. It was concluded that pollen is capable of forming an intracellular glass, both in desiccation-tolerant and -sensitive species (*chapter 1*). The ageing kinetics was found to change around a temperature that could be associated with the T_g , indicating a role for intracellular glasses in the storage stability of the pollen (*chapter 2*).

In **Section II**, the molecular mobility in the cytoplasm of several tissues is characterized using various applications of spin probe EPR spectroscopy. It was found that the molecular mobility of a spin probe localised in the cytoplasm increased with increasing water content and temperature. The rotational motion was found to increase more rapidly upon melting of the intracellular glass (*chapters 3 and 5*). Apart from the detection of rotational motion of the spin probe, it was established that the spin probe also undergoes librational motion in the intracellular glass (*chapter 4*).

After it was confirmed that intracellular glasses are correlated with storage behaviour and that EPR spectroscopy could be used to measure rotational motion in the cytoplasm, **Section III** focusses on the relationship between cytoplasmic molecular mobility and storage stability. Using *Typha latifolia* pollen as a model system, it was concluded that the rate of ageing and rotational motion of a spin probe inserted into the pollen were related to one another, by manipulation of the water content and temperature (*chapter 6*). A similar conclusion was drawn from a study on oily lettuce seeds (*chapter 7*). For several species, a linear relationship was established between the rotational mobility of a spin probe in the cytoplasm and the longevity or vigour, suggesting that cytoplasmic mobility could influence the rate of ageing. From this linear relationship, it was possible to describe and predict the storage stability of germplasm (*chapter 8*). These predictions point to the existence of

an optimum water content for storage that shifted to higher values with decreasing storage temperatures, indicating that too far drying will negatively affect the longevity of germplasm. **Section IV** is devoted to the composition and biophysical properties of intracellular glasses, with special emphasis on the role of oligosaccharides in glass formation, because these sugars are thought to play a role in storage stability. Changing the oligosaccharide content through an osmo-priming treatment did not influence the T_g or molecular mobility in the cytoplasm of seeds (*chapters 9 and 10*). It was concluded that the reduced longevity after priming of the seeds could not be ascribed to changes in the cytoplasmic molecular mobility (*chapter 10*). Based on several biophysical measurements, it was concluded that sugars are not the predominant glass formers in intracellular glasses. Sugar glasses appeared to exhibit a second increase in the kinetics of molecular mobility at a defined temperature (T_c) above T_g , which was found to correspond with the so-called crossover temperature, where the dynamics changes from solid-like to liquid-like. For intracellular glasses, this critical temperature was at least 50°C above T_g , and this high T_c is expected to provide excellent long-term stability to dry organisms, maintaining a slow molecular motion in the cytoplasm even at temperatures far above T_g (*chapter 11*).

Section I

Intracellular Glasses

Overview

The presence of glasses in seeds has been established over a decade ago. A role for glasses was proposed in desiccation tolerance as well as in storage stability of seeds. Because glasses are likely to occur in all dry desiccation-tolerant organisms we investigated the occurrence of intracellular glasses in pollen of different plant species. After it was established that upon drying the cytoplasm of pollen transforms into a glassy state, the role of the intracellular glass in desiccation tolerance and storage stability was investigated.

The application of differential scanning calorimetry revealed that intracellular glasses are formed in both desiccation-tolerant and -intolerant pollen at around 10% moisture during drying at room temperature. However, desiccation-intolerant pollen loses its viability during drying before intracellular glasses are formed, indicating that the formation of glasses during drying is not reason for desiccation tolerance.

By studying the temperature and water content dependence of the ageing kinetics of cattail pollen it was shown that drying below an optimum water content that corresponded to the BET monolayer value was detrimental to storage longevity. For each storage temperature, optimal longevity was achieved under storage conditions of 11-15% RH. The corresponding water contents shifted to higher values with decreasing storage temperatures, implying that care has to be taken not to overdry pollens when storing them at low temperatures. The water contents of optimal storage for each temperature were found to be below the glass transition temperature, indicating that under these conditions the cytoplasm of the pollen was in a glassy state. The activation energy of ageing changed around a temperature where the glassy state melted, suggesting that the presence of intracellular glasses affects the storage stability of the pollen.

1

Calorimetric properties of dehydrating pollen: analysis of a desiccation-tolerant and an - intolerant species

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and Olivier Leprince**

The physical state of water in the desiccation-tolerant pollen of *Typha latifolia* L. (cattail) and the desiccation-sensitive pollen of *Zea mays* L. (maize) was studied using differential scanning calorimetry in an attempt to further unravel the complex mechanisms of desiccation tolerance. Melting transitions of water were not observed at water content values less than 0.21 (cattail) and 0.26 (maize) g H₂O g dw⁻¹. At moisture levels at which melting transitions were not observable, water properties could be characterized by changes in heat capacity. Three hydration regions could be distinguished with the defining water content values changing, changing as a function of temperature. Shifts in baseline power resembling second-order transitions were observed in both species and were interpreted as glass-to-liquid transitions, the glass transition temperatures being dependent on water content. Irrespective of the extent of desiccation tolerance, both pollens exhibited similar state diagrams. The viability of maize pollen at room temperature decreased gradually with the removal of the unfrozen water fraction. In maize, viability was completely lost before grains were sufficiently dried to enter into a glassy state. Apparently, the glassy state per se cannot provide desiccation tolerance. From the existing data, we conclude that, although no major differences in the physical behaviour of water could be distinguished between desiccation-tolerant and -intolerant pollens, the physiological response to the loss of water varies between the two pollen types.

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Living processes are dependent on water, which regulates biological reactions, serves as a fluid medium and stabilises macromolecular structure (reviewed in Vertucci and Farrant, 1995). Therefore, removal of water from biological tissues affects the ability to function and may induce deleterious effects eventually leading to cell death. Nevertheless, numerous organisms have the ability to survive removal of a major part of their cellular water (reviewed in Crowe et al., 1992 and Crowe et al., 1997a). These organisms form suitable model systems to study properties of water in biological tissues, since they remain viable even at low water contents.

Previous studies of proteins (Rupley et al., 1983; Rupley and Careri, 1991) and desiccation-tolerant organisms (Clegg, 1986; Vertucci, 1990) have indicated that the motional and physical properties of water change as a function of water content. This led to the assignment of hydration levels distinguishable by the changes in these properties with water content (Rupley et al., 1983; Clegg, 1986; Vertucci, 1990). Five hydration levels could be identified in seeds (Vertucci, 1990) and the metabolic response of the tissue was related to these hydration levels (reviewed by Vertucci and Farrant, 1995). Recently, glassy states were found to occur in dry anhydrobiotes at ambient temperature (Williams and Leopold, 1989; Bruni and Leopold, 1992; Sun and Leopold, 1993, Sun et al, 1994), and it has been suggested that glassy states are an important factor for the survival of desiccated tissues (reviewed in Leopold et al., 1994).

The majority of pollens are desiccation tolerant, i.e. they can be dried to water contents of less than about $0.05 \text{ g H}_2\text{O g dw}^{-1}$ without loss of viability (Hoekstra, 1986). However, some desiccation-intolerant species can be found among the Gramineae (Goss, 1968). For instance, maize pollen does not permit extensive drying without viability loss. The present study was undertaken to describe the physical properties of water in a desiccation-tolerant pollen species (cattail) and a moderately desiccation-sensitive one (maize) to ascertain whether these two species exhibit different water characteristics.

Materials and methods

Plant materials and water content determinations

Mature male inflorescences of *Typha latifolia* L. (cattail) were collected from field populations near Heteren, The Netherlands, in 1993 and spread on the laboratory bench. After dehiscence pollen was sieved and dried to about $0.07 \text{ g H}_2\text{O g dw}^{-1}$, and subsequently stored at -20°C until used. *Zea mays* cv. Gaspé Flint (maize) was grown in a greenhouse in Fort Collins, CO. Whole tassels that were about to shed their pollen were collected and kept in the laboratory under conditions of 100% RH for 4 h. After removal from the high humidity, anthers simultaneously dehisced, and the harvested pollen, which contained approximately $1 \text{ g H}_2\text{O g dw}^{-1}$, was immediately used in the experiments.

Water contents of cattail pollen was manipulated by equilibration over saturated salt solutions at room temperature for 48 h for both viability assays and differential scanning calorimetry (DSC) measurements (Rockland, 1960; Winston and Bates, 1960;

summarised by Vertucci and Roos, 1993b). In the case of maize, the freshly collected pollen was flash-dried in a stream of dry air at room temperature (Pammenter et al., 1991) for various lengths of time to obtain the desired water contents for the viability assay. For DSC measurements, fresh maize pollen was equilibrated over the saturated salt solutions for 48 h. Water contents were calculated from the difference in weight before and after heating the pollens at 95°C for 24 h, a time sufficient to achieve constant weight. Water content was expressed on a dry weight basis as $\text{g H}_2\text{O g dw}^{-1}$.

Viability assays

Viability of cattail pollen was determined by counting the percentage of empty, cytoplasm-less grains in 4 replicates of 50 pollen tetrads 24 h after placing the pollen on a germination medium at 24°C (Hoekstra et al., 1992a). In nongerminated grains the cytoplasm is still present whereas in the germinated grains it has moved into the pollen tube. Maize pollen was dusted on a drop of semi-solid germination medium containing salts according to Brewbaker and Kwack (1963), 15% sucrose and 0.125% agar, and then placed in an open petri-dish on the laboratory bench. After 30 min the germinated grains were counted (4 replicates of 50 grains each). To prevent imbibitional damage, pollen grains were prehydrated in water vapour at room temperature for 2 h prior to the germination assay.

DSC

Pollens, adjusted to different water contents, was hermetically sealed into standard aluminum DSC pans. Grains with less than $0.1 \text{ g H}_2\text{O g dw}^{-1}$ were packed into non-hermetically sealing pans to improve thermal conductivity.

Phase transitions in pollen at various water contents were determined using a Perkin-Elmer (Norwalk, CT) DSC-4 or DSC-7, calibrated for temperature with methylene chloride (-95°C) and indium (156.6°C) standards, and with indium (28.54 J g^{-1}) for energy. Baselines were determined using an empty pan, and all thermograms were baseline-corrected. The presence of phase transitions was determined from cooling and heating thermograms recorded between -150 and +100°C at a rate of $10^\circ\text{C min}^{-1}$. The onset temperature of the melting and freezing transitions was determined from the intersection between the baseline and a line drawn from the steepest portion of the transition peak. The enthalpy (ΔH) of the transition was determined from the area encompassed by the peak and the baseline. The T_g values, detected in pollen with low water contents, were determined by the midpoint of the temperature range over which the change in specific heat occurred. All analyses were performed using Perkin Elmer software.

Enthalpies of exotherms and endotherms expressed on a per g dry weight basis were regressed with the water content of the pollen. The slope of the linear relationship corresponds to $\Delta H \text{ g}^{-1} \text{ H}_2\text{O}$ and the x-intercept gives the water content below which melting or freezing transitions could not be observed (Vertucci, 1990).

Another calorimetric property of water in the pollens was quantified by changes in heat capacity (C_p) at different water contents between 0.01 and $0.30 \text{ g H}_2\text{O g dw}^{-1}$ and three different temperatures (30, -10, -60°C) for maize and six different temperatures (30, 10, -10, -20, -40, and -60°C) for cattail. Displacement from the DSC isothermal baseline was evaluated while samples were heated 4°C at 4°C min^{-1} , e.g. -12 to -8°C for the -10°C determination. Heat capacity was measured at the highest temperature first and then samples were cooled at $10^\circ\text{C min}^{-1}$ to the next-lower assay

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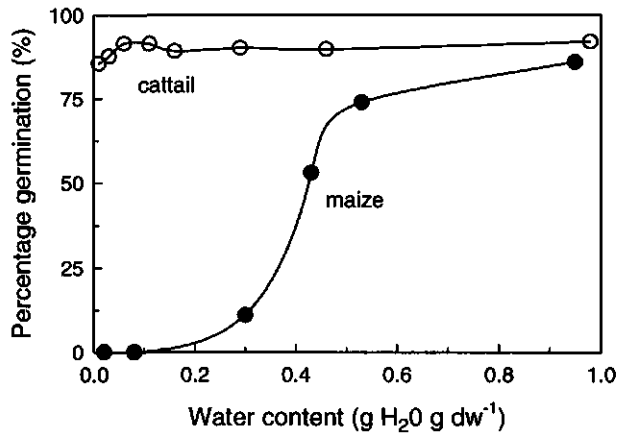
temperature and allowed to equilibrate until a steady baseline was achieved (usually within about 5-10 min). The DSC was calibrated for C_p measurements using benzoic acid as a standard (Ginnings and Furukawa, 1953).

Results and Discussion

Viability and water content

Pollen of cattail and maize are known to differ in their desiccation tolerance (Goss, 1968; Hoekstra, 1986; Hoekstra et al., 1989). To quantify this difference, percentage germination *in vitro* was measured after pollen grains were adjusted to different water contents (Fig. 1.1). Germination percentages of cattail pollen were constant at about 90% for the entire water content range. In contrast, the viability of maize decreased sharply if grains were dried to water contents less than about $0.4 \text{ g H}_2\text{O g dw}^{-1}$, and no germination was observed when grains were dried to $0.1 \text{ g H}_2\text{O g dw}^{-1}$.

Figure 1.1. The germination percentage determined *in vitro* for cattail (open circles) and maize (closed circles) pollen dried to different water contents.



Phase behaviour of water at different water contents

The calorimetric properties of water in pollen of cattail and maize were measured as a function of water content and temperature. In Fig. 1.2 a selection of these thermograms is presented. Several transitions were observed within the temperature range of -80 to 70°C . At water contents greater than about $0.22 \text{ g H}_2\text{O g dw}^{-1}$, a broad endothermic peak at about -30 to -12°C was observed during warming runs for both pollens (Fig. 1.2A and B, w). The area and temperature of the peak increased with increasing water content. These endothermic peaks were considered to be water melting transitions (Vertucci, 1990). Exothermic peaks were observed in cooling runs at temperatures about 20°C lower than the corresponding melting transitions (hysteresis effect), and we interpret these as water-freezing transitions (thermograms not shown). The area encompassed by the exothermic peaks

were considerably smaller (about 30 J g dw^{-1}) than the corresponding endothermic peaks. As has been found using seed tissues (Vertucci, 1990), the onset temperatures of the freezing and melting transitions were considerably lower than for pure water or dilute solutions.

At low water contents, a small endothermic peak was observed at about -20°C for cattail and -25°C for maize (Fig. 1.2C and D, nl). We interpret this peak as the melting of neutral lipids because the size of the peak and transition temperature seemed to be independent of the water content and are similar to triglyceride transitions reported for seed tissues (Vertucci, 1989, 1990, 1992; Hoekstra et al., 1991).

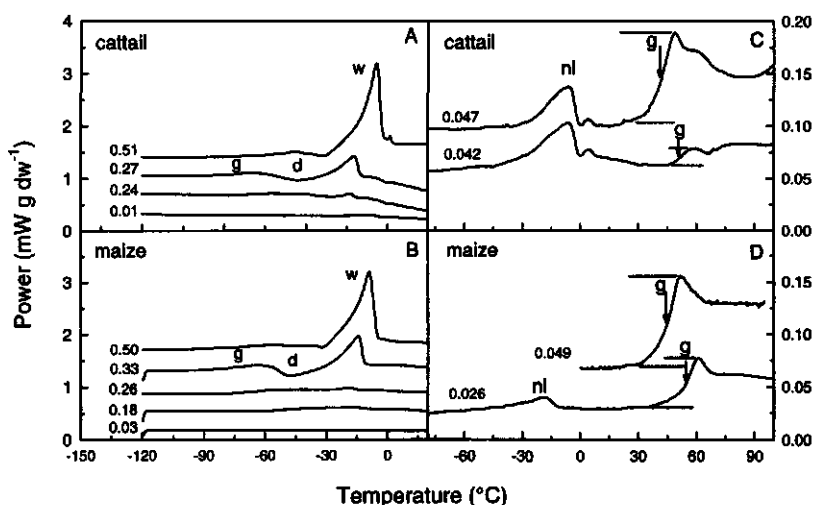


Figure 1.2. Heating thermograms of cattail (A and C) and maize (B and D) pollen with varying water contents made using DSC. Samples were scanned at $10^\circ\text{C min}^{-1}$ from -120 to 20°C (A and B) or from -80 to 100°C (C and D). w and nl, Water or neutral lipid melting transition, respectively. D, Devitrification event. G, Glass-to-liquid state transition. Numbers by the traces indicate water content ($\text{g H}_2\text{O g dw}^{-1}$). Arrows indicate the midpoint temperature of the glass transition.

In addition to the apparent first-order transitions described above, shifts in baselines resembling second-order transitions were observed in warming thermograms of both pollen species (Fig. 1.2, g). The temperatures at which these shifts occurred increased with decreasing water contents. For example, in cattail pollen of $0.27 \text{ H}_2\text{O g dw}^{-1}$ the baseline shift occurred at -75°C (Fig. 1.2A, g), whereas in samples of $0.042 \text{ g H}_2\text{O g dw}^{-1}$ (Fig. 1.2C, g) this shift occurred at 50°C . An endothermic peak superimposing the baseline shift was observed in samples with water contents between 0.02 and $0.06 \text{ g H}_2\text{O g dw}^{-1}$, and is the "overshoot" commonly observed during glass transitions (Berens and Hodge, 1982; Green and Angell, 1989; Perez, 1994; Leprince and Walters-Vertucci, 1995). At water contents between about 0.25 and $0.35 \text{ g H}_2\text{O g dw}^{-1}$, the baseline shifts were observed at subzero temperatures and were

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accompanied by exothermic reactions considered as devitrification (ice crystal formation) events (Franks, 1982; Vertucci, 1989) (Fig. 1.2A and B, g and d). The baseline shifts, occurring at elevated temperature with reduction in water content, the "overshoot" and the devitrification events are typical of glass to liquid transitions (Franks, 1982; Perez, 1994). Glasses are a common feature of food and polymers (Slade and Levine, 1991b) and have also been detected in seed tissues by thermally stimulated depolarisation current (Bruni and Leopold, 1992; Sun and Leopold, 1993; Sun et al., 1994) and DSC (Vertucci, 1989; Williams and Leopold, 1989; Leprince and Waiters-Vertucci, 1995). As with seeds, the baseline shifts in pollen were not detected at water contents greater than about $0.35 \text{ g H}_2\text{O g dw}^{-1}$, probably because the $10^\circ\text{C min}^{-1}$ cooling rate was not rapid enough to allow for glass formation at high water contents (Vertucci, 1989). At water contents between 0.08 and $0.15 \text{ g H}_2\text{O g dw}^{-1}$, the shifts in the baseline were not measurable, probably because the melting transition of the triglycerides and the baseline shift occurred over the same temperature range and the former event masked the latter one.

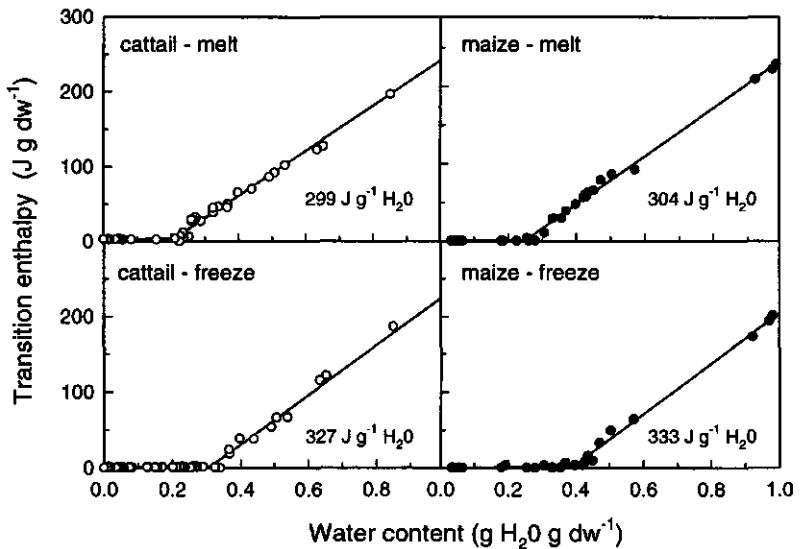


Figure 1.3. The relationship between the water content and the ΔH and freezing transitions for pollen of cattail (left panels) and maize (right panels). The lines drawn represent the least-squares best fit. The slopes of the lines represent the ΔH of the transition on a per g water basis. Areas of peaks were determined from thermograms similar to those in Fig. 1.2.

Plotting the ΔH of the freezing and melting transitions of water versus the water content of the dehydrating pollen may give insight in the different properties of water (Fig. 1.3). The $\Delta H \text{ g}^{-1} \text{ H}_2\text{O}$ of the melting and freezing transitions, calculated from the slopes of the linear relationship between $\Delta H \text{ g dw}^{-1}$ versus water content ($r^2 > 0.99$ for all regressions), was approximately 300 and $330 \text{ J g}^{-1} \text{ H}_2\text{O}$, respectively, for both pollen species. A slight reduction

in the value of $\Delta H \text{ g}^{-1} \text{ H}_2\text{O}$ compared to the value for pure water ($333 \text{ J g}^{-1} \text{ H}_2\text{O}$) is expected when the melting temperature is reduced due to the presence of solutes (Gekko and Satake, 1981). In contrast to these pollens, an unusually low ΔH of about $100 \text{ J g}^{-1} \text{ H}_2\text{O}$ has been observed in orthodox and minimally recalcitrant seeds at water contents between 0.15 and $0.45 \text{ g H}_2\text{O g dw}^{-1}$ (Vertucci, 1990; Vertucci et al., 1994a).

The amount of unfrozen water in both pollen species, calculated from ΔH of the freezing and melting transitions versus water content relationships (Fig. 1.3), was 0.27 and $0.36 \text{ g H}_2\text{O g dw}^{-1}$ for cattail and maize, respectively, if determined from freezing transitions, or was 0.21 and $0.26 \text{ g H}_2\text{O g dw}^{-1}$, respectively, if determined from melting transitions. These values correspond well to values reported for seed tissues (Vertucci, 1990; Pammenter et al., 1993) and maize pollen (Kerhoas et al., 1987). At the water content where freezing and melting transitions are not observable, water molecules may be sufficiently immobilised to limit the molecular reorganisation necessary for crystallisation events.

The viability of maize pollen declined if it was dried to water contents between 0.4 and $0.2 \text{ g H}_2\text{O g dw}^{-1}$ (Fig. 1.1). This water content range coincides with the removal of the unfrozen water fraction and suggests that this water fraction is important to the structural stability of desiccation-sensitive cells. A similar relationship has been described for recalcitrant embryos (Pammenter et al., 1991, 1993). However, it is doubtful whether *in vitro* germination tests at such low water contents of the pollen would give an accurate estimation of viability, since *in vivo* tests, i.e. the analysis of successful tube growth in the silks, showed much higher viability under these low water contents (Hoekstra, 1986). Pollination in maize is generally successful with pollen having water contents in the range of 0.10 to $0.15 \text{ g H}_2\text{O g dw}^{-1}$ (Barnabas et al., 1988; Hoekstra et al., 1989; Shi and Tian, 1989; Digonnet-Kerhoas and Gay, 1990), which indicates that a part of the unfrozen water can be removed without viability loss. Further removal of the unfrozen water fraction to water contents less than 0.10 to $0.15 \text{ g H}_2\text{O g dw}^{-1}$ leads to a loss in fertility. For the tolerant cattail pollen, removal of unfrozen water is tolerated to much lower water contents.

C_p at low water contents

At water contents less than about $0.3 \text{ g H}_2\text{O g dw}^{-1}$, water freezing and melting transitions were not observed (Figs. 1.2 and 1.3). To investigate the properties of water at these low water contents, C_p was measured in both pollen species at temperatures between $+30^\circ\text{C}$ and -60°C (Fig. 1.4). The C_p of pollen grains was a function of water content and temperature. The C_p changed sigmoidally as a function of water content, and three hydration regions (a, b and c) could be distinguished (Fig. 1.4). Only two hydration regions were observed at -60°C . The water contents at which changes in the C_p -water content relationship were evident (i.e. a change in slope) were calculated from the points of intersection of pairs of lines. The values of these pivotal water contents increased with

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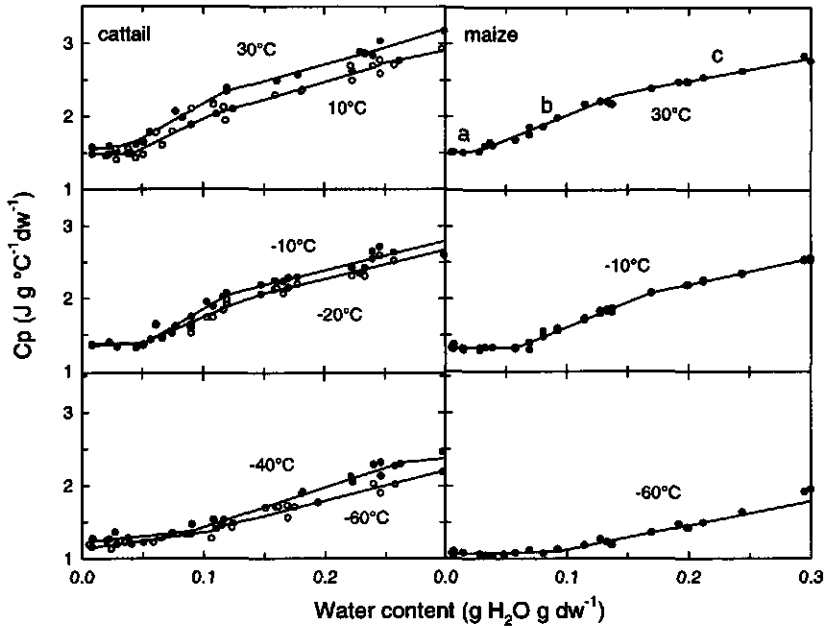


Figure 1.4. The relationship between the water content and the heat capacity of cattail and maize pollen at temperatures between 30 and -60°C . Heat capacities were calculated from the ordinate displacement when samples were heated 4°C at $4^{\circ}\text{C}/\text{min}$. The lines are drawn according to the least-squares best fit of all the data.

decreasing temperatures (Fig. 1.4). Such changes in the properties of water with water content and temperature have been predicted on the basis of thermodynamical considerations (Vertucci, 1993; Vertucci and Roos, 1993b).

The slopes of lines drawn for the C_p -water content relationship (Fig. 1.4) within each hydration region gave the C_p of the water in $\text{J } ^{\circ}\text{C}^{-1} \text{g}^{-1} \text{H}_2\text{O}$ for that region. The C_p of water in pollen containing less than $0.3 \text{ g H}_2\text{O g dw}^{-1}$ and held at different temperatures differed from what has been reported for pure water in the liquid and hexagonal ice state (Fig. 1.5). In the first hydration region (Fig. 1.4, a), the C_p of water ranged between 0.1 and $2.2 \text{ J } ^{\circ}\text{C}^{-1} \text{g}^{-1} \text{H}_2\text{O}$, depending on temperature, for both pollens. Except for the -60 and -40°C measurements in cattail, the C_p of water in region a is considerably lower than the C_p of pure water in any state (Fig. 1.5). Although the significance of water with such low C_p has not been established, it has been demonstrated that the longevity of seeds (Vertucci and Roos, 1990, 1993b; Vertucci et al., 1994b) and cattail pollen (J. Buitink, unpublished data) stored in this hydration range decreases with decreasing water content.

At intermediate water contents (region b), the C_p of water in the pollens ranged between 4 and $9 \text{ J } ^{\circ}\text{C}^{-1} \text{g}^{-1} \text{H}_2\text{O}$, depending on temperature, and was considerably higher than the C_p of pure liquid water (ranging from 4 to $5 \text{ J } ^{\circ}\text{C}^{-1} \text{g}^{-1} \text{H}_2\text{O}$ at temperatures between -20 and $+40^{\circ}\text{C}$) (Fig. 1.5). In this region glass-to-liquid transitions were observed as power shifts in the baseline (Fig. 1.2).

Values of C_p that were higher than that of liquid water have also been reported for seed tissues (Vertucci, 1990).

As the water content of pollen grains is increased to between 0.12 and 0.20 g H₂O g dw⁻¹ for temperatures between +30 and -20°C, respectively, the C_p of water approaches that of pure liquid water. This third hydration region (region c) was not observed at -60°C, perhaps because the water content in this region c is close to where freezing and melting transitions become detectable. The viscosity of food polymers at comparable water contents is much lower than that of the glassy state, but it is considerably higher than that of liquid water (Slade and Levine, 1991b; Kalichevsky et al., 1992; Angell et al., 1994). This condition is commonly referred to as a "rubbery state" (Slade and Levine, 1991b; Peleg, 1993).

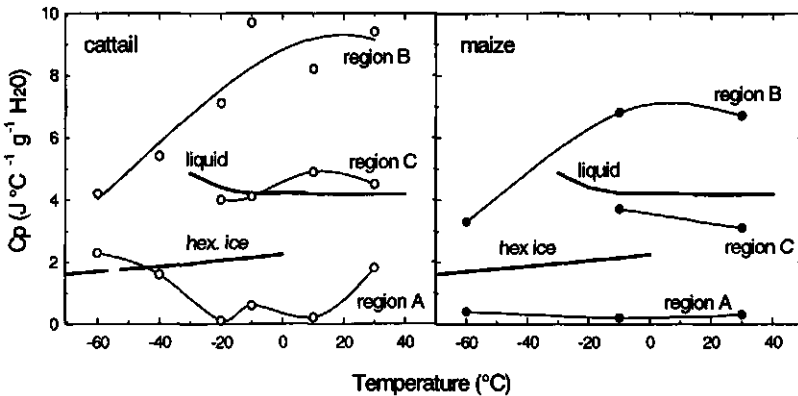


Figure 1.5. The heat capacity of water measured in cattail and maize pollen at different water contents and temperatures. C_p values were calculated from the slopes of regression lines given in Fig. 1.4. Values of the C_p of pure water were taken from Angell (1982) (ice); Ginnings and Furukawa (1953), Wakabayashi and Franks (1986) (liquid).

State diagrams: implication for desiccation tolerance

In previous studies, the impact of temperature and water content on the formation of glassy states and on the freezing behaviour of water in seeds have been displayed in state/phase diagrams (Williams and Leopold, 1989; Bruni and Leopold, 1992; Vertucci et al., 1994a). In Fig. 1.6 such state/phase diagrams are depicted for cattail and maize pollen. For both pollens, melting transitions of water (T_{melt}) were observed at higher temperatures and lower water contents than freezing transitions (T_{freeze}), but no obvious differences could be observed between the pollens. The T_g , derived from an apparent shift in the thermogram baseline (Fig. 1.2), decreased with increasing water contents. The temperatures at which the glass transition occurs in these two pollen species is lower than has been reported for seeds (approximately 10 to 20°C difference; Leopold et al., 1994), with maize pollen having a slightly higher T_g than cattail pollen.

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Also included in the state/phase diagrams are breaks from the C_p curves of Fig. 1.4, which are indicative of changing water properties. The changes in C_p of water followed the water content-temperature relationship of the T_g . Region b, which was identified by an anomalously large C_p of water of the system, coincided with the glass to liquid (or perhaps rubbery) transition. This unusually high C_p might be due to alteration of the molecular structure of the water in the system when it is changing from the glassy state to liquid (rubbery) state.

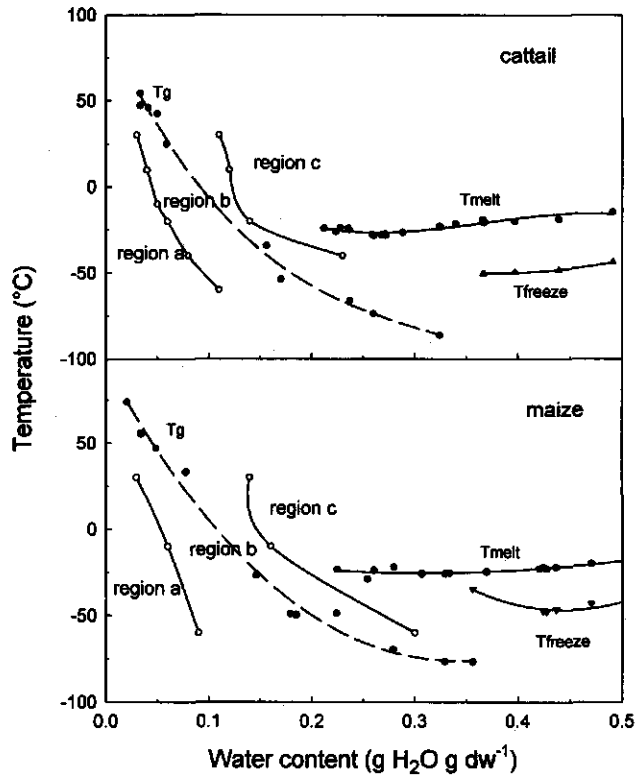


Figure 1.6. State/phase diagrams for pollen of cattail and maize, describing the interaction between water content and temperature on glass transition temperature, changes in apparent C_p , and freezing and melting events. The open circles represent the water contents at which changes in the slopes of the C_p versus water content relationships could be detected at various temperatures (Fig. 1.4). Onset temperature for water transitions were determined from thermograms similar to those in Fig. 1.2. The curve used to illustrate the T_g – water content relationship is a third-order polynomial function fit to the datapoints.

Considering sugars as major determinants of glass formation (Franks, 1982; Slade and Levine, 1991b; Angell et al., 1994) it is interesting to note that the dry pollens contain sucrose as the major (around 95%) intrinsic soluble sugar (12 and 23% of dw for maize pollen and cattail pollen, respectively; Hoekstra et al., 1989; 1992a), whereas maize and soybean embryos have, in

addition to sucrose, considerable amounts of oligosaccharides (Koster and Leopold, 1988; Kuo et al., 1988). Oligosaccharides generally result in higher T_g values for a given water content (Slade and Levine, 1991b), which may explain the higher T_g values of the embryos (compare data from Williams and Leopold [1989] and Sun and Leopold [1993] with T_g values given in Fig. 1.6). However, comparisons remain questionable, since the chemical composition may influence the actual concentration of the sugars and other glass forming components in the cytoplasm; for example, oil bodies or starch grains may contribute considerably to the dry weight but limit the space available for sugars.

In both desiccation-tolerant and -sensitive pollen species, a glass was detected with approximately similar state diagrams. Apparently, the presence of the glassy state is not in itself sufficient for full desiccation tolerance. However, the glassy state cannot be reached in maize pollen at room temperature without exceeding the critical minimum water content for viability (compare Fig. 1.6 with Barnabas et al., 1988; Hoekstra et al, 1989; Shi and Tian, 1989; Digonnet-Kerhoas and Gay, 1990). Thus, these pollen cannot benefit from the characteristics of glasses such as high viscosity and reduction of deteriorative processes.

Glass formation has been demonstrated to be important in cryopreservation of seed tissues (Vertucci, 1989, 1993; Wesley-Smith et al., 1992), and frozen foodstuffs (Slade and Levine, 1991b), and the same arguments can be applied to recalcitrant pollen. A general procedure for cryopreserving maize pollen is to dry grains at room temperature to water contents where desiccation damage is slight and then lower the temperature to -76 or -196°C (Barnabas and Rajki, 1976, 1981; Shi and Tian, 1989). Examination of the state diagram in Fig. 1.6 shows that this procedure probably results in the formation of glasses and limits the structural damage induced by ice-crystal formation.

Conclusions

We have investigated the physical state of water interacting with the pollen dry matter as a function of water content and temperature. Viability of maize pollen is gradually lost when the unfrozen water is progressively removed from cells. In contrast, viability of cattail pollen is unaffected by moisture contents as low as $0.01 \text{ g H}_2\text{O g dw}^{-1}$. The physical state of water changes as a function of both water content and temperature in both pollen species. Both pollens had comparable state diagrams and no major differences were found in C_p , T_g , or ΔH of melting and freezing of water between desiccation-tolerant and -intolerant pollen. We conclude that even though the state of water is similar for desiccation-tolerant and -sensitive pollens, the response to loss of different states of water varies for desiccation-tolerant and -intolerant species.

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Storage behaviour of *Typha latifolia* pollen at low water contents: Interpretation on the basis of water activity and glass concepts

Julia Buitink, Christina Walters, Folkert A. Hoekstra, Jennifer Crane

Germplasm must be stored under optimal conditions to maximise longevity and efficiently maintain genetic resources. In order to identify optimal storage conditions, we investigated the effects of temperature (-5 to 45°C) and water content (<0.17 g H₂O g dw⁻¹) on longevity of *Typha latifolia* L. pollen. Longevity was highest at water contents corresponding to storage relative humidity (RH) of 11-15% which corresponded to the shoulder of the water sorption isotherms. Also coinciding with this shoulder were abrupt changes in heat capacity of water present in the pollen. Consistent with changes in isotherms with temperature and the concept of critical RH for storage, optimum water contents increased with decreasing temperature. An attempt was made to explain the ageing behaviour according to the glass concept. The water content-temperature combinations of optimal storage were found to be below the glass transition curve, indicating that optimum storage conditions are achieved when intracellular glasses are present. We also found a change in activation energy of ageing in Arrhenius plots around T_g, demonstrating a change in ageing kinetics when the glassy state is lost. We conclude that T_g curves can not be used solely to predict precise conditions of optimum storage, but might be useful for predictions of storage longevity above optimum water contents. The data imply that too much drying reduces longevity and should be avoided, particularly when cryogenic storage is considered.

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An important goal of germplasm facilities is the long-term preservation of viability. Because most seeds and pollens are able to endure severe desiccation, they are particularly suitable for this purpose. Increased longevity is achieved by lowering storage relative humidity (RH) and temperature. However, in spite of the long lifespans that are achievable by drying to low water content, these desiccation-tolerant cells eventually lose viability. Genebank operators must know when the loss of viability commences in order to regenerate samples. Therefore, they need to have effective tools to predict longevity under given storage conditions and to minimise damaging germplasm. Although some predictive tools are available, i.e. viability equations, more studies are needed in order to explain and predict seed storage behaviour at low RH.

Viability equations fail to predict longevity at low water contents because there is a limit to the beneficial effects of drying seeds (Ellis et al., 1989, 1990b, Vertucci and Roos, 1990). The value of this limit, also denoted as the critical water content, is a matter of some debate (Ellis et al., 1991, 1995; Vertucci and Roos, 1991, 1993a; Smith, 1992). It was also suggested that the critical water content was dependent on temperature (Vertucci and Roos, 1993a,b; Vertucci et al., 1994b).

The present study was initiated to clarify the role of water and temperature on longevity of a model anhydrobiotic system (pollen). We made attempts to (1) determine whether there is a critical water content and if so, whether this water content is temperature dependent; (2) determine whether there is a detrimental effect of drying below the critical water content. Additionally, we will examine two different approaches to unravel the interaction of temperature and water content on storage behaviour and longevity. In addition to the equilibrium approach, in which water absorption isotherms may be used for prediction of optimal storage conditions, the glass concept may be used to understand and possibly predict the relationship between water content and temperature on reaction rates (Slade and Levine, 1991a).

A glass is defined as an amorphous metastable state that resembles a solid, brittle material, but retains disorder and physical properties of the liquid state (Franks, 1994a; Slade and Levine, 1994). Formation of a glass during drying results in an increase of viscosity of several orders of magnitude, thereby severely slowing down molecular diffusion and decreasing the probability of chemical reactions (Burke, 1986; Green and Angell, 1989). The presence of intracellular glasses has been established in seeds (Williams and Leopold, 1989; Bruni and Leopold, 1992; Sun et al., 1994; LePrince and Walters-Vertucci, 1995) and *Typha latifolia* (cattail) pollen (Buitink et al., 1996). Since water content and temperature determine the formation and properties of glasses, the extended longevity of dry tissues may be due to the presence of intracellular glasses. Therefore, long-term storage of seed and pollen might be improved by drying or cooling them into a glassy state. The glass concept suggests that reactions occurring in a glassy matrix follow Arrhenius kinetics and that there will be a marked discontinuity in Arrhenius behaviour at temperatures around T_g (Karmas et al., 1992; Nelson and Labuza, 1994).

Investigating ageing kinetics using orthodox seeds is difficult because their inherent longevity necessitates long-term experiments, lasting several years to decades. Due to its relatively short lifespan (Hoekstra, 1995), pollen permits extensive ageing experiments on a realistic time scale. Furthermore, in vitro germination can be used as a viability test. Changes in rate of radicle growth, used as a more sensitive assay of ageing (Vertucci and Roos, 1993b), have been criticised as being not reflective of ageing kinetics as measured by percentage germination (Ellis et al., 1991; Ellis et al., 1995).

The present storage experiments on cattail pollen were conducted to investigate the influence of RH and temperature on longevity. The effects of water content and temperature on ageing behaviour will be examined with respect to the characteristics of intracellular glasses and water sorption.

Materials and methods

Mature male inflorescences of *Typha latifolia* L. (cattail) were collected from field populations near Heteren, The Netherlands, in 1993 and spread on the laboratory bench. After dehiscence, pollen was sieved and dried to about 0.07 g H₂O g dw⁻¹ and subsequently stored at -20°C until use.

To determine the effects of storage conditions on ageing rates, viability was monitored in pollen that was kept in the dark at different RH and temperatures for up to 261 days. The RH under which the pollen samples were stored was controlled and maintained by P₂O₅ (<0.5% RH) and various saturated salt solutions: ZnCl₂ (5.5% RH), LiCl (11-15% RH), K(C₂H₃O₂) (20-25% RH), MgCl₂ (31-33.5% RH), Ca(NO₃)₂ (43-61% RH) and NaNO₂ (62-66% RH) (Vertucci and Roos, 1993b). Storage temperatures ranged from -5 to 45°C ± 1°C at 10°C intervals. The water content obtained ranged from 0.002 to about 0.17 g H₂O g dw⁻¹ and was dependent on the RH and temperature.

Germination was determined by counting the percentage of empty, cytoplasm-less grains 24 h after the pollen was placed in culture. Nongerminated grains are still filled with cytoplasm. The culture medium was a 0.6% agar medium containing 0.2 M sucrose, 1.6 mM H₂BO₃, 1.3 mM Ca(NO₃)₂·4H₂O, 0.8 mM MgSO₄·7H₂O, 1.0 mM KNO₃ and 2 mM citric acid adjusted to a pH of 5.9 with KOH. To prevent imbibitional damage, pollen was prehydrated in saturated water vapour at room temperature for 4 h prior to the germination assay. This prehydration time was sufficient to prevent imbibitional damage even in the driest pollen sample. For the duration of the experiment, samples stored at -15°C and approximately 0.07 g H₂O g dw⁻¹ were included as controls in every germination assay. The percentage germination of the controls remained constant at 89 ± 4% throughout the ageing experiments, indicating that the conditions of germination were comparable at each sampling interval. Each germination assay used 50 tetrads (200 grains) and was replicated four times.

Time course data for viability decline (5 to 20 sample periods) for each water content temperature combination were fitted to a probit curve, and a survival plot was constructed by linear regression. The point where the survival plot intersects the 50% level was estimated as the mean viability period (P₅₀) for the treatment (Roberts, 1972). Ageing rates were quantified as the slope of the probability curves.

Values of water content, expressed as g H₂O g dw⁻¹, were determined by comparing the fresh and dry weight of a sample for each RH/temperature treatment

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throughout the storage experiment. Dry weights were measured after heating the pollen for 24 hours at 95°C, which is sufficiently long to achieve constant weight.

Isotherms were experimentally determined at -5 to 35°C. Data represent means of at least nine replicates. The isotherm at 45°C was constructed by extrapolating van 't Hoff isochores ($\ln [RH/100]$ vs T^{-1}) drawn for water contents from 0.005 to 0.15 at 0.005 intervals. For each water content, there was a linear relationship (data not shown) between $\ln(RH/100)$ and T^{-1} . This allowed us to construct isotherms outside the experimental range by extrapolation (Vertucci and Roos, 1993b). Because of their usefulness in predicting optimum storage conditions for foods (Labuza, 1980), Brunauer, Emmet and Teller (BET) parameters were calculated from pollen isotherms by regressing $RH/100 \times [wc \times (1-RH/100)]^{-1}$ by $RH/100$, where wc is the water content of the pollen for each RH/temperature combination. The y-intercept of the regression line is equal to $a_1^{-1}c^{-1}$ and the slope is equal to $(c-1) \times a_1^{-1}c^{-1}$, where a_1 is the BET monolayer and c is related to the enthalpy of sorption.

Results

The relationship between RH and water content of the pollen at temperatures between -5 and 45°C is depicted by water sorption isotherms (Fig. 2.1). Construction of the isotherms assumes that water content is equilibrated to the RH, and this assumption is justified by a constant water content for over 250 days (data not shown). The water sorption isotherms exhibited the expected reverse sigmoidal shape as previously described for seeds (Vertucci and Leopold, 1987; Vertucci and Roos, 1993b). Temperature influenced the relationship between water content and RH in such a manner that at any given

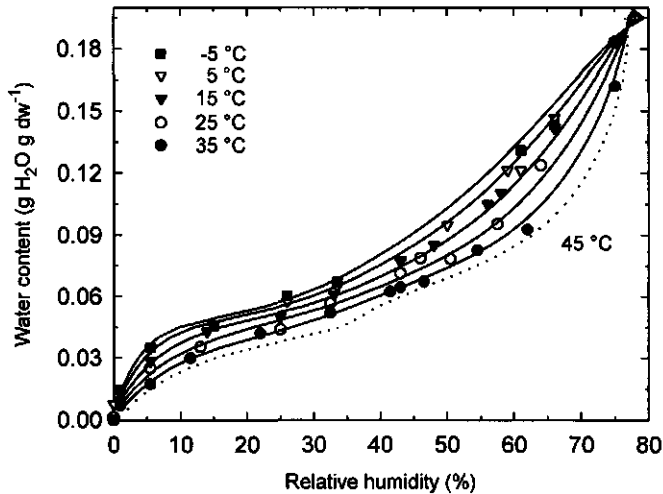


Figure 2.1. Water sorption isotherms of cattail pollen for various temperatures. Water contents for -5 to 35°C were experimentally determined using saturated salt solutions. Data points represent the mean of nine replicates. Curves are calculated from van 't Hoff isochores drawn for water contents between 0.005 and 0.15 g H₂O g dw⁻¹ at intervals of 0.005 H₂O g dw⁻¹ (Vertucci and Roos, 1993b). The isotherm of 45°C was constructed by extrapolating van 't Hoff isochores.

RH, water content increased when temperature decreased. However, the change in water content with temperature was much less in cattail pollen than reported for orthodox seeds (Vertucci and Leopold, 1993b). The water content corresponding to the BET monolayer decreased with increasing temperature while the enthalpy of sorption calculated from the BET model appeared to increase with increasing temperature.

Table 2.1 Parameters of the Brunauer, Emmet and Teller (BET) isotherm calculated for water sorption isotherms of pollen constructed at -5 to 45°C.

Isotherm temperature	a_1 (monolayer value)	c (∞ enthalpy)
-5	0.053	19.1
5	0.045	21.4
15	0.033	25.1
25	0.023	30.6
35	0.027	30.8

The degree of deterioration during storage of cattail pollen was evaluated by reductions of the percentage of germinated pollen with storage time (Fig. 2.2). Data were plotted on a probability scale (Roberts, 1972; Priestley et al., 1985). Lines representing linear regressions showed a reasonably good fit, with $r^2 > 0.8$ in most cases. Under all storage conditions, including extremely low water content, loss of germination potential was progressive with time, demonstrating that we were measuring ageing effects and not imbibitional injury (Hoekstra et al., 1992a). The extent of the decrease in survival was dependent on both RH and temperature of storage. Samples stored at 45°C (all storage RH) were inviable after 13 days. The same level of deterioration was observed after about 100 days and 225 days in samples stored at 35 and 25°C, respectively. Complete loss of germination was not observed in all the samples stored at 15 to -5°C within the time frame of the experiment. For 5 and 15°C at least 50% reduction was noted in all samples, except for the most slowly deteriorating sample at 5°C (RH=15%), which showed a 20% loss in percentage germination. Pollen stored at -5°C only showed more than 50% reduction in germination when stored at low RH (<5%) and high RH (>61%). For each temperature studied, deterioration was fastest in samples stored over the NaNO₂ solution (62-66% RH) and slowest in samples stored over the LiCl solution (11-15% RH).

Half-viability periods (P_{50}) were calculated from the probability plots in Fig. 2.2 (Fig. 2.3). For each temperature, the P_{50} was highest when pollen was stored at water contents between 0.024 and 0.053 g H₂O dw⁻¹ (11-15% RH). Above and below this optimum water content range, P_{50} decreased sharply. For pollen stored at 11% RH at 5°C, the P_{50} could not be determined without resorting to extrapolation. However, even without a precise value of this P_{50} , it is clear that it exceeds the P_{50} of pollen stored at 5% (0.033 g H₂O g dw⁻¹) and

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35% RH (0.066 g H₂O g dw⁻¹). The P₅₀ of pollen stored at -5°C could be obtained without extrapolation only for storage at low (below 5.5%) and high RH (above 66%).

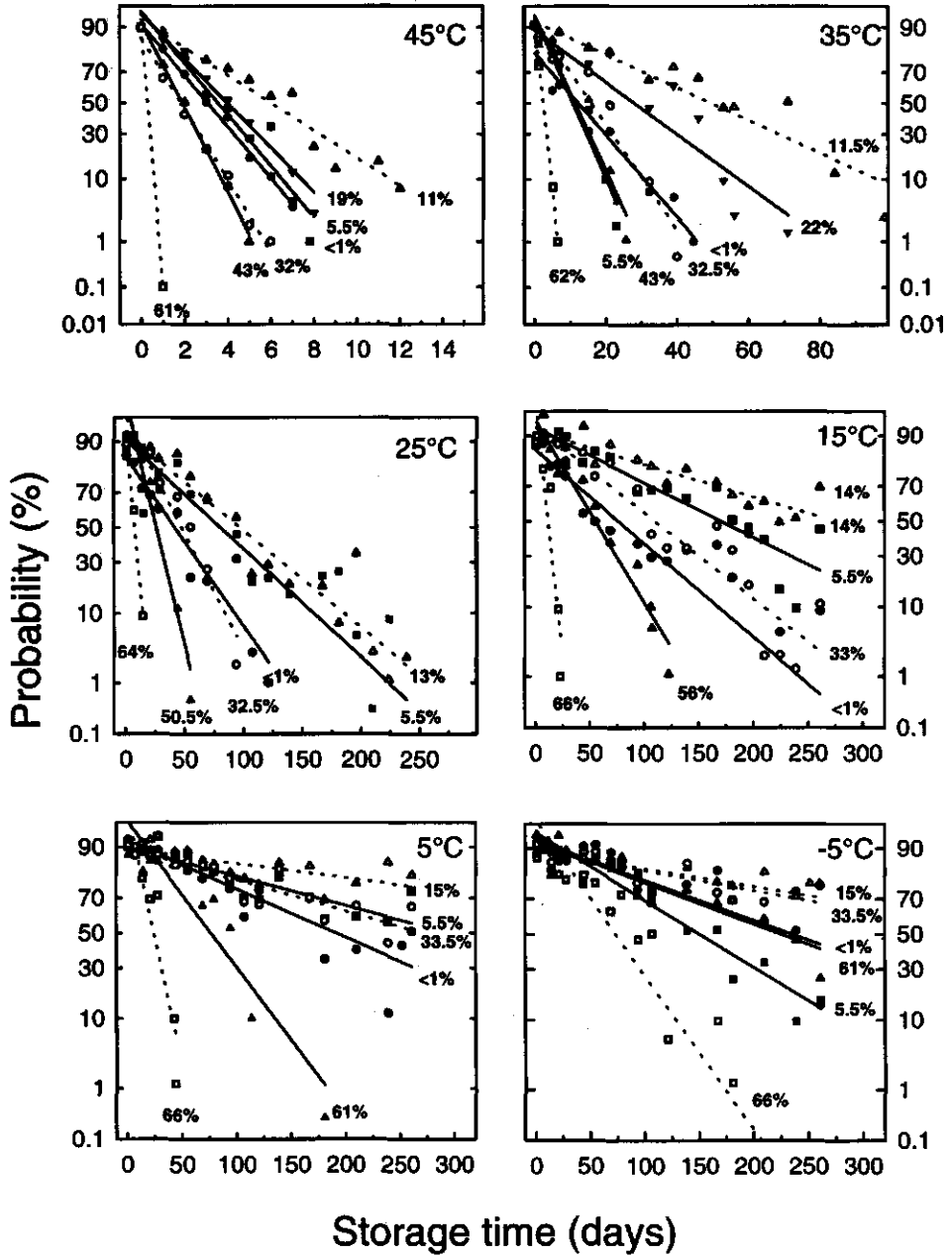


Figure 2.2. Probability curves of viability of cattail pollen, stored at different RH and temperatures. Lines represent first order regressions.

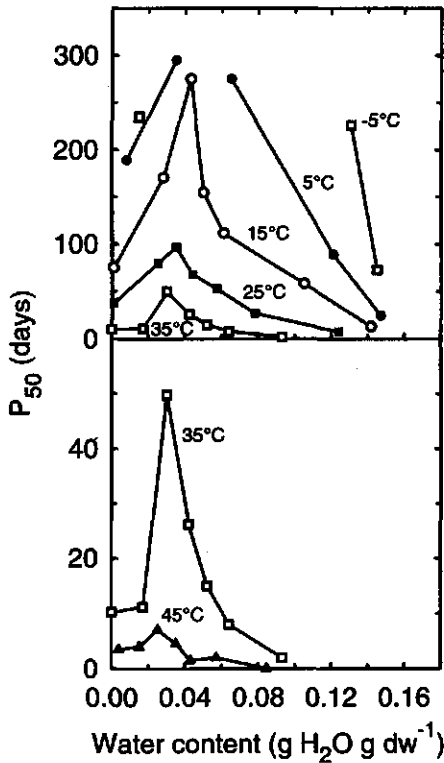


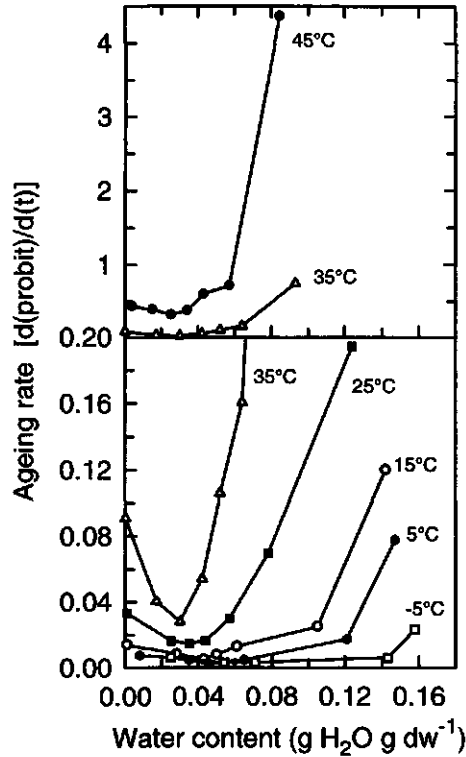
Figure 2.3. Half-viability periods (P_{50}) of cattail pollen after storage at different RH. Water contents are determined from water sorption isotherms (Fig. 2.1). P_{50} represents the days of storage at which the probability plots reached 50% (Fig. 2.2).

Ageing rates of the pollen were calculated from the slope of the probability curves shown in Fig. 2.2. When these ageing rates were plotted against water content for each temperature (Fig. 2.4), the curves exhibited a water content at which the ageing rate was minimum. The water content at which ageing rate was minimal appeared to increase with decreasing storage temperature, but the RH at which ageing rate was minimal was constant at about 11-15% for all storage temperatures (compare minimum water content values at each temperature with isotherms given in Fig. 2.1).

The optimum storage conditions of the pollen were related to changes in water properties, obtained from changes in calorimetric data (Buitink et al., 1996). Figure 2.5 summarises the combined data in a state diagram. Using differential scanning calorimetry we previously determined the glass transition temperature (T_g) in the pollen at various water contents. The T_g curve shows that the T_g decreased with increasing water content. The curve representing the optimum water content/temperature combinations giving maximal storage stability (obtained from Fig. 2.4) was situated below the T_g curve, indicating that under these storage conditions glasses are present in the pollen. The T_g curve is flanked by two other curves, Cp_1 and Cp_2 , which represent the water content at which sudden changes in the apparent heat capacity of the pollen take place (Buitink et al., 1996). The water content at which these sudden changes appear shifted with temperature. The optimum water content curve corresponded

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Figure 2.4. Ageing rates of cattail pollen stored at different temperatures and water content. Ageing rates were quantified as the negative of the value of the slopes of the probability plots.



closely to the C_{p1} curve. The BET monolayer value also corresponded closely with the C_{p3} curve (Fig. 2.5).

To test the glass concept that a break in the Arrhenius behaviour of ageing rate occurs at or near T_g , \ln (ageing rates) were plotted against T^{-1} ($K^{-1} \times 1000$) for pollen stored at constant water content (Fig. 2.6). For each water content, ageing rates were obtained from interpolation of the data in Fig. 2.4. Estimates of Arrhenius plots of pollen stored at water contents from 0.05 to 0.08 $g H_2O g dw^{-1}$ showed two lines with a break near the T_g (arrows, Fig. 2.6A-D). Above T_g , the activation energy (E_a) was higher (161-215 $kJ mol^{-1}$) than below T_g (52-84 $kJ mol^{-1}$). At water contents of 0.05 and 0.06 $g H_2O g dw^{-1}$, the data point at $-5^\circ C$ appeared to deviate from the line drawn for 25 to $5^\circ C$ (Fig. 2.6C,D). This deviation might result from the uncertainty in calculating ageing rates when only slight changes in germination percentages were observed during the storage period. Alternatively, Arrhenius behaviour may not apply as the water content approaches the optimum. The optimum water content for storage at $25^\circ C$ or less was 0.038 $g H_2O g dw^{-1}$ or greater (see Figs. 2.3 and 2.4) and Arrhenius plots of ageing rates for pollen stored at temperatures less than $25^\circ C$ and water contents less than the optimum were curvilinear (Fig. 2.6E and F show Arrhenius plots of pollen with water contents of 0.04 and 0.03 $g H_2O g dw^{-1}$). Consistent with this hypothesis, the deviation from Arrhenius behaviour at $-5^\circ C$ was observed for 0.06 and 0.05 $g H_2O g$

dw^{-1} (Fig. 2.6C and D). These water contents are close to the optimum water content of $0.053 \text{ g H}_2\text{O g dw}^{-1}$ (Fig. 2.4) at -5°C . Although the data set presented here is extensive, it still gives only a few temperature points upon which to evaluate Arrhenius behaviour. Thus, interpretations of breaks in Arrhenius behaviour are made with caution.

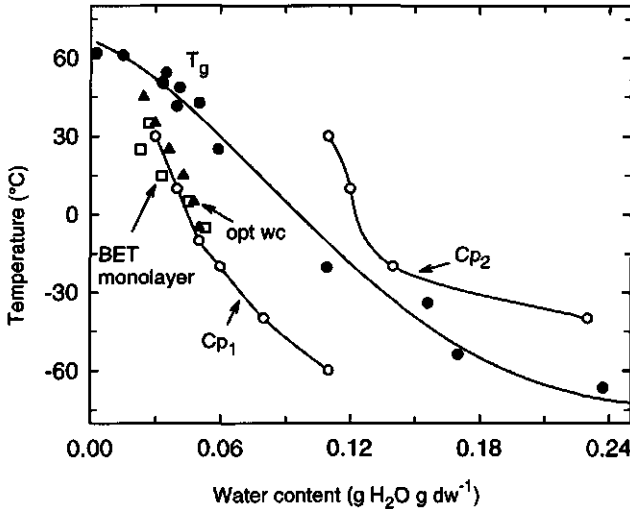


Figure 2.5. Supplemented state diagram of cattail pollen. Cp_1 and Cp_2 , water content/temperature conditions of first and second break in the heat capacity (open circles), respectively; T_g , glass transition temperature (closed circles). Data are according to Butink et al. (1996). Water content/temperature combinations giving optimal storage (closed triangles) were derived from Fig. 2.4 and BET monolayer values (open squares) are given in Table 2.1.

Discussion

Optimum moisture level of storage

In an attempt to understand the interaction of temperature, RH and water content on longevity of cattail pollen, we compared the decline in viability, expressed as percentage germination, during storage under defined conditions with water sorption isotherms and state diagrams.

For all storage temperatures studied, deterioration was slowest in pollen samples stored over saturated LiCl solutions (Fig. 2.2). This solution gives a RH of about 11-15%, depending on temperature. The water content corresponding to this optimum level varied with temperature, decreasing from about $0.053 \text{ g H}_2\text{O g dw}^{-1}$ at -5°C to about $0.024 \text{ g H}_2\text{O g dw}^{-1}$ at 45°C (Figs. 2.3 and 2.4). Our data on the effect of temperature and water content on ageing of pollen are mostly consistent with those obtained by Vertucci and Roos (1990) and Vertucci et al. (1994b) on seeds. Their ageing data were based on measurements of rates of germination and radicle elongation (vigour), whereas

2

our data were derived from percentages of germination. Whereas Vertucci and colleagues found optima in correspondence to about 22% RH in pea, we detect an optimum at about 11-15% RH. This may appear to be in closer agreement with the critical RH suggested by Ellis and colleagues (Ellis et al., 1989, 1990b) for seeds. However, our conclusions differ from the conclusions made by those authors. While Ellis et al. (1989, 1990b) suggest that the critical water content obtained at 11% RH and 20°C is similar for all the storage temperatures and that there is no deleterious effect of overdrying, we demonstrate that in cattail pollen the critical water content corresponds to 14% RH at each temperature and that drying below this value has damaging effects on the longevity. In other words, we suggest that an optimum water content exists and the value is not constant but varies with temperature. This temperature dependence is illustrated by the water sorption isotherms (Fig. 2.1). The change in optimum water content with temperature for pollen is considerably less than that reported for pea seeds (Vertucci et al., 1994b). However, the change in water content with temperature, given constant RH, is also considerably less for pollen compared to pea. Our study supports the concerns that ultra-dry storage is counter-productive to long-term storage of dry systems.

Possible negative effects of extreme drying on longevity of pollen (and seed) might not have been recognised properly in the early literature, because they were mistakenly attributed to imbibitional stress (e.g. Hoekstra, 1986,

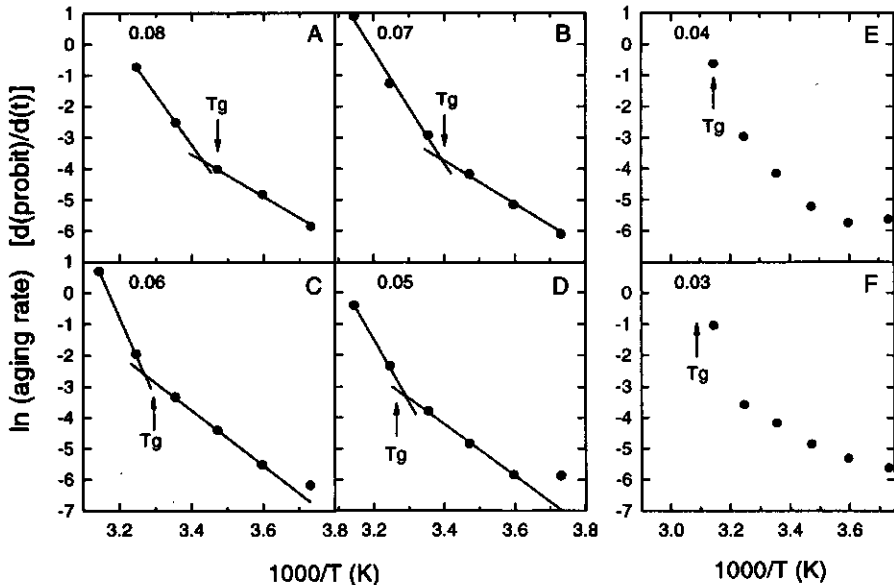


Figure 2.6. Arrhenius plots of ageing rate (slope of probability analysis) versus $1000/T$. Arrows denote the T_g of the pollen at the corresponding water content obtained from Fig. 2.5. Water content values ($\text{g H}_2\text{O g dw}^{-1}$) are indicated. Lines represent linear regressions (A-D). Data points are interpolated from Fig. 2.4.

1995; Ellis et al., 1990c). However, in the present study imbibitional damage was avoided by carefully prehydrating the grains from the vapour phase before germination. This does not rule out the possibility that ageing leads to an increased susceptibility to imbibitional stress during storage as described for cattail pollen by Van Bilsen et al. (1994).

Relationship between calorimetric properties and optimal storage conditions

We have identified the storage conditions for maximal longevity of cattail pollen and the influence of temperature and water content on longevity. We subsequently attempted to relate the storage behaviour of pollen to cytoplasmic glass formation that has a comparable dependence of water and temperature. The presence of glasses has profound beneficial effects on the stability of various food materials (Lim and Reid, 1991; Karmas et al., 1992; Roos, 1995 and references therein) and pharmaceutical biomolecules (Chang et al., 1996). Intracellular glasses in seeds are hypothesised to play a role in storage stability (Burke, 1986; Leopold et al., 1994; Maki et al., 1994). We previously characterized the water content/temperature combinations at which glasses occur in cattail pollen (Buitink et al., 1996). Here we show (Fig. 2.5) that the water content/temperature combinations at which storage longevity is maximal are positioned about 10 to 30°C below the T_g curve, i.e. when the pollen is in a glassy state. Similar results have been shown with food systems and pharmaceuticals where degradative reactions still occur below T_g and conditions for maximum storage stability are considerably less than T_g (Lim and Reid, 1991; Nelson and Labuza, 1994; Hancock et al., 1995; Chang et al., 1996). In line with glassy concepts used in the food and pharmaceutical literature, we conclude that ageing reactions in pollen can occur in the glass, albeit at lower rates compared with ageing rates above T_g (Fig. 2.6).

The curve representing optimal storage conditions of the pollen deviates to a certain extent from the T_g curve. Several speculations based on recent glass literature can be put forward to explain this deviation. Diffusion of harmful molecules in the glassy matrix may be higher than expected on the basis of viscosity (Liu and Oppenheim, 1996; Perera and Harrowell, 1996). Small molecules such as free radicals or oxygen, which may be involved in the loss of viability may diffuse more freely through the glassy matrix than larger molecules (Karel and Saguy, 1991; Le Meste et al., 1991). At suboptimal water contents, there may be a greater production of harmful free radicals, their mobility may be less restricted for some reason, or there may be greater interactions between these small molecules and macromolecules. Alternatively, detrimental reactions may occur in the lipid phases of the cell where the glassy matrix is not present. Indeed, many of the mechanisms hypothesised to cause ageing involve physical and chemical alterations of the lipids in membrane systems (Priestley, 1986; Thompson et al., 1987; McKersie et al., 1988; Senaratna et al., 1988; Hendry, 1993; Van Bilsen and Hoekstra, 1993; Van Bilsen et al., 1994).

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Besides the observation that optimum storage occurs at constant RH, it is remarkable that the optimum storage conditions follow so closely the first break in the heat capacity measurements (Fig. 2.5). The physical explanation for a change in heat capacity is not clear, although its correspondence with the BET monolayer value may be significant. Heat capacity measurements may assist in predicting the water content/temperature combination for optimal longevity.

Ageing kinetics of cattail pollen

We demonstrate that ageing kinetics of the pollen follow similar water content/temperature dependencies as the storage stability of various food systems (Karmas et al., 1992; Nelson and Labuza, 1994). Presuming that the presence of glasses in pollen suppresses the mobility of molecules which, in turn, suppresses ageing rates, ageing rates should increase considerably when the glassy matrix melts (Karmas et al., 1992; Nelson and Labuza, 1994). In pollen, breaks in Arrhenius plots around T_g (Fig. 2.6) substantiate the expected change in ageing kinetics. Similar observations have been made for seeds, where the high humidity/high temperature conditions of "accelerated ageing" or "controlled deterioration" experiments, bring the tissues above their T_g and cause very rapid loss of viability (Priestley, 1986; Sun and Leopold, 1993). Although the evidence for this is limited, an additional deviation from Arrhenius behaviour is suggested when pollen is dried below the optimum water content for storage (Fig. 2.6C-F). The optimum water content coincided with a change in the heat capacity of the pollen (C_p) and the BET monolayer value.

Practical implications for storage protocols

The results of the present experiments are relevant to recent discussions regarding whether preservation protocols should be based on energetic considerations, in which water activity is the operative parameter (Labuza, 1980; Roberts and Ellis, 1989; Vertucci and Roos, 1990, 1993b; Chirife and del Pilar Buera, 1994) or dynamic considerations in which glass formation is important (Slade and Levine, 1991; Williams et al., 1993; Leopold et al., 1994; Sun and Leopold, 1994).

Water sorption in seed and pollen is one of the main factors affecting their physical state and stability. When water sorption isotherms of pollen and seed are compared with their longevity at different water contents, it becomes apparent that for both tissues the optimum RH for storage is situated around the "shoulder" of the sorption isotherm (compare Vertucci and Roos, 1990 and Fig. 1.1). The water content around this shoulder often has been found to correspond to the optimum water content for stability of low-moisture foods (Labuza et al., 1970; Labuza, 1980). Also, this amount of water has been proposed to be necessary for maintenance of structural integrity of desiccation-tolerant organisms (Clegg, 1986; Vertucci and Leopold, 1987; Sun et al., 1997). Water sorption isotherms show an inverse relationship between

temperature and water content if RH is held constant. Thus, if the RH giving optimum storage is constant with temperature, then the water content giving maximum longevity varies with temperature (Vertucci and Roos, 1993b). Therefore, water sorption isotherms produced at several temperatures are helpful in predicting the optimal storage conditions for biological tissues.

In accordance with concepts of the glassy state, we found that we had to store the pollen below their T_g for optimal storage longevity. However, the precise conditions at which the pollen exhibits maximum storage longevity are not revealed by the T_g curve. Therefore, predictions on optimal storage conditions can not be made solely on the basis of T_g . Nonetheless, the glass concept cannot be discarded when there is a need to explain, and possibly, predict ageing kinetics of pollen and seed. We showed that ageing kinetics change when glasses disappear, which is indicated by a break in the Arrhenius plots when pollen is stored above its T_g . The change in activation energy around the T_g warns us not to simply extrapolate kinetic data obtained by ageing seeds above the T_g (high humidity or high temperature conditions) to lower temperatures or water contents (Franks, 1994b).

We demonstrate here that there exists an optimum water content of storage that changes with temperature, as was reported previously for seeds. Because of the increase in optimum water content with lower temperatures, too much drying should be avoided when lower temperature storage is planned. The beneficial effects of the low temperature will then be partially lost.

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Section II

Molecular Mobility

Overview

To increase our understanding of the role of intracellular glasses in storage stability, measurements of molecular mobility are indispensable. Previous studies of molecular mobility in glasses of sugars and polymers involved Electron Paramagnetic Resonance (EPR) spectroscopy. In this section, we applied spin probe EPR spectroscopy to determine the molecular mobility in biological tissues.

The proper choice of spin probe is essential when performing EPR spectroscopy on biological tissues. To ascertain that the spin probe resides in the cytoplasm upon drying, a polar spin probe has to be utilised. Upon drying, the shape of the EPR spectra of the spin probe 3-carboxy-proxyl (CP) indicated immobilisation of CP. The change in distance between the outer extrema of the solid state spectrum of CP in dry organisms could be ascribed to librational motion, as confirmed by Echo Detected EPR. Whereas conventional EPR spectroscopy could be used to derive the rotational correlation time τ_r of the spin probe in hydrated tissues, Saturation Transfer EPR spectroscopy has to be employed to obtain the τ_r in the solid state, i.e. in dry biological tissues.

Drying of embryonic axes of pea seeds resulted in a decrease in τ_r of seven orders of magnitude, confirming that upon drying the cytoplasm transforms into a solid state. Melting of the glassy state resulted in an increase in rotational motion of the spin probe, with the activation energy of mobility increasing a factor two to three. The increase in molecular mobility of the spin probe upon melting of the intracellular glass was moderate compared to the increase found previously for the melting of sugar glasses.

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Influence of water content and temperature on molecular mobility and intracellular glasses in seed and pollen

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Although the occurrence of intracellular glasses in seeds and pollen has been established, physical properties such as rotational correlation times and viscosity have not been studied extensively. Using electron paramagnetic resonance spectroscopy, we examined changes in the molecular mobility of the hydrophilic nitroxide spin probe 3-carboxy-proxyl during melting of intracellular glasses in embryonic axes of pea (*Pisum sativum* L.) seeds and cattail (*Typha latifolia* L.) pollen. The rotational correlation time of the spin probe in intracellular glasses of both organisms was approximately 10^3 s. Using the distance between the outer extrema of the electron paramagnetic resonance spectrum ($2A_{zz}$) as a measure of molecular mobility, we found a sharp increase in mobility at a definite temperature during heating. This temperature increased with decreasing water content of the samples. Differential scanning calorimetry data on these samples indicated that this sharp increase corresponded to melting of the glassy matrix. Molecular mobility was found to be inversely correlated with storage stability. With decreasing water content, the molecular mobility reached a minimum, to increase again at very low water contents. Minimum mobility and maximum storage stability occurred at similar water contents. This correlation suggests that storage stability might be partially controlled by molecular mobility. At low temperatures, when storage longevity cannot be determined on a realistic time scale, $2A_{zz}$ measurements can provide an estimate of the optimum storage conditions.

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Deterioration of seed and pollen during storage involves many physical and chemical changes, such as disrupted intracellular integrity, decreased activities of enzymes, lipid peroxidation and deesterification, and Maillard reactions (Priestley, 1986; Wilson and McDonald, 1986; Wettlaufer and Leopold, 1991; Van Bilsen and Hoekstra, 1993; Van Bilsen et al., 1994). Since the formation of glasses in dehydrating biological tissues has been established, this physical phenomenon has been put forward as a prominent factor in the control of deterioration rates during storage (Burke, 1986; Williams and Leopold, 1989; Leopold et al., 1994; Leprince and Walters-Vertucci, 1995; Buitink et al., 1996). A glass is a thermodynamically unstable solid-state with an extremely high viscosity (Franks et al., 1991). Its formation is promoted by low water content of the tissue and by low temperatures. Both factors are also known to extend the longevity of seeds and pollens (Roberts, 1972; Roberts and Ellis, 1989; Vertucci and Roos, 1993b; Buitink et al., 1998b), and improved storage stability was observed when glasses were present (Sun and Leopold, 1994; Sun, 1997; Buitink et al., 1998b). It is assumed that the high viscosity of intracellular glasses decreases molecular mobility and impedes diffusion within the cytoplasm, thus slowing down deleterious reactions and changes in structure and chemical composition during ageing (Sun and Leopold, 1993; Sun, 1997). However, the molecular mobility and viscosity in biological glasses has received little attention.

Molecular mobility has been studied using EPR spectroscopy by labelling polymers and food materials with a suitable, stable spin probe (Steffen et al., 1992; Blackburn et al., 1996; Dzuba, 1996; Hemminga and Van den Dries, 1998). From the EPR spectra of the spin probe, the rotational correlation time τ_r can be assessed (Kumler and Boyer, 1976; Kovarskii et al., 1978; Spielberg and Gelerinter, 1982; Ohta and Kuwata, 1985; Roozen and Hemminga, 1990; Roozen et al., 1991; Dzuba et al., 1993). Whereas τ_r values can be calculated from conventional EPR spectra in the order of 10^{-12} to 10^{-9} s (Knowles et al., 1976), ST-EPR spectroscopy further expands this range to very slow (10^{-6} to 10^{-3} s; Hemminga, 1983) and ultra slow (10^{-3} to 10^2 s; Van den Dries et al., 1998a) molecular motions. ST-EPR spectroscopy has been successfully applied to determine τ_r values of spin probes in sugar glasses (Roozen et al., 1991; Van den Dries et al., 1998a) and organic liquids at low temperatures (Ito, 1983). In glassy sucrose-water and malto-oligosaccharide mixtures, τ_r decreased by several orders of magnitude upon approaching T_g (Roozen et al., 1991).

The present paper is aimed at gaining insight into changes in the molecular mobility that accompany glass formation in anhydrobiotes. We used EPR and ST-EPR spectroscopy to characterize the molecular motion of the polar nitroxide spin probe CP that we incorporated in embryonic axes of pea (*Pisum sativum* L.) seeds and cattail (*Typha latifolia* L.) pollen. We show that the distance between the outer extrema in powder spectra produced by conventional EPR spectroscopy can be used to detect changes in molecular motion, and that ST-EPR spectroscopy allows an estimation of τ_r . We discuss

the possible relationship between glasses, molecular mobility and storage stability in these organisms.

Materials and methods

Plant material and treatments

Mature male inflorescences of cattail (*Typha latifolia* L.) were collected from field populations near Wageningen, The Netherlands, in 1996 and allowed to shed their pollen in the laboratory. Pollen (94% germination) was cleaned by sieving through a fine copper mesh, dried in dry air to 0.05 to 0.08 g H₂O g dw⁻¹ and stored at -20°C until use. Pea (*Pisum sativum* cv Karina) seeds (99% germination) were obtained from Nunhems Zaden (Haalen, The Netherlands) and stored at 5°C until use.

The polar nitroxide spin probe 3-carboxy-proxyl (CP, Sigma) was used for spin labelling of pea seeds and cattail pollen. Pollen (3 g) was prehydrated in water vapour for 16 h at 5°C to about 0.7 g H₂O g dw⁻¹ and then mixed at 25°C with 6 ml of liquid germination medium containing 2.5 mM CP. The germination medium consisted of 1.6 mM H₃BO₃, 1.3 mM Ca(NO₃)₂·4H₂O, 0.8 mM MgSO₄·7H₂O, 1.0 mM KNO₃ and 0.2 M sucrose in 2 mM sodium-phosphate-citrate buffer, pH 5.9. After a few minutes, an additional 20 ml of the germination medium was added, and the pollen was recovered by filtration. The pollen was then mixed with 20 ml of a solution of 1 mM CP and 120 mM of the broadening agent potassium ferricyanide. Ferricyanide broadens spin probe signals in the solution surrounding the cells to invisibility (Golovina and Tikhonov, 1994; Golovina et al., 1997). After 5 more min, the pollen was recovered by filtration, spread out in a large Petri dish, and rapidly dried in a flow of dry air (3% RH) in a box to water contents of less than 0.05 g H₂O g dw⁻¹.

The pollen retained high viability after labelling and drying. To determine the τ_r at high water contents (1.5 to 0.5 g H₂O g dw⁻¹), pollen samples were taken regularly during drying for EPR measurements. After drying, the pollen was stored over various saturated salt solutions or P₂O₅ (Winston and Bates, 1960) at 25°C for at least 3 d to obtain various water contents.

Pea seeds were rolled in germination paper and imbibed in tap water overnight at 15°C, and then embryonic axes were isolated. Subsequently, the axes were incubated in a solution containing 1 mM CP and 200 mM ferricyanide at room temperature. After 45 min, the axes were rinsed with distilled water and dried at 35% RH for 3 d. To determine the τ_r at high water content (1.5 to 0.5 g H₂O g dw⁻¹), pea axes were regularly sampled during drying for EPR measurements. After drying, the axes were stored over the saturated salt solutions or P₂O₅ at 25°C to obtain various water contents.

With every sample taken for EPR measurements, a sample treated similarly was taken for determination of water content. Water contents were analysed by weighing the samples before and after heating at 96°C for 36 to 48 h (Buitink et al., 1996) and calculating the water loss on a dry weight basis.

EPR and ST-EPR Measurements

EPR spectra were recorded with an X-band EPR spectrometer (Model 300E, Bruker Analytik, Rheinstetten, Germany). Microwave power was kept low (200 μ W or 2 mW) to avoid saturation. Modulation amplitude was 0.4 G for pea axes and 1 G for cattail pollen.

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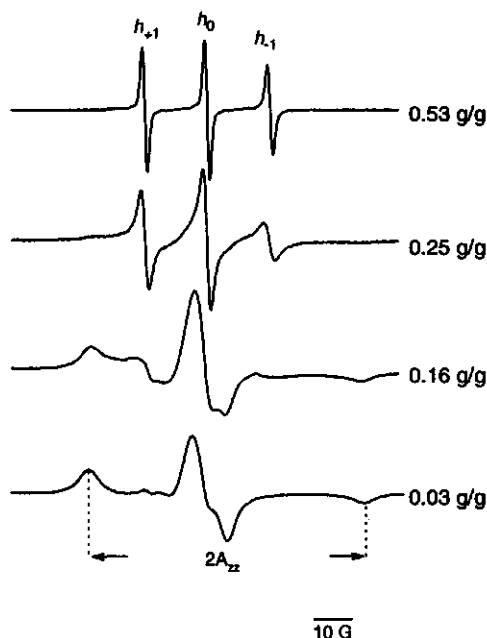
Samples of various water contents were loaded into a 3-mm diameter EPR capillary. For each measurement, the capillary was filled for a length of 5 cm with pollen or with two isolated pea axes. To prevent water loss during the measurements, the capillaries were sealed at both sides. Temperature was controlled using a controller with liquid nitrogen vapour as the coolant. Samples were rapidly cooled to -150°C and allowed to equilibrate for 30 min, after which scans were recorded at 10°C increments with equilibration for 5 min after each increment.

Conventional EPR spectroscopy can detect changes in τ_r of spin probes ranging from 10^{-12} to 10^{-9} s, which corresponds to the lifetime of the probe in a given orientation. In this motional range, the EPR spectrum of nitroxides consists of three lines (Fig. 3.1, top spectrum), and τ_r can be calculated according to the method of Knowles et al. (1976):

$$\tau_r = 6.5 \times 10^{-10} \Delta B_0 [(h_0/h_{\pm 1})^{1/2} - 1] \quad (3.1)$$

where $h_{\pm 1}$ and h_0 are the heights of the high-field and central lines in the EPR spectra, respectively and ΔB_0 is the line width of the central line in Gauss. The rotational motion of the spin probe was assumed to be isotropic. The τ_r of partially hydrated (above approximately $0.25 \text{ g H}_2\text{O g dw}^{-1}$) pollen and pea axes was determined using equation (3.1).

Figure 3.1. EPR spectra of CP in cattail pollen at various water contents (g/g, g H₂O g dw⁻¹). Spectra were recorded at room temperature. The $h_{\pm 1}$, h_0 and $h_{\pm 1}$ peaks are shown in the top spectrum. The distance between the two outer extrema, $2A_{zz}$, is indicated in the bottom spectrum.



The characteristic τ_r for spin probes in organic glasses near and above T_g is approximately 10^{-9} s or shorter (Dzuba et al., 1984; Roozen et al., 1991; Van Dries et al., 1998a). These τ_r values can not be determined using equation 3.1, because the line shapes of the spectra change due to the appearance of a powder spectrum (Fig. 3.1, below $0.25 \text{ g H}_2\text{O g dw}^{-1}$). In such powder spectra, a temperature dependence of the distance between the two outer extrema ($2A_{zz}$) was observed for a number of glass-forming substances at and above their T_g (Kumler and Boyer, 1976;

Kovarskii et al., 1978; Spielberg and Gelerinter, 1982; Ohta and Kuwata, 1985; Dzuba et al., 1993b) (Fig. 3.1). We made use of this parameter to obtain a qualitative measurement of the molecular mobility of CP.

From the τ_R one can derive the viscosity of the matrix in which the spin probe is rotating, according to the modified Stokes-Einstein equation (Roozen et al., 1991):

$$\tau_R = (\eta V/k_b T) k + \tau_0 \quad (3.2)$$

where η is the solvent viscosity, k_b is Boltzmann's constant, V is the volume of the rotating molecule, T is the absolute temperature, τ_0 is the zero viscosity rotational correlation time, and k is a dimension-less slip parameter.

ST-EPR spectroscopy was used in the motional region for $\tau_R > 10^{-7}$ s. This method is based on the diffusion and recovery of saturation between different portions of the powder spectrum in competition with field modulation (Hemminga, 1983). For ST-EPR spectroscopic measurements, the second harmonic quadrature absorption signal was detected under the following conditions: field modulation amplitude of 5 G, microwave power of 100 mW, and field modulation frequency of 50 kHz (Hemminga et al., 1984). The phase was set with the self-null method (Thomas et al., 1976).

In ST-EPR spectroscopy, τ_R values are usually obtained empirically using reference material with known viscosity. Here, spectra of CP in anhydrous glycerol were used to construct a calibration curve according to the method of Van den Dries et al. (1998a). Because the viscosity for anhydrous glycerol is known over a broad temperature range, τ_R of CP in glycerol can be obtained from equation 3.2. Spectra of CP in anhydrous glycerol were recorded every 3°C, and the values of the line shape parameters L^*/L and C'/C (explained in Fig. 3.5) were calculated for each temperature (data not shown). From the curves representing the line shape parameters of CP in glycerol against τ_R , the τ_R values of CP in the axes and pollen were obtained by interpolation of the corresponding line shape parameters.

Differential scanning calorimetry

Pollen and pea embryonic axes with different water contents were hermetically sealed in aluminum pans for differential scanning calorimetry. Second-order transitions of the samples were determined using a differential scanning calorimeter (Pyris-1, Perkin-Elmer) calibrated for temperature with indium (156.6°C) and methylene chloride (-95°C) standards and for energy with indium (28.54 J g⁻¹). Baselines were determined using an empty pan, and all thermograms were baseline corrected. Scans were taken from -100°C to 120°C at a rate of 10°C min⁻¹. The T_g values were determined by the onset and midpoint of the temperature range over which the change in specific heat occurred. All analyses were performed with Perkin-Elmer software.

Results

Molecular motion in pollen and seed

Figure 3.1 shows representative EPR spectra of CP in cattail pollen at different water contents recorded at room temperature. When the water content is decreased from 0.53 to 0.25 g H₂O g dw⁻¹ (top two spectra), the relative amplitudes of the outer spectral lines decreased and the width of the central

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peak increased. Using equation 3.1, the τ_r of CP in hydrated pollen and pea embryonic axes (approximately $1.2 \text{ g H}_2\text{O g dw}^{-1}$) was calculated to be approximately 10^{-11} s (data not shown). In the partially dehydrated state ($0.53 \text{ g H}_2\text{O g dw}^{-1}$), τ_r of CP in cattail pollen was calculated as $1.6 \cdot 10^{-10} \text{ s}$ (Fig. 3.1, top spectrum). At $0.25 \text{ g H}_2\text{O g dw}^{-1}$ (Fig. 3.1, second spectrum from the top), τ_r of CP in pollen was $7.9 \cdot 10^{-10} \text{ s}$. The τ_r of CP in pea embryonic axes at $0.5 \text{ g H}_2\text{O g dw}^{-1}$ was $3.6 \cdot 10^{-10} \text{ s}$ (data not shown). At a water content below $0.25 \text{ g H}_2\text{O g dw}^{-1}$, a powder spectrum (characterized by the two broad peaks at the extremes) overlapped the mobile spectrum (three sharp lines separated by a distance of 14.5 G). The bottom spectrum shown in Fig. 3.1 has the characteristic shape of a powder spectrum, indicative of slow molecular mobility of the spin probe, with τ_r longer than 10^{-8} s . With the appearance of this powder spectrum, below $0.25 \text{ g H}_2\text{O g dw}^{-1}$, the τ_r of CP can not be calculated using equation 3.1, because the line shapes are distorted. The distance between the two broad peaks at the extremes is referred to as $2A_{zz}$ (Fig. 3.1, bottom spectrum). Similar spectra were obtained for pea embryonic axes in relation to water content (data not shown).

Figure 3.2 shows EPR spectra of CP in dry pea embryonic axes and cattail pollen (both having $0.07 \text{ g H}_2\text{O g dw}^{-1}$) at a range of temperatures. At least two overlapping spectra contributed to the total spectrum observed; a powder spectrum at all temperatures, and a mobile spectrum above 20°C , the contribution of which increased with increasing temperature. Note that the contribution of the mobile component to the total spectrum is considerably larger for pollen at 70°C than for pea embryonic axes at 90°C .

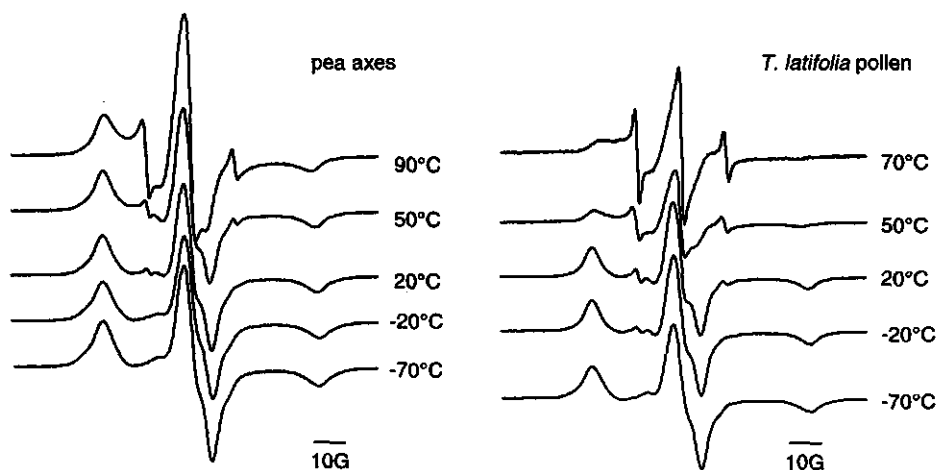


Figure 3.2. EPR spectra of carboxy-proxyl in pea embryonic axes and cattail pollen recorded at various temperatures. Both tissues contained approximately $0.07 \text{ g H}_2\text{O g dw}^{-1}$.

At -150°C , we assumed that the motion of the probe was completely immobilised and therefore we took the corresponding $2A_{zz}$ values as the maximum values. At this low temperature, the value of $2A_{zz}$ gives information

about the polarity of the spin probe's environment in the tissue (Knowles et al., 1976). In pea embryonic axes, the maximum $2A_{zz}$ decreased with decreasing water content from 74 to 70 G, whereas in cattail pollen, it changed from 72.5 to 71.5 G (Fig. 3.3).

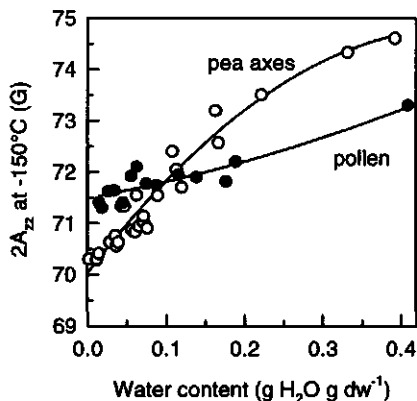


Figure 3.3. Change in the distance between the outer extrema of the EPR spectra ($2A_{zz}$) of CP in pea embryonic axes (open symbols) and cattail pollen (closed symbols) at -150°C as a function of water content.

Because we observed a powder spectrum of CP in pea embryonic axes and cattail pollen at low water contents ($<0.2 \text{ g H}_2\text{O g dw}^{-1}$), τ_R cannot be directly calculated from the EPR spectra using equation 3.1. However, the change in $2A_{zz}$ with temperature can be used as an estimate of molecular motion (Van et al., 1974; Dzuba, 1996). A decrease in $2A_{zz}$ is indicative of an increase in molecular mobility. Figure 3.4 shows these changes in $2A_{zz}$ with temperature. When the temperature of pea embryonic axes and pollen increased, the $2A_{zz}$ slowly decreased, then abruptly decreased above a definite temperature. With increasing water contents, this abrupt decrease in $2A_{zz}$, denoting an abrupt increase in molecular mobility, commenced at lower

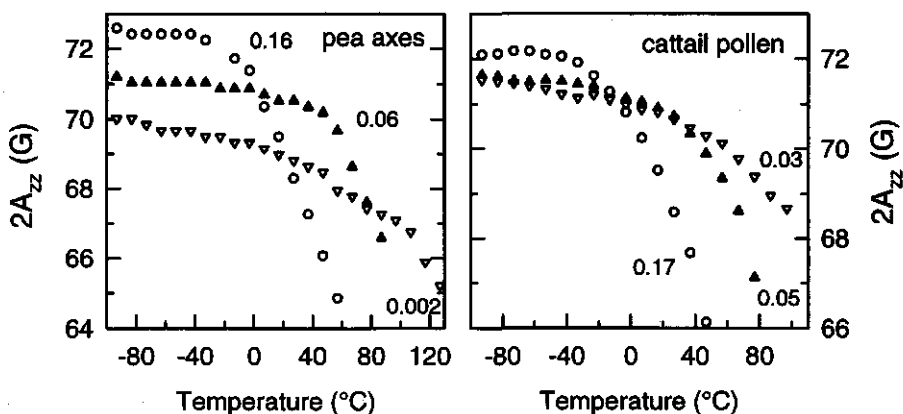


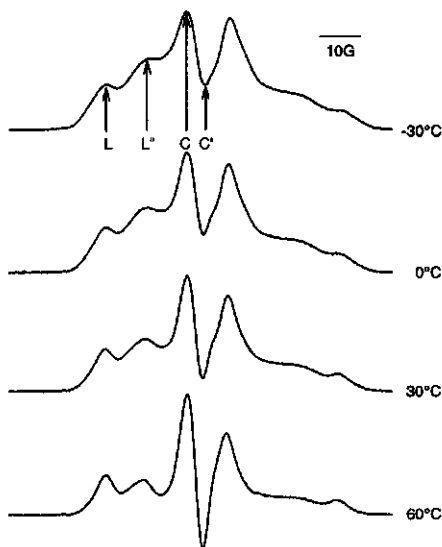
Figure 3.4. Distance between the outer extrema of the EPR spectra ($2A_{zz}$) of CP in pea embryonic axes and cattail pollen at different water contents plotted as a function of temperature.

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temperatures. At a water content of $0.002 \text{ g H}_2\text{O g dw}^{-1}$ in pea embryonic axes, the decrease in $2A_{zz}$ was less clear.

We used ST-EPR to obtain an indication of the magnitude of change in τ_R (reflecting the lifetime of the probe in a given orientation) that corresponds to the change in $2A_{zz}$ with temperature. This technique is based on recording spectra under saturation conditions, which yields line shapes that are sensitive to τ_R below 10^{-7} s (Hyde and Dalton, 1979). The ST-EPR spectra can be well characterized by independent line shape parameters, such as the line height ratio L'/L and C'/C (Fig. 3.5). These ratios are dependent on the τ_R of the spin

Figure 3.5. ST-EPR spectra of CP in pea embryonic axes at $0.08 \text{ g H}_2\text{O g dw}^{-1}$ recorded at various temperatures. Scans were recorded at 100 mW, modulation frequency 50 kHz, and a modulation amplitude of 5 G.



probe. To determine the τ_R of CP corresponding to a certain line height ratio, anhydrous glycerol was used as a reference solvent [this solvent was previously used as a reference in sugar glasses in Roozen et al. (1991) and Van den Dries et al. (1998a)]. Although the composition of the cytoplasm of seed and pollen is not comparable to glycerol, at present, the use of glycerol will give the best approximation of the relationship between the line height ratios and τ_R . Extrapolation of the τ_R of CP in seed and pollen to the corresponding viscosity is not valid, because the Stokes-Einstein law fails at temperatures below approximately $1.3 \times T_g$ (Liu and Oppenheim, 1996).

As the viscosity for glycerol is known over a broad temperature range, the corresponding τ_R could be calculated according to equation 3.2. Subsequently, the two line height ratios were obtained from ST-EPR scans of anhydrous glycerol for a range of temperatures and plotted against the corresponding τ_R . Those calibration curves were used to obtain τ_R from line height ratios calculated from ST-EPR scans of CP in pea embryonic axes (Fig. 3.5). The τ_R values for pea embryonic axes ($0.08 \text{ g H}_2\text{O g dw}^{-1}$) calculated according to both line height ratios and plotted against the temperature are shown in Fig. 3.6. Thus, τ_R values of CP in pea embryonic axes were in the

range of 2×10^{-4} to 10^{-6} s. The τ_R values of CP in the pollen were in the same range (data not shown). From Fig. 3.6 it can be seen that there is a difference in the τ_R derived from the L^*/L ratio and the C'/C ratio. Furthermore, it can be observed that the τ_R obtained from the C'/C ratio shows an increase in mobility with increasing temperature comparable to the $2A_{zz}$ curves (Fig. 3.4). One can speculate on what causes the difference in τ_R obtained from both line height ratios. The τ_R obtained from the L^*/L ratio probably reflects overall isotropic

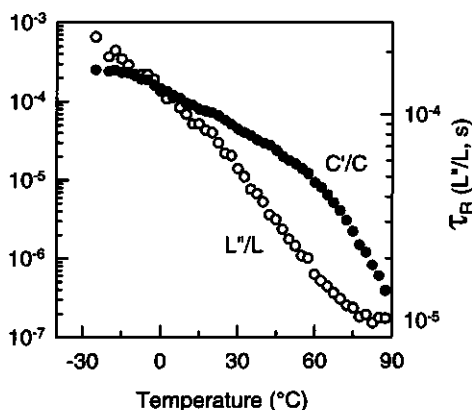


Figure 3.6. Rotational correlation time (τ_R) of CP in pea embryonic axes at $0.08 \text{ g H}_2\text{O g dw}^{-1}$ as a function of temperature. The τ_R values were obtained by comparing the L^*/L ratio and the C'/C ratio with those of CP in anhydrous glycerol.

rotational motion, whereas the τ_R derived from C'/C also reflects some anisotropic motion. According to Dzuba (1996), the change in $2A_{zz}$ can be described by a librational model. Unlike rotational motion, in which the spin probe rotates entirely randomly, librational motion assumes that the spin probe rotates within a cone given by a small angle α . Therefore, it could be that the anisotropic motion seen in the τ_R obtained from the C'/C ratio arises from librational motion. Whether the change in $2A_{zz}$ with temperature is due to librational motion is currently under investigation. As ST-EPR is a laborious technique, more investigations are needed to make full use of this technique as a method to determine molecular mobility. Meanwhile, we will consider the measurements of $2A_{zz}$ as an estimate of molecular motion.

Molecular motion and intracellular glasses

As shown in Fig. 3.4, a sharp increase in the molecular motion of CP was noticeable when the temperature of the sample was increased. Because the temperature corresponding to this sharp increase depended on water content, an attempt was made to explain this behavior according to the glass theory. From the plot of $2A_{zz}$ against temperature we derived two characteristic temperature points (see inset in Fig. 3.7 for details): at the intercept (midpoint T_g) and at the point of deviation from a straight line (onset T_g). Figure 3.7 shows plots of the temperature at which the breaks occurred compared with the water content in pea embryonic axes. For cattail pollen a similar type of plot was

3

obtained (data not shown). At low water contents ($<0.002 \text{ g H}_2\text{O g dw}^{-1}$) the characteristic temperature points were difficult to determine exactly, as the legs below and above the glass transition did not show a sharp drop in $2A_{zz}$. The curves in Fig. 3.7 are remarkably similar to state diagrams of intracellular glasses in seeds (Leopold et al., 1994; Leprince and Walters-Vertucci, 1995) and pollen (Buitink et al., 1996).

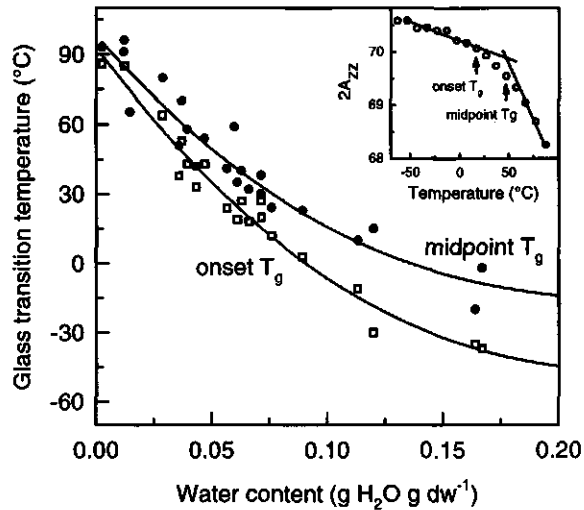


Figure 3.7. Effects of water content on the onset and midpoint of abrupt changes in the distance between the outer extrema of EPR spectra of CP in pea embryonic axes. The inset shows how the onset and midpoint temperatures were determined. Onset T_g was taken as the point of deviation from a straight line, and midpoint T_g as the intercept of the two lines (the line drawn through the data points is meant as an aid to visualisation).

To ascertain whether the sharp changes in molecular motion were, indeed due to the melting of intracellular glasses, the onset T_g values were measured by DSC and compared with the onset T_g values as determined by EPR (Fig. 3.8). Both curves closely matched one another, the EPR data being slightly lower than the DSC data. The T_g measured by DSC has been found to occur above the T_g measured at a molecular level (Kalichevsky et al., 1992). The midpoint of T_g from EPR measurements was situated slightly above the midpoint of the T_g measured by DSC (data not shown). The T_g for dry pollen (62°C) was lower than that for dry pea embryonic axes (92°C), which may be related to the high level of oligosaccharides in the latter (Amuti and Pollard, 1977; Saleki-Gerhardt and Zografis, 1994). The constant value of T_g measured by DSC when the last 2% of water was removed might indicate that the first small amount of water does not contribute to plasticization of the glass. It is possible that this water is not present in the glass but, rather, is located in some other part of the tissue (e.g. cell walls).

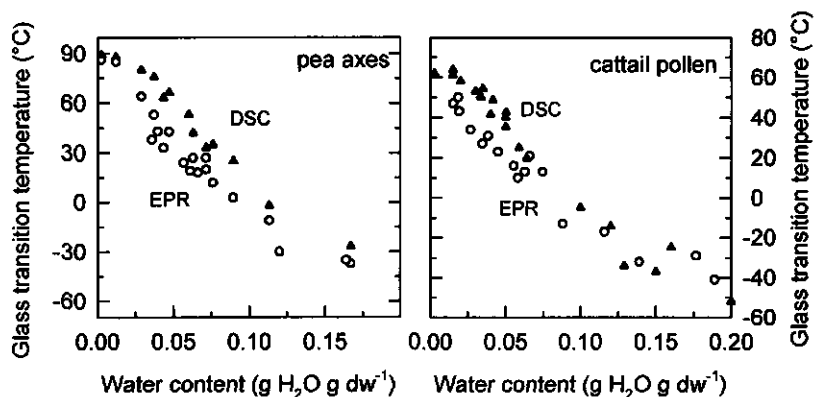


Figure 3.8. The relationship between T_g and water content in pea embryonic axes and cattail pollen. The open symbols show the T_g determined as the onset of the second-order like transitions measured by DSC during heating with a scanning rate of $10^\circ\text{C min}^{-1}$. The closed symbols show the onset of the abrupt decrease in $2A_{zz}$ (derived from Fig. 3.4).

The change in molecular mobility during melting of intracellular glasses can be measured as a function of temperature or water content, the relationship being reflected by the state-diagram (Fig. 3.8). To determine the change in molecular mobility as a function of water content instead of temperature, it is necessary to correct for the polarity change of the environment in which CP is present for each water content (Fig. 3.3). Therefore, using curves similar to those shown in Fig. 3.4, the mobility at a certain water content was expressed as the difference between the maximum $2A_{zz}$ (at -150°C , where the spin probe is assumed to be immobilised) and the $2A_{zz}$ measured at the desired temperature. We refer to this parameter as ΔA_{zz} . Thus, an increase in ΔA_{zz} represents a relative increase in molecular mobility compared with the completely immobilised situation at -150°C (i.e. the more the value departs from zero, the higher the molecular mobility). Figures 3.9 and 3.10 show the dependence of the molecular mobility (ΔA_{zz}) on water content in pea embryonic axes and pollen, respectively. Between approximately 0.2 and 0.1 $\text{g H}_2\text{O g dw}^{-1}$, the mobility decreased with decreasing water content at all temperatures analysed. When the tissues reached approximately 0.1 to 0.05 $\text{g H}_2\text{O g dw}^{-1}$, the ΔA_{zz} reached a constant value indicating that the mobility reached a minimum. When water contents were decreased further below approximately 0.05 $\text{g H}_2\text{O g dw}^{-1}$, mobility increased again for pollen (Fig. 3.10). For pea embryonic axes, the mobility slightly increased again or reached a constant level at very low water content (Fig. 3.9). The water content corresponding to the minimum mobility (lowest ΔA_{zz}) shifted to higher values with decreasing temperatures.

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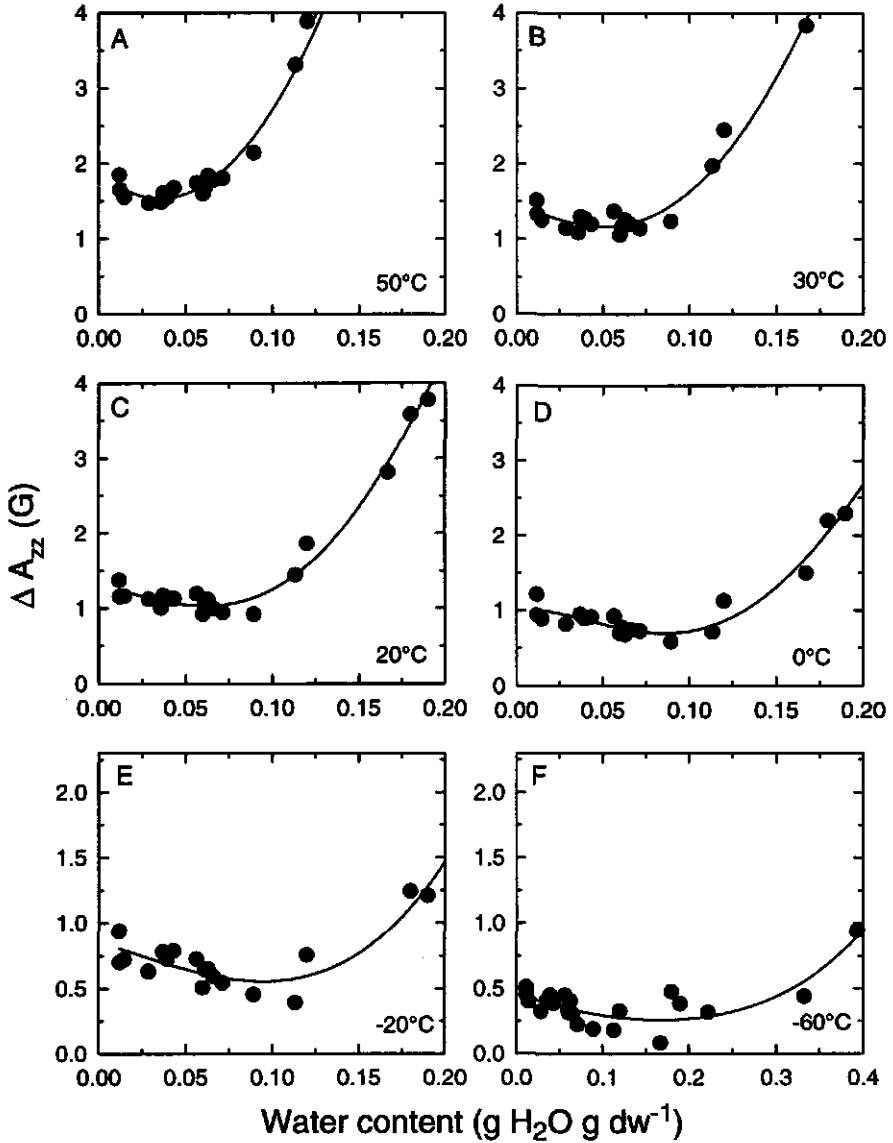


Figure 3.9. Change in ΔA_{zz} , calculated as the difference between the maximum $2A_{zz}$ (-150°C) and $2A_{zz}$ at the indicated temperatures, with water content for spectra of CP in pea embryonic axes. Curves were fitted with a third order polynomial.

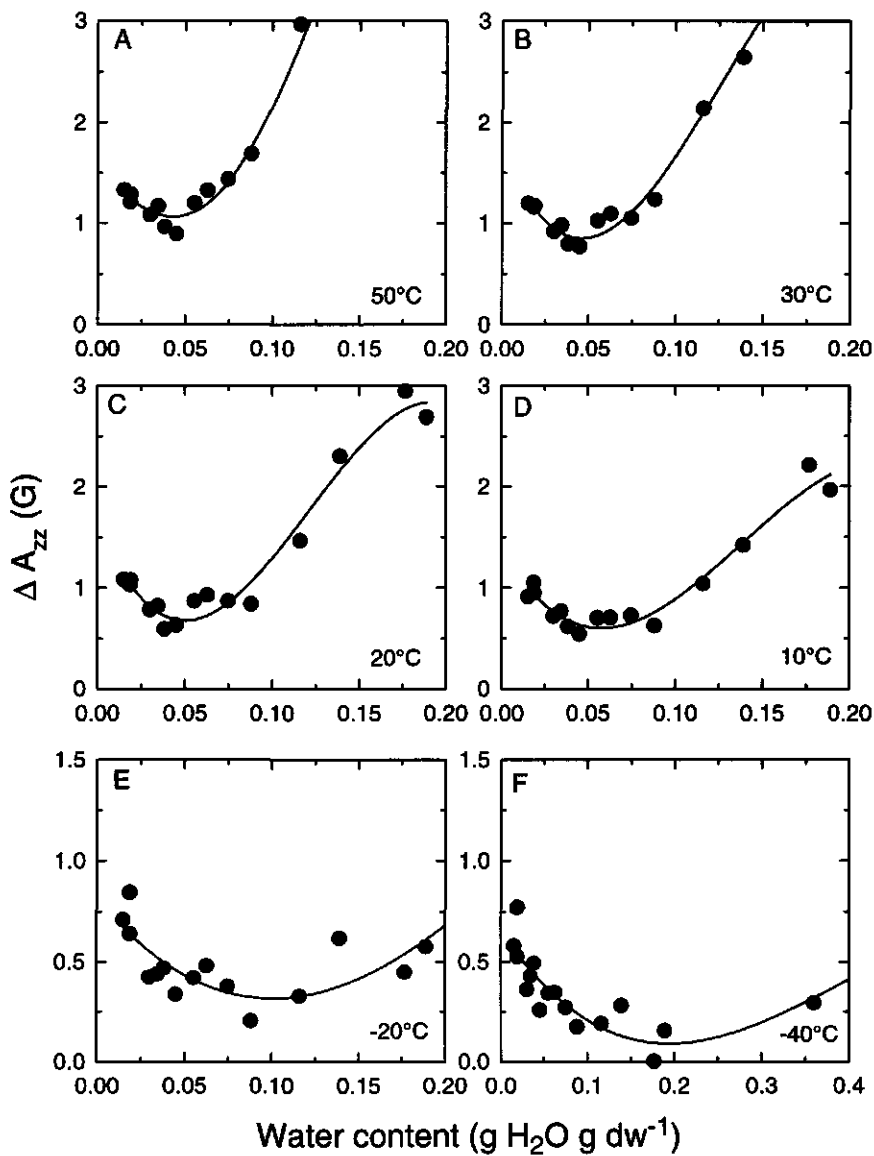


Figure 3.10. Change in ΔA_{zz} , calculated as the difference between the maximum $2A_{zz}$ (at -150°C) and $2A_{zz}$ at the indicated temperatures, with water content for spectra of CP in cattail pollen. Curves were fitted with a third order polynomial.

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Discussion*Behaviour of nitroxide spin probes in dehydrating organisms*

To characterize molecular motion using EPR, a spin probe must be introduced into the material. Depending on the polarity of the spin probe, it will partition into the apolar oil phase, polar aqueous cytoplasm, or both. Several amphipathic spin probes such as TEMPO and TEMPONE completely partition into the lipid phase during drying of cattail pollen (Hoekstra and Golovina, 1998). We found that the more polar TEMPOL partially partitioned into the lipid phase of cattail pollen and pea embryonic axes during drying (data not shown). Because we were interested in the molecular mobility in the cytoplasm rather than in the lipid phase, we avoided the use of these spin labels and instead used the polar nitroxide spin probe CP.

There are several reasons why we believe that CP was not present in the lipid phase in the dry organisms. Upon melting of the oil, which occurred in cattail pollen at approximately -20°C and in pea embryonic axes at -40°C and -20°C as determined by DSC (data not shown), we never observed an abrupt appearance of a mobile EPR spectrum. Furthermore, during drying of pollen at 25°C , when the oil is liquid (Fig. 3.1), we did not observe the typical lipid signal with a hyperfine splitting constant of 14.5 G (Knowles et al., 1976). The resolution of the spectrum was high enough to distinguish a possible oil contribution from the aqueous contribution. Indeed, when samples of elevated water content were heated above 80°C , two peaks were observed in the high field part of the spectrum, one attributable to the water signal and the other to the lipid signal (data not shown).

During drying, the hyperfine splitting constant of CP decreased from 17 to 15.5 G (Fig. 3.1). A decrease in hyperfine splitting constant is associated with a decrease in polarity of the environment of the spin probe (Knowles et al., 1976). During drying the apparent polarity of the cytoplasmic environment decreased, possibly because of the decrease of the dielectric constant of the environment associated with the loss of water. This is also substantiated by the observation of the decrease in $2A_{zz}$ with water loss at -150°C (Fig. 3.3). It is interesting to note that cattail pollen and pea embryonic axes differ in the extent of their $2A_{zz}$ decrease. Therefore, it seems evident that changes in water content are not solely responsible for this difference and that some intrinsic factors (e.g. salts or proteins) also contribute to the decrease in $2A_{zz}$ during drying.

Molecular mobility in biological glasses

Although the presence of intracellular glasses in seeds and pollen has been established, little is known about their viscosity and molecular mobility upon melting. We investigated the molecular motion of a spin probe in the cytoplasm of dry tissues to establish the relationship between glasses, molecular mobility and storage stability.

During drying of cattail pollen and pea embryonic axes, the τ_r of CP increased from 10^{-11} s in the hydrated state to 10^{-3} s in the dry state. These values are consistent with values obtained in organic and inorganic glasses (Ito, 1983; Dzuba et al., 1984; Roozen et al., 1991), but contradict a previous study on molecular mobility in dry soybean axes, in which the τ_r of TEMPO was found to be approximately 10^{-10} s (Bruni and Leopold, 1991). Referring to the tendency of this spin probe to partition into oil during drying, we attribute this contradiction to the fact that these authors might have observed TEMPO in oil bodies. They nevertheless observed an abrupt change in molecular mobility around T_g . It is interesting that TEMPO in the lipid phase seems to be able to "sense" the glass transition of the cytoplasmic surroundings.

Molecular mobility was assessed in relation to water content and temperature. The change in distance between the two outer extrema of the powder spectrum ($2A_{zz}$) with increasing temperature revealed a sharp increase in molecular mobility at a certain temperature that depended on the sample water content. This sharp increase was closely associated with the T_g as measured by DSC. When τ_r was monitored during melting of the glass, there was a decrease of three orders of magnitude, from 10^{-3} to 10^{-6} s.

We found a close correlation between changes in τ_r , determined by the C'/C ratio of ST-EPR spectra and changes in $2A_{zz}$ derived from conventional EPR spectra during melting of intracellular glasses (compare Figs. 3.4 and 3.7). This correlation indicates that a change in $2A_{zz}$ represents a change in molecular mobility. One can speculate on the type of molecular mobility that gives rise to the change in $2A_{zz}$. Echo-detected EPR spectroscopy of nitroxide spin probes dissolved in organic glasses has revealed that the nitroxides undergo librational motions (Dzuba, 1996). This type of motion is described by a motional model that assumes that spin probe rotates within a cone given by a small angle, α (Dzuba et al., 1992; Dzuba, 1996). Since we measured a τ_r for CP in intracellular glasses of approximately 10^{-3} to 10^{-4} s, it seems unlikely that the change in $2A_{zz}$ was due to a change in overall rotational motion. Most likely it represents a change in librational motion, as has been established in wheat embryos (Dzuba et al., 1996). This is further supported by our ST-EPR study, in which some indication for anisotropic motion arising from libration comes from the different values of τ_r deduced from the line height ratios L'/L and C'/C (Fig. 3.6). For the interpretation of our results, however, a motional model that describes the change of $2A_{zz}$ is not needed.

Storage stability in relation to molecular mobility and intracellular glasses

It has been shown that upon formation of glasses, the storage stability of seeds and pollen improves (Sun and Leopold, 1994; Sun, 1997b; Buitink et al., 1998b). The impact of intracellular glasses on the storage behaviour of seeds and pollen has been ascribed to the high viscosity in the glass. Indeed, glasses are known to slow down detrimental reactions, such as the rate of browning reactions (Karmas et al., 1992) and to increase the stability of enzymes (Chang et al., 1996). Although the presence of glasses has been associated with

3

increased storage stability of seeds and pollen, there is not much known about the relationship between molecular mobility and storage stability. Our data on molecular motion in pea embryonic axes and cattail pollen in relation to water content and temperature enable a comparison with storage behaviour, which is known also to depend on water content and temperature (Vertucci et al., 1994b; Buitink et al., 1998b).

We found a close relationship between the molecular mobility of CP and storage behaviour in both pollen and seed embryonic axes. With decreasing water content, molecular mobility, expressed as a change in the outer extrema of the EPR spectra (ΔA_{zz}), decreased, whereas storage stability increased. Figure 3.11 clearly demonstrates that molecular mobility and storage stability are linked; the water content of optimum storage at various temperatures corresponds closely to the water content at which minimum mobility is observed (data from Vertucci et al., 1994b; Buitink et al., 1998b). Although the

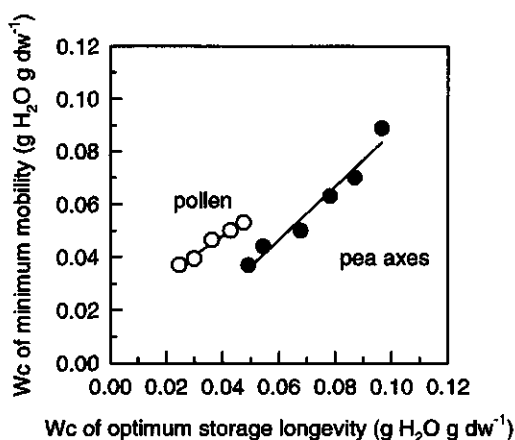


Figure 3.11. Correlation between water content of optimum storage longevity for pea seeds and for cattail pollen and the water content of minimum mobility (lowest value of ΔA_{zz}). Water contents (wc) of optimum storage longevity for cattail were determined by Buitink et al. (1998b). The water content of optimum storage longevity was found to increase with decreasing temperatures of storage (45, 35, 25, 15 and 5°C). Water contents of optimum storage longevity for pea seeds stored in the light were derived from Vertucci et al. (1994). With decreasing storage temperatures (45, 35, 25, 15, 5, -5°C), the water contents of optimum storage increased. The water content at which minimum mobility was observed was determined from the minima of the third order polynomial equations derived from curves similar to Figs. 3.9 and 3.10 at temperatures comparable to the storage temperatures (45, 35, 25, 15, 5 and -5°C).

curves do not converge exactly with the 1:1 line, the slight deviation might be explained from errors in the determination of water content or a lack of an exact determination of the water content for optimum storage stability. Furthermore, the water content of minimum mobility was determined from the minima of the third-order polynomial equations. Especially at lower temperatures, the exact water content of minimum mobility is difficult to

assess; there seems to be a plateau of minimum mobility present. To present the data clearly, we calculated a single value of minimum mobility, which should be considered with caution.

At low water contents, molecular mobility seems to increase again. A similar observation has been made by Seitz et al. (1981) who observed that in *Artemia* cysts at lower hydration levels, the water self-diffusion coefficients increased slightly. Clegg et al. (1982) suggested from NMR studies on *Artemia* cysts that the increased mobility of water at a low water content might be due to a displacement of the water from polar binding sites in the cell by sugars. Another explanation is that at these low water contents the spin probe partitions into a more mobile environment. However, it should be noted that this environment can not be the lipid phase. CP in these phases will rotate faster than 10^8 s at room temperature. Therefore, the spectrum of CP in the oil phase will show the characteristic three sharp lines of a mobile spectrum and does not contribute to the $2A_{zz}$ of the powder spectrum.

Optimum storage conditions predicted on account of molecular mobility

Recent studies report a water-content limit below which seed longevity did not increase further (Ellis et al., 1989, 1990b), or had an adverse effect on seed viability and seed vigour (Vertucci and Roos, 1993b; Vertucci et al., 1994b; Buitink et al., 1998b). It is thought that the removal of the last remaining water molecules may destabilise biological structures (Sun, 1997b; Buitink et al., 1998b) or enhance the life-time of free radicals due the loss of water as a quencher (Karel, 1975). However, we observed an increase in molecular mobility for cattail pollen (which also occurred to a lesser extent in pea embryonic axes) when they were dried to very low water content. This increase in molecular mobility also might be responsible for the decreased storage stability observed at these low water contents.

In some cases, state diagrams can be used to predict the optimal storage conditions (Sun, 1997). For pea embryonic axes, the optimum water contents of storage at a certain temperature were found to coincide with T_g (Fig. 3.8; Vertucci et al., 1994b). However, other seed species show a divergence of the optimal storage stability from the T_g curve (Sun, 1997b). This is also true for cattail pollen (Buitink et al., 1998b). Here, we found that the optimum storage conditions for cattail pollen coincided with the water content at which molecular mobility was minimum (Fig. 3.11). This minimum mobility occurred below T_g . Therefore, we propose that measurements of molecular mobility rather than state diagrams, be used to predict optimum storage conditions.

Based on the relationship between the minimum molecular mobility and the water content of optimal storage, we made an attempt to predict the optimum storage conditions at subzero temperatures: for pea embryonic axes at -20°C and -60°C (Fig. 3.9E and F) and for cattail pollen at -20°C and -40°C (Fig. 3.10E and F). In practice it will not be possible to analyse storage behaviour at these low temperatures on a realistic time scale. At -20°C , the

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minimum molecular motion in pea can be estimated at approximately 0.10 g H₂O g dw⁻¹, and at -60°C between 0.14 and 0.2 g H₂O g dw⁻¹. A similarly elevated optimum water content for low-temperature storage was predicted by Vertucci and Roos (1993b) on the basis of thermodynamic considerations. At -20°C the minimal molecular motion in pollen was approximated at 0.1 g H₂O g dw⁻¹, and at -40°C between 0.15 to 0.2 g H₂O g dw⁻¹. With lower temperatures, an increase in the water contents at which minimum mobility was observed became evident. This implies that too much drying increases mobility and reduces longevity, and should be avoided, particularly when cryogenic storage is considered. Where determinations of optimal storage conditions of seeds and pollen by germination assays take too long to perform, measurements of 2A_{zz} might be considered instead.

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4

Pulsed EPR spin-probe study of intracellular glasses in seed and pollen

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EPR spectra of 3-carboxy-proxyl (CP) in dry biological tissues exhibited a temperature dependent change in the principal value A'_{zz} of the hyperfine interaction tensor. The A'_{zz} value changed sharply at a particular temperature that was dependent on water content. At elevated water contents, the break occurred at lower temperatures, and appeared to be associated with the melting of the cytoplasmic glassy state. To investigate the reason for the change in A'_{zz} we employed echo-detected EPR (ED EPR) spectroscopy. The shape of the ED EPR spectrum revealed the presence of librational motion of the spin probe, a motion typically present in glassy materials. The similarities in temperature dependency of A'_{zz} and librational motion of CP in pea seed axes indicated that the change in A'_{zz} arose from librational motion. ED EPR measurements of 3-carboxy-proxyl as a function of water content in *Typha latifolia* pollen showed that librational motion decreased with decreasing water contents until a plateau or minimum was reached. ED EPR spectroscopy is a valuable technique to characterize the relation between molecular motion and storage kinetics of dry seed and pollen.

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The presence of intracellular glasses in biological systems has been established for over a decade (Burke, 1986; Williams and Leopold, 1989; Leopold et al., 1994). The formation of glasses has been correlated with the ability to endure the dry quiescent state for a long time, enabling these biological systems to maintain their viability until they resume life when they become hydrated (Leopold et al., 1994; Sun, 1997b). The decreased molecular mobility within the glassy cytoplasm has especially been implied to restrict reactions leading to the loss of viability (Sun, 1997b; Buitink et al., 1998a). To obtain a better understanding of the kinetics of detrimental reactions responsible for the loss of viability, an in-depth study of molecular motions in seed and pollen is desirable.

A powerful technique for studying molecular motions in seeds and pollens is EPR spectroscopy (Buitink et al., 1998a). This technique has been applied in the study of rotational and librational motions of spin probes in glass-forming substances, such as supercooled ethanol (Dzuba et al., 1992), polymers (Kovarskii et al., 1978), sugar-water systems (Dzuba, 1996; Hemminga and van den Dries, 1998) and biological materials (Buitink et al., 1998a, 1999). Echo-detected EPR (ED EPR) of nitroxide spin probes in organic glasses revealed that the molecules undergo librational motion (orientational oscillations of a molecule) (Dzuba et al., 1992; Paschenko et al., 1999). This kind of motion appeared to be a general property of guest molecules in glass-forming liquids (Van et al., 1974; Dzuba et al., 1992).

Previously, the molecular motion of a polar spin probe, 3-carboxy-proxyl (CP) (Fig. 4.1), was determined in pea axes using continuous wave EPR (CW EPR) and saturation transfer EPR. This highly polar spin probe was used to exclusively obtain a signal of the spin probe in the cytoplasm. More apolar spin probes have the tendency to partition into the lipid phase with drying (Buitink et al., 1998a). Saturation transfer EPR revealed that the rotational correlation time of CP was in the order of 10^{-6} to 10^{-3} s (Buitink et al., 1999). CW EPR spectroscopy has shown that a change in the principal value A'_{zz} of the hyperfine interaction tensor for CP in pea seed axes and cattail (*Typha latifolia*) pollen was dependent on water content and temperature (Buitink et al., 1998a). A relation was found between the A'_{zz} and storage stability of these biological tissues as a function of temperature and water content. This correlation suggested that storage stability might be controlled by molecular mobility. However, the question was raised what type of molecular motion was responsible for the changes in A'_{zz} , because it is unlikely that rotational motion is involved.

It has been shown that the A'_{zz} temperature dependence may be induced by librational motion (Dzuba, 1996). A'_{zz} is linked to the mean-squared amplitude of motion $\langle\alpha^2\rangle$ by the relation

$$A'_{zz} = A_{zz} + (A_{zz} - A_1) \langle\alpha^2\rangle \quad (4.1)$$

where A_{zz} and A_1 are the principal values of the hyperfine interaction tensor for immobilised nitroxide (assuming that A_{xx} and A_{yy} are close and therefore may be

substituted by their averaged value A_{\perp}). It is furthermore assumed in equation 4.1 that the motional axis lies in the xy plane of the molecular framework (see Fig. 4.1). Recent studies have shown that the A'_{zz} temperature dependence is a general property of molecular glasses (Paschenko et al., 1999). In this study, we used ED EPR spectroscopy to confirm this motional model in axes of pea seeds and pollen of cattail.

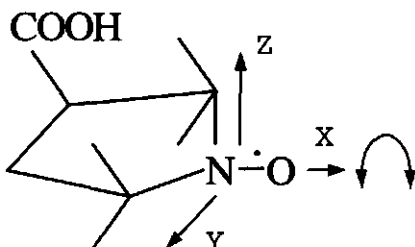


Figure 4.1. Structure of the nitroxide spin probe 3-carboxyproxyl (CP). The principal axes of the nitroxide hyperfine interaction tensor are indicated by x , y , and z . The probable librational motion is indicated.

Material and methods

Mature male inflorescences of *Typha latifolia* L. (cattail) were collected from field populations near Wageningen, The Netherlands, in 1996 and allowed to shed their pollen in the laboratory. Pollen (94% germination) was cleaned by sieving through a fine copper mesh, dried to $0.05 - 0.08 \text{ g H}_2\text{O g dw}^{-1}$, and stored at -20°C until use. Pea seeds (*Pisum sativum* cv Karina) (99% germination) were obtained from Nunhems Zaden (Haalen, The Netherlands) and stored at 5°C until use.

For spin labelling of these organisms, the polar nitroxide spin probe 3-carboxyproxyl (CP, Sigma) was used (Fig. 4.1). Because of its high polarity, CP is present in the cytoplasm of the tissues (Buitink et al., 1998a). Cattail pollen and pea axes were labelled according to Buitink et al. (1998a). Briefly, the tissues were hydrated in water until their water content reached approximately $1 \text{ g H}_2\text{O g dw}^{-1}$. The tissues were then incubated in a solution of 1 mM CP and 200 mM of the broadening agent potassium ferricyanide for 60 min. After labelling with CP and drying, samples were subsequently stored over various saturated salt solutions or phosphorus pentoxide (P_2O_5) at 25°C for at least 7 days to obtain various water contents. For EPR measurements, samples were hermetically sealed in 2-mm diameter capillaries to prevent changes in water contents. After the EPR measurements, samples were taken out of the capillary and water contents were analysed by weighing the samples before and after heating at 96°C for 36 to 48 h, and calculating the water loss on a dry weight basis.

Continuous wave EPR spectra were recorded with a Bruker X-band ESP 300E EPR spectrometer. A low microwave power ($200 \mu\text{W}$) was used to avoid saturation. Temperature was controlled using a temperature controller with liquid nitrogen vapour as the coolant. Samples were rapidly cooled to -150°C and allowed to equilibrate for 30 min. Subsequently, spectra were recorded with 10°C increments in temperature, equilibrating the sample for 5 min after each increment.

Echo-detected EPR spectra were obtained with a pulsed Bruker X-band ESP-380 FT EPR spectrometer. Electron spin echo was generated by two microwave pulses, with duration of 40 ns and 80 ns, respectively. The pulse amplitude was adjusted to provide a $\pi/2 - \pi$ pulse sequence. ED EPR spectra were taken by scanning magnetic field, while the time delay τ between the two pulses was kept constant. The

4

temperature was maintained with a thermocontrol unit ER4111VT, with an accuracy of $\pm 0.5^\circ\text{C}$. Measurements at -196°C were performed in a quartz dewar vessel filled with liquid nitrogen.

Results

Typical CW EPR spectra of CP in dry pea axes are shown in Fig. 4.2, in their usual form of the first derivative of the resonance absorption with respect to the magnetic field. The A'_{zz} values were determined from the separation between the two outer peaks. This separation decreased when the temperature was increased. The high polarity of the spin probe causes it to be present in the glass-forming aqueous cytoplasm (Buitink et al., 1998a). However, at high temperatures, a second, small component appeared in the spectrum (Fig. 4.2, top spectrum) which could be attributed to CP partitioning into the lipid phase present in the pea axes (Buitink et al., 1998a; Golovina and Tikhonov, 1994).

Figure 4.2. CW EPR spectra of CP in pea seed axes containing $0.07 \text{ g H}_2\text{O g dw}^{-1}$ at different temperatures. Two vertical lines are given to illustrate the decrease of the principal value $2A'_{zz}$ of the hyperfine interaction tensor with increasing temperature.

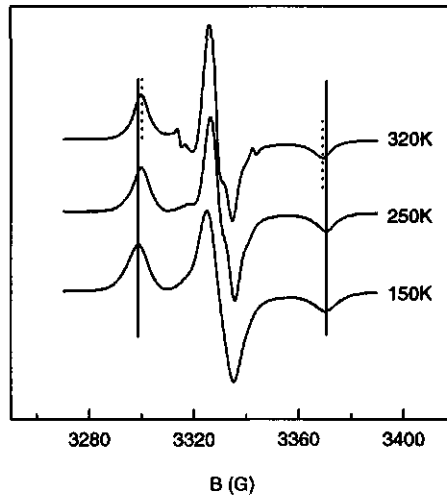


Figure 4.3 shows the temperature dependence of the A'_{zz} values of CP in pea axes with water contents of 0.07 or $0.12 \text{ g H}_2\text{O g dw}^{-1}$. At high temperatures a remarkable departure from a linear dependence was observed. When the water content of the pea axes was lower, the break at which the deviation occurred commenced at higher temperatures. Arrows indicate the glass transition temperature (T_g) as measured by differential scanning calorimetry (DSC), obtained from Buitink et al. (1999). The sharp decrease in A'_{zz} coincided with the T_g (Fig. 4.3), as was found previously (Buitink et al., 1998a). At the temperatures investigated, the possible influence of rotational molecular motion on the CW EPR spectra can be ruled out because the rotational motion it is too slow in this region (Buitink et al., 1998a; Dzuba et al., 1984). Therefore, the abrupt change in A'_{zz} around T_g is likely to be associated with librational motion. To further investigate this phenomenon, we performed ED EPR spectroscopy on similar biological samples.

ED EPR spectra are obtained by detecting the electron spin echo signal as a function of the external magnetic field. The time separation τ between two echo-forming pulses was kept constant during the measurement but was varied from one measurement to another. In this way, the relaxation rate for different

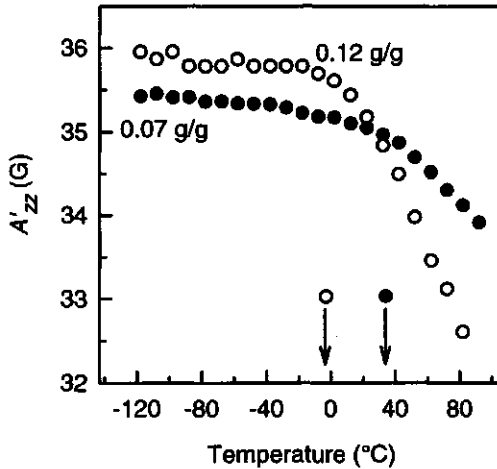


Figure 4.3. Temperature dependence of the A'_{zz} values of CP in pea seed axes having water contents (g/g) of 0.07 g H_2O g dw^{-1} (closed symbols) or 0.12 g H_2O g dw^{-1} (open symbols). Arrows indicate the onset T_g measured by DSC, obtained from Buitink et al. (1999).

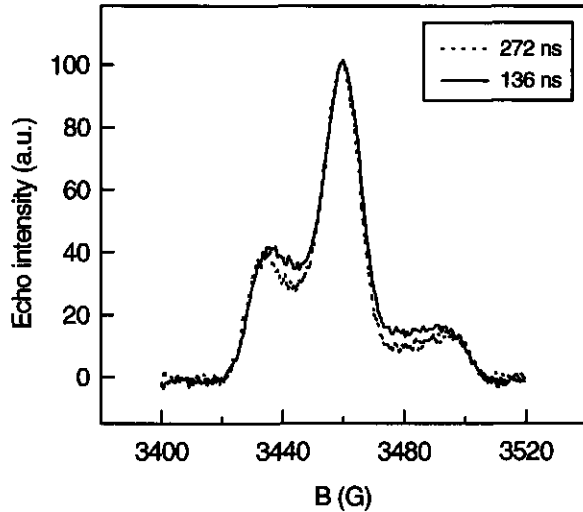
field positions was studied. Figure 4.4 depicts the echo-detected spectra of CP in dry cattail pollen at different times τ (136 and 272 ns) between the echo-forming pulses. The spectra were normalised to their maximum amplitude in order to exclude all field independent relaxation mechanisms. Notable changes were observed in the shapes of the low-field and high-field components. These changes are determined by the different rates of magnetic phase relaxation of the nitroxide for the different orientations with respect to the external magnetic field (Dzuba et al., 1992). The reason of such a behaviour has been suggested to be the result of molecular librations (Dzuba et al., 1992; Dzuba, 1996). Qualitatively this may be understood as follows. The orientational dependence of phase relaxation arises as a result of different variation of the resonant magnetic field, B_{res} , when the nitroxide librates. Molecular motion moves a spin off resonance providing a phase relaxation pathway. The larger the effect of motion on B_{res} , the more effective the relaxation pathway. This variation, $dB_{res}/d\theta$, where θ is the angle between the molecular z axis and the external magnetic field, is larger for the low-field and high-field components. Also, it tends to zero for canonical orientations of the nitroxide (Dzuba et al., 1992; Dzuba, 1996). So for these orientations the relaxation must be slower than for others. This is indeed observed for the outer edges which correspond to the parallel orientation (Fig. 4.4).

Changing time τ may result in an additional field-dependent relaxation mechanism, so-called "instantaneous spectral diffusion", which also influences the ED EPR line shape (Dzuba et al., 1993a). Another way of performing ED EPR experiments, which is free of this influence, is keeping the time τ constant

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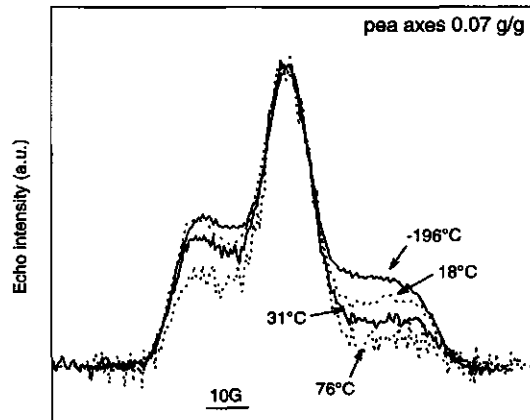
for all measurements but varying the temperature, as shown in Fig. 4.5. The ED-EPR spectra were strongly influenced by temperature. All spectra were obtained for the same time separation between microwave pulses ($\tau = 136$ ns) and were normalised to their maximum amplitude. Analogous data sets were also obtained for CP in pea axes containing $0.12 \text{ g H}_2\text{O g dw}^{-1}$ and for CP in cattail pollen (data not shown).

Figure 4.4. ED-EPR spectra of CP in cattail pollen at different times τ between the echo-forming pulses, recorded at room temperature. Water content of the pollen was $0.04 \text{ g H}_2\text{O g dw}^{-1}$. $\tau = 136$ ns, solid line; $\tau = 272$ ns, dashed line. Spectra were normalised to the same maximum amplitude.



In order to quantify the temperature dependence of the shape of the spectra, the ratio of the line height in the low field region was determined at -196°C ($h_{-196^\circ\text{C}}$) and at higher temperatures (h) (see inset Fig. 4.6). Note that ED EPR line shape depends on the parameter $\langle \alpha^2 \rangle \tau_c$ where τ_c is the correlation time of motion (Dzuba et al., 1992; Dzuba, 1996). This parameter increases when the low-field component and high-field component decrease. The line height of the low field of ED EPR spectra at -196° was taken as 1, assuming

Figure 4.5. ED EPR spectra of CP in pea seed axes with $0.07 \text{ g H}_2\text{O g dw}^{-1}$ (g/g), recorded at different temperatures. All spectra were obtained for the same time separation between microwave pulses ($\tau = 136$ ns) and were normalised to the same maximum amplitude.



that at this temperature motion was frozen out. This was confirmed by recording a CW EPR spectrum at the same temperature and digitally integrating the spectrum. Comparison of the CW EPR spectrum with the ED EPR spectrum showed that they coincided, implying that motion was frozen out at -196°C (data not shown). The ratio of $h / h_{-196^{\circ}\text{C}}$ of CP in pea axes decreased with increasing temperature, indicating an increase in librational motion during warming (Fig. 4.6). Arrows indicate the T_g as measured by DSC. The sharp decrease in the ratio occurred at a similar temperature as that found for the A'_{zz} (Fig. 4.3), implying that both parameters were related to the same type of molecular motion.

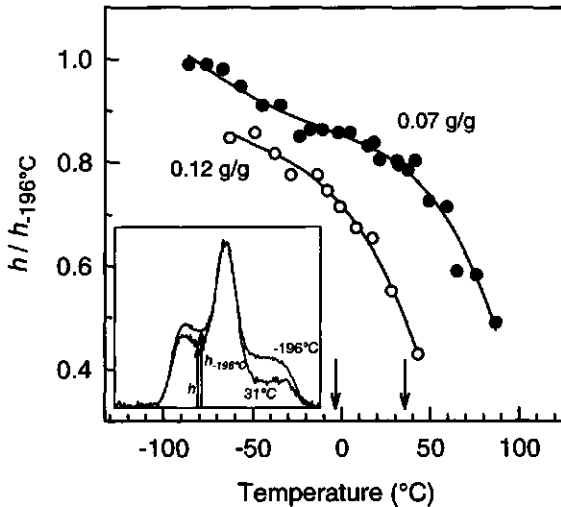


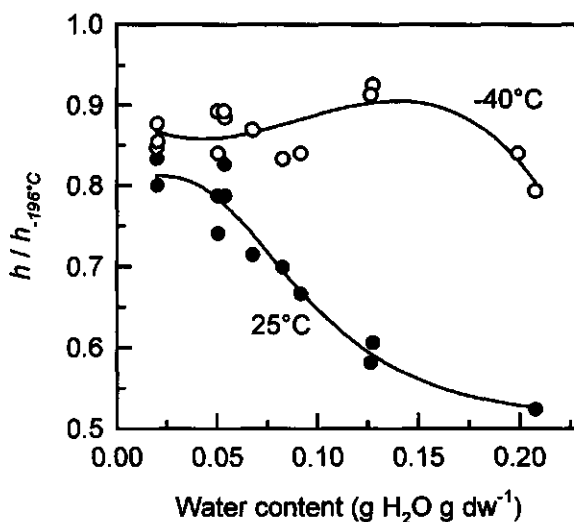
Figure 4.6. Changes in the line height ratio ($h / h_{-196^{\circ}\text{C}}$) in dependence of temperature for CP in pea seed axes at two water contents. Water contents (g/g) were $0.07 \text{ g H}_2\text{O g dw}^{-1}$ (closed symbols) or $0.12 \text{ g H}_2\text{O g dw}^{-1}$ (open symbols). Inset shows the calculation of changes in librational motion as a function of temperature, using the difference between the height of the low-field region at -196°C ($h_{-196^{\circ}\text{C}}$) and the height of the low-field region of spectra recorded at temperatures above -196°C (h).

The A'_{zz} is also known to change in dependence of the water content of the sample, when measured at a constant temperature (Buitink et al., 1998a). To ascertain whether these changes in A'_{zz} were also due to changes in libration motion, ED EPR spectra were recorded for CP in pollen at different water contents and three constant temperatures (25 , -40 and -196°C) (Fig. 4.7). At 25°C , the ratio $h / h_{-196^{\circ}\text{C}}$, calculated as shown in the inset in Fig. 4.6, increased with decreasing water contents from 0.53 at $0.22 \text{ g H}_2\text{O g dw}^{-1}$ to 0.83 at $0.05 \text{ g H}_2\text{O g dw}^{-1}$. At water contents lower than $0.05 \text{ g H}_2\text{O g dw}^{-1}$, the ratio remained constant. At -40°C , the data were more scattered (Fig. 4.7). From 0.22 to approximately $0.15 \text{ g H}_2\text{O g dw}^{-1}$, the ratio $h / h_{-196^{\circ}\text{C}}$ appeared to increase slightly. Below approximately $0.15 \text{ g H}_2\text{O g dw}^{-1}$, the ratio decreased or remained constant. In general, the water content at which a minimum in

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librational motion (max. $h / h_{-196^\circ\text{C}}$) was found shifted to higher values with decreasing temperature.

Figure 4.7. Changes in the line height ratio ($h / h_{-196^\circ\text{C}}$) in dependence of water content for CP in cattail pollen. Line height ratios were determined at -40°C (open symbols) and 25°C (closed symbols).



Discussion

In this study, we report a change in the principal values of the hyperfine interaction of CP in the axes of pea seeds in relation to temperature as was found previously for cattail pollen (Buitink et al., 1998a) and impatiens seeds (chapter 10). The characteristic time of rotational molecular motion for spin probes near or above T_g is in the order of 10^{-6} to 10^{-4} s (Buitink et al., 1999). These motions are too slow to account for changes observed in the principal values of the hyperfine interaction that are determined from CW EPR spectra. Modelling of ED EPR spectra of TEMPONE in glucose and trehalose glasses has shown that the nitroxide molecules undergo orientational oscillations about some axis fixed in the molecular frame (librational motion) (Dzuba, 1996). The analogous shape of the echo-detected EPR spectra obtained in this study revealed that it was indeed librational motion that was detected in biological tissues. This type of spectral transformation was found to be common for disordered systems and supports the concept that the local microscopic structure in glassy biological cells is comparable to other glass forming substances (Dzuba et al., 1993a; Leopold et al., 1994; Sun, 1997b; Buitink et al., 1998a).

The temperature dependence of ED EPR spectra of CP in pea axes revealed that librational motion increased with increasing temperature. An abrupt change in A'_{zz} was observed above a temperature corresponding to the melting of the glassy matrix (Fig. 4.3). The association with the glassy state was further corroborated by the increase in temperature at which the break was observed when the water content of the pea axes was decreased from 0.12 to 0.07 g H₂O g dw⁻¹. The abrupt change in the $h / h_{-196^\circ\text{C}}$ ratio was comparable to that observed for the change in A'_{zz} (compare Figs. 4.6 and

4.3). Because the parameter $\langle\alpha^2\rangle\tau_c$ increases when the ratio of the low-field component decreases, melting of the glass results in a sharp increase in $\langle\alpha^2\rangle\tau_c$. Similar observations were made on spectra of nitroxide spin probes present in trehalose and glucose glasses (Dzuba, 1996).

The A'_{zz} is also known to change in dependence of the water content of the sample when measured at a constant temperature (Buitink et al., 1998a). In order to determine the change in A'_{zz} as a function of water content instead of temperature, a correction was made for the polarity change of the environment in which CP is present in cattail pollen for each water content (Buitink et al., 1998a). The resulting mobility parameter, expressed as the difference between the A_{zz} (at -150°C , where the spin probe is assumed to be immobilised) and the A'_{zz} measured at the desired temperature (ΔA_{zz}), was shown to exhibit a minimum when plotted as a function of water content. The water content corresponding to this minimum mobility shifted to higher values with decreasing temperatures. In this study, similar results were found by ED EPR measurements (Fig. 4.7). Although it is not clear whether there is a plateau in librational motion at low water contents rather than a minimum, both ED EPR and CW EPR measurements confirm that below a certain water content, librational motion does not decrease anymore.

The implications of these findings are important for seed storage preservation. Ageing rates are thought to be influenced by the molecular motion in the cytoplasm (Leopold et al., 1994; Sun, 1997b, Buitink et al., 1995a, 1999). A decrease in librational or rotational motion in the cytoplasm is likely to result in decreased ageing rates that take place in this cytoplasm. Determination of molecular motions under various conditions of temperature and water content might reveal the storage conditions under which molecular mobility is minimised. ED EPR measurements show that there is a sharp increase in librational motion when the cytoplasm is heated above its T_g . Therefore, storage of seeds is recommended below T_g . In addition, we found that at a low temperature (-40°C), the water content at which the librational motion is minimised is as high as 0.1 to 0.15 g H₂O g dw⁻¹. A further reduction in the water content did not decrease the librational motion any further. This finding implies that when tissues are stored at these ultra-low temperatures, they do not have to be dried to very low water contents in order to maintain their viability (Vertucci and Roos, 1993b; Vertucci et al., 1994b).

In this study, ED EPR measurements revealed the occurrence of librational motions of CP in biological tissues. As this kind of motion is thought to be a general property of molecular glasses (Dzuba et al., 1993a; Dzuba, 1996; Paschenko et al., 1999) the data obtained provide additional evidence for the existence of intracellular glasses in seed and pollen. The librational motion changed along with changes in A'_{zz} , indicating that A'_{zz} measurements reflect librational motion. The change in librational motion in dependence of temperature and water content of the biological samples can be used to obtain information on the rate of detrimental ageing reactions that take place in these organisms during storage.

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Characterization of molecular mobility in seed tissues: an EPR spin probe study

Julia Buitink, Marcus A. Hemminga, Folkert A. Hoekstra

The relationship between molecular mobility (τ_R) of the polar spin probe 3-carboxy-proxyl and water content and temperature was established in pea axes by electron paramagnetic resonance (EPR) and saturation transfer EPR. At room temperature, τ_R increased during drying from 10^{-11} s at $2.0 \text{ g H}_2\text{O g dw}^{-1}$ to 10^{-4} s in the dry state. At water contents below $0.07 \text{ g H}_2\text{O g dw}^{-1}$, τ_R remained constant upon further drying. At the glass transition temperature, τ_R was constant at $\sim 10^{-4}$ s for all water contents studied. Above T_g , isomobility lines were found that were approximately parallel to the T_g curve. The temperature dependence of τ_R at all water contents studied followed Arrhenius behaviour with a break at T_g . Above T_g the activation energy for rotational motion was $\sim 25 \text{ kJ mol}^{-1}$ compared to 10 kJ mol^{-1} below T_g . The temperature dependence of τ_R could also be described by the WLF equation, using constants deviating considerably from the universal constants. The temperature effect on τ_R above T_g was much smaller in pea axes as found previously for sugar and polymer glasses. Thus, although glasses are present in seeds, the melting of the glass by raising the temperature will cause only a moderate increase in molecular mobility in the cytoplasm as compared to a huge increase in amorphous sugars.

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The longevity of seeds is determined by the conditions under which they are stored, major factors being temperature and water content. Predictions of the longevity of seeds or the optimum storage conditions that have to be chosen to obtain maximum longevity are valuable assets in maintaining a seed collection. In desiccation-tolerant organisms, such as seeds and pollen, it has been found that the cytoplasm enters into a glassy state when those organisms are stored at low water contents and/or low temperatures (Williams and Leopold, 1989; Leopold et al., 1994; Sun and Leopold, 1994; Leprince and Walters-Vertucci, 1995; Sun, 1997b; Buitink et al., 1998b). A glass is a solid-like liquid with an extremely high viscosity (Franks, 1994a). The formation of glasses in seeds is thought to be responsible for the prolonged survival of these tissues in the dry state. It was found that the lifespan of biological materials such as seeds and pollen was increased profoundly during storage under conditions in which the cytoplasm was brought into a glassy state (Sun and Leopold, 1994; Sun, 1997; Buitink et al. 1998b).

In many glass-forming substances, melting of the glass results in a dramatic increase in translational and rotational motion (Soesanto and Williams, 1981; Roozen et al., 1991; Steffen et al., 1992; Blackburn et al., 1996; Deppe et al., 1996; Champion et al., 1997; Hemminga and Van den Dries, 1998; Van den Dries et al., 1998a). It has been known for a long time that stabilisation of many 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 (see Roos, 1995, for a review). In the pharmaceutical industry, it is currently recognised that the presence of an amorphous phase has very important implications for storage of pharmaceutical dosage forms (Hancock and Zografi, 1997). Recently, Hancock et al. (1995) suggested the use of molecular mobility measurements below T_g in the prediction of shelf lives of amorphous drugs, assuming a direct correlation between the molecular mobility and the degradation of the product.

The physical properties of water have been studied in complex biological systems that are able to survive the removal of their water, such as seeds (Vertucci, 1990; Bruni and Leopold, 1992; Konsta et al., 1996) and *Artemia* cysts (Seitz et al., 1981). The restricted mobility of water at low hydration levels has been attributed to the formation of intracellular glasses (Williams and Leopold, 1989). However, little is known about the viscosity or molecular mobility of molecules other than water in these systems. Detrimental processes associated with ageing that take place in the cytoplasm of seeds are likely to be restricted by slow molecular motion of molecules in the cytoplasm. Therefore, characterization of molecular mobility in seeds as a function of temperature and water content might aid in understanding the kinetics of seed ageing during storage. Soluble sugars present in the cytoplasm of seeds are thought to be the major component responsible for the formation of intracellular glasses. Comparison of the behaviour of intracellular glasses with sugar glasses will shed light on the nature of intracellular glasses.

An elegant technique for the study of rotational motion is electron paramagnetic resonance (EPR) spectroscopy, which measures the rotational

correlation time (τ_r) of spin probes dissolved in samples. Continuous wave (CW) EPR can detect changes in the τ_r of spin probes ranging from 10^{-12} to 10^{-8} s, and has been applied previously to biological systems (Bruni and Leopold, 1990; Buitink et al., 1998a; Leprince and Hoekstra, 1998). Saturation transfer EPR (ST-EPR) can detect τ_r on the order of 10^{-7} to 10^2 s and has been successfully applied to determine τ_r of spin probes in sugar glasses (Roozen and Hemminga, 1990; Roozen et al., 1991, Hemminga and Van den Dries, 1998; Van den Dries et al., 1998a) and organic liquids at low temperatures (Ito, 1983). It has been shown previously that the τ_r of spin probes provides a unique and simple parameter for the characterization of the state of the cytoplasm of seeds (Buitink et al., 1998a).

For many systems, the temperature dependence of reaction rates or mobility below T_g can be described by the Arrhenius equation (Levine and Slade, 1988; Karmas et al., 1992; Nelson and Labuza, 1994). Above T_g , it has been shown that the temperature dependence of viscosity, translational or rotational relaxation times of model glasses such as sugar-systems and polymers cannot be described by an Arrhenius-like relationship. For such systems it turns out that the effect of increasing temperature on relative relaxation times above T_g can be successfully predicted by the Williams-Landel-Ferry (WLF) equation, an empirical equation, the form of which was originally derived from the free volume interpretation of the glass transition (Williams et al., 1955; Ferry, 1980; Soesanto and Williams, 1981; Chan et al., 1986; Roos and Karel, 1991; Steffen et al., 1992; Champion et al., 1997). Fitting of the rotational mobility in pea axes as a function of temperature according to the Arrhenius and WLF equations will enable us to compare mobility in intracellular glasses with that of model systems. In addition, modelling the temperature effect of mobility could aid predictions of shelf life.

This study was performed to obtain insight into how molecular mobility of molecules in the cytoplasm of seed changes, depending on water content and temperature. Different EPR techniques were used to determine the rotational mobility of spin probes in the fast and very slow motional regions. The temperature dependence of τ_r was assessed in relation to Arrhenius and WLF behaviour, and reference was made to other glass-forming substances.

Materials and methods

Plant material and sample preparation

Highly viable pea (*Pisum sativum* L. cv karina) seeds were obtained from Nunhems zaden (Haelen, The Netherlands). They were allowed to imbibe for 16 h at 15°C, after which the axes were excised and incubated in 10 ml of a solution of 1 mM 3-carboxy-proxyl (CP, Sigma). After 45 min, potassiumferricyanide was added to a final concentration of 200 mM, and the axes were incubated for another 15 min. The ferricyanide 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 dried in dry air (3% RH) for 24

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hours. After drying, axes were stored over several saturated salt solutions (Winston and Bates, 1960) for 7 days to obtain various water contents.

Two to three axes of the same treatment were sealed in a 2-mm diameter capillary for EPR measurements. After the measurements, the axes were removed from the capillaries and water contents were determined. For samples that were heated above 50°C during the EPR measurements, similar samples equilibrated to the same water content were taken for water content determination. Water contents were analysed by weighing the samples before and after heating at 96°C for 36 to 48 h.

Molecular motion in the fast motional region (CW EPR)

EPR spectra were recorded using a Bruker X-band EPR spectrometer (Bruker Analytik, Rheinstetten, Germany, model 300E). Rotational correlation times in the fast motional region were determined from the line shapes of CW EPR spectra according to the method of Knowles et al. (1976) for isotropic tumbling:

$$\tau_R = 6.5 \times 10^{-10} \Delta B_0 \{(h_c/h_h)^{1/2} - 1\} \quad (5.1)$$

where B_0 is the width of the center field component in Gauss, and h_c and h_h are the amplitudes of the central and high field components of the three-line nitroxide radical spectrum, respectively.

Molecular motion in the slow motional region (ST-EPR)

At low water contents and temperatures, τ_R of CP in seed axes becomes slower than 10^{-8} s, resulting in the appearance of a powder spectrum (Buitink et al., 1998a). Under these conditions, τ_R cannot be calculated according to equation 5.1. However, ST-EPR further expands the motional region from 10^{-7} s to 10^2 s (Hyde and Dalton, 1979; Van den Dries et al., 1998). 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 characterized by independent line shape parameters, such as the line-height ratios L^*/L and C'/C (see Fig. 5.1 for details). 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)

$$\tau_R = (\eta V/k_b T) k + \tau_0 \quad (5.2)$$

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. 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 at 3°C increments. For each temperature the values of the line shape parameters L'/L and C'/C were 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 seed axes were obtained by interpolation of the corresponding line shape parameters.

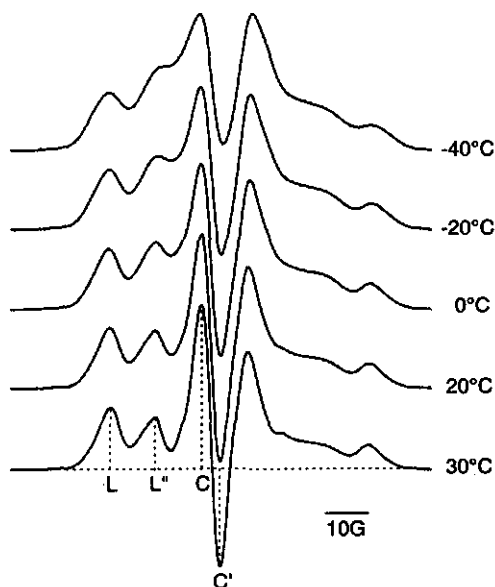


Figure 5.1. ST-EPR spectra of CP in pea axes with a water content of $0.09 \text{ g H}_2\text{O g dw}^{-1}$, recorded at different temperatures.

DSC

The glass transition temperature is conventionally the temperature at which a change in the heat capacity can be detected by DSC. Two to three pea axes of different water contents were hermetically sealed into aluminum DSC pans. Second order transitions of the samples were determined using a Perkin-Elmer (Norwalk, CT) Pyris-1 DSC, calibrated for temperature with indium (156.6°C) and methylene chloride (-95°C) standards and for energy with indium (28.54 J g^{-1}). Baselines were determined using an empty pan, and all thermograms were baseline-corrected. Scans were taken from -100°C to 120°C at a rate of $10^{\circ}\text{C min}^{-1}$. The T_g values were determined as the onset of the temperature range over which the change in specific heat occurred. All analyses were performed with Perkin-Elmer software.

Results and Discussion

Rotational motion of CP in pea axes in relation to temperature and water content

ST-EPR spectra of CP in pea axes ($0.09 \text{ g H}_2\text{O g dw}^{-1}$) at different temperatures are displayed in Fig. 5.1. It has been shown previously that to exclusively obtain spectra of the spin probe in the cytoplasm, a nitroxide spin probe of high

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polarity has to be selected. More apolar spin probes have the tendency to partition into the lipid phase with drying (Buitink et al., 1998a; Golovina et al., 1998). With the use of a broadening agent such as potassium ferricyanide, the CP signal was completely removed from the intercellular spaces and is therefore exclusively of intracellular origin. Although CP is expected to be present in a heterogeneous environment consisting of a mixture of ions, sugars and proteins, its signal in dry seeds appears to be a single component spectrum (Fig. 5.1). Only at high temperatures may a small second component appear in the spectrum that can be attributed to CP partitioning into the lipid phase. However, the resulting small distortion in some parts of the spectrum did not influence the calculations of the line shape parameters from the ST-EPR spectra. Spectra in which a clear distortion of the line shapes was observed were omitted from the analysis.

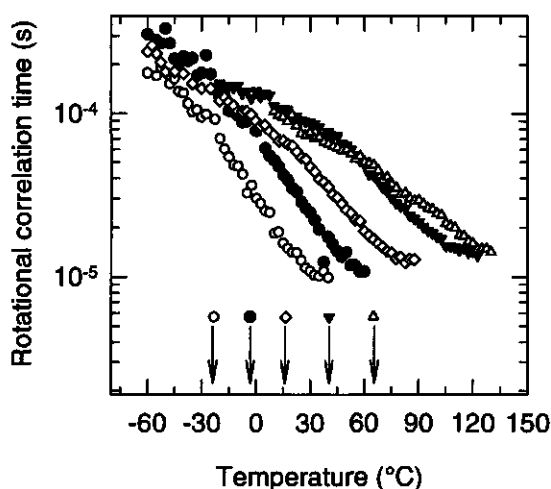


Figure 5.2. τ_r of CP in pea axes as a function of temperature. τ_r was calculated from the line-height ratio L'/L . The different curves represent pea axes with different water contents ($\text{g H}_2\text{O g dw}^{-1}$): 0.048 (open triangle), 0.07 (closed triangle), 0.085 (open diamond), 0.12 (closed circle), 0.16 (open circle). Arrows indicate the onset T_g as measured by DSC at a scanning rate of $10^\circ\text{C min}^{-1}$.

The τ_r of CP in pea axes was derived from spectra as shown in Fig. 5.1. With increasing temperature, the line height ratios (L'/L and C'/C) decreased, indicating an increase in rotational motion. The saturation transfer in the central part of the spectrum is more extensive than that in the low field region, especially at higher temperatures. If the motion were isotropic, then the two peak height ratios would give the same correlation time, because overall rotation would modulate all the spectral anisotropies, giving rise to saturation transfer throughout the entire spectrum (Marsh, 1980). However, there appears to be some motional anisotropy around the z -axis, which modulates the anisotropy of the g -tensor in the x - y plane, giving rise to saturation transfer in

the central part of the spectrum. Because this effect complicates the interpretation of the results from the C/C measurements, only the τ_R derived from the line height ratio L'/L will be utilised in the analysis of the ST-EPR spectra.

The temperature dependence of τ_R of CP is shown in Fig. 5.2 for pea axes. The different curves represent pea axes with decreasing water contents from left to right. The arrows denote the onset of T_g as measured by DSC. Below T_g , τ_R followed a linear behaviour with temperature. Around T_g , a change occurred in the relationship between τ_R and temperature. Above T_g , the τ_R increased more sharply with temperature from 10^{-4} s to $\sim 10^{-5}$ s over the next 50°C temperature increase. At temperatures $\sim 50^\circ\text{C}$ above T_g , the increase in τ_R with temperature levelled off.

The increase in mobility of CP in pea axes above T_g is not as dramatic as found for glycerol or other glass-forming sugars (Williams et al., 1955; Soesanto and Williams, 1981; Chan et al., 1986; Roozen and Hemminga, 1990; Roozen et al., 1991). For 20% wt sucrose-water mixtures, τ_R increased by about four orders of magnitude in the first 20°C above T_g (Roozen and Hemminga, 1990). In a maltoheptaose glass, dry or stored at 33% relative humidity, the τ_R increased by three orders of magnitude over the same temperature interval (Roozen et al., 1991). In comparison, the increase in τ_R for CP in pea axes from T_g to 20°C above T_g was only a factor of six (Fig. 5.2). Thus, although glasses are present in seeds, the effect of melting the glass by raising the temperature will not dramatically increase the mobility of molecules in the cytoplasm. Considering the survival of seeds in their natural habitat, the relatively small change in mobility above T_g might render them fairly insensitive to fluctuations in the environmental conditions that drive their cytoplasm out of the glassy state.

During drying of pea axes at 25°C , τ_R of CP in the cytoplasm increased from 7×10^{-11} s at $2.2 \text{ g H}_2\text{O g dw}^{-1}$ to 10^{-4} s at $0.07 \text{ g H}_2\text{O g dw}^{-1}$ (Fig. 5.3).

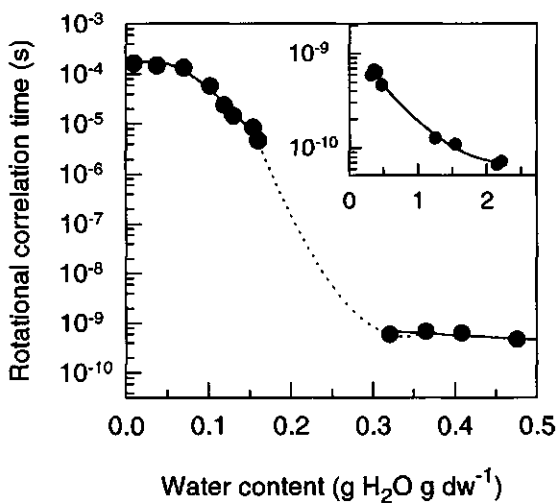


Figure 5.3. τ_R of CP in pea axes as a function of water content at 25°C . Values of $\tau_R > 10^{-6}$ s were calculated from the L'/L ratio of ST-EPR spectra. For $\tau_R < 10^{-9}$ s equation 5.1 was used with the EPR spectra. The inset shows the decrease in τ_R during drying from high water contents.

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Upon further drying τ_R remained constant. The inset shows the decrease in rotational correlation time during drying determined according to equation 5.1, where the water contents were sufficiently high to obtain a sharp three-line spectrum. The rotational mobility decreased when the tissues were dried below $2.2 \text{ g H}_2\text{O g dw}^{-1}$. The dotted line indicates the range of magnitude in which τ_R cannot be measured with either methods. Nonetheless, it can be seen that between 0.3 and $0.2 \text{ g H}_2\text{O g dw}^{-1}$, τ_R strongly increases. This sharp increase in τ_R coincides with the water content range in pea axes at which the water remains unfrozen as measured by DSC (Vertucci, 1990). The change in τ_R upon drying is on the order of more than seven orders of magnitude. The slow molecular mobility at low water contents is thought to have a protective effect on the structural and functional stability of enzymes and other molecules in the cytoplasm (Burke, 1986; Leopold et al., 1994; Leprince and Walters-Vertucci, 1995). Indeed, the considerable decrease in molecular mobility that we found with drying argues in favour of this hypothesis. The preservation of the cellular components in the dry state is likely to prolong the survival of the seeds in the dry state (Leopold et al., 1994; Sun and Leopold, 1994; Sun, 1997b, Buitink et al., 1998a,b).

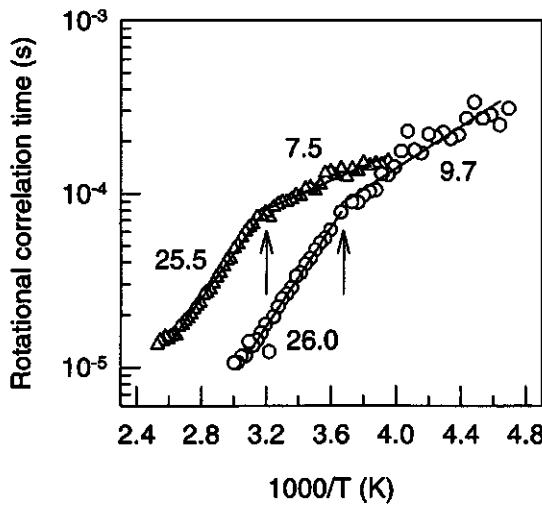


Figure 5.4. Arrhenius plots of the temperature dependence of τ_R of CP in pea axes. The τ_R was calculated from the line-height ratio L''/L . The different curves represent pea axes with different water contents ($\text{g H}_2\text{O g dw}^{-1}$); 0.07 (open triangles), 0.12 (open circles). Arrows indicate the onset T_g as measured by DSC at a scanning rate of $10^\circ\text{C min}^{-1}$. Lines represent linear regressions. Values represent the activation energy expressed as kJ mol^{-1} .

Modelling of molecular mobility in pea embryonic axes

If the rate of reactions were controlled by the mobility of molecules in the cells, characterizing the temperature dependence of molecular mobility would aid in

predicting shelf life. In addition, it allows us to compare the behaviour of rotational motion in intracellular glasses with that of sugar and polymer glasses. The temperature dependence of reaction rates is often described by the Arrhenius equation

$$k = k_0 \exp(-E_a/RT) \quad (5.3)$$

where k is the rate constant at temperature T , k_0 is a pre-exponential factor, R is the ideal gas constant and E_a is the activation energy. Fig. 5.4 shows Arrhenius plots of τ_r of CP in pea axes at two water contents. The arrows indicate the onset of T_g as measured by DSC. The temperature dependence of τ_r of CP in pea axes followed Arrhenius behaviour below T_g , with a break in the plot at T_g . Above T_g , Arrhenius behaviour with a higher activation energy was found compared to below T_g . At temperatures ~ 40 to 60°C above T_g , the data deviated from Arrhenius behaviour, as became apparent from the deviation of the data points from a straight line. Activation energies from the slopes of the Arrhenius plots below T_g and for the first 40 to 60°C above T_g for pea axes containing different water contents are summarised in Fig. 5.5. For τ_r of CP in

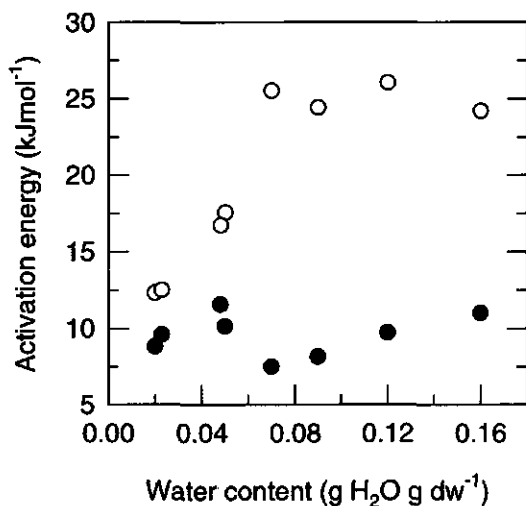


Figure 5.5. Activation energies (kJ mol^{-1}) for the temperature dependence of τ_r below (closed circles) and above T_g (open circles) for pea axes of different water contents. The datapoints were derived from the Arrhenius plots as shown in Fig. 5.4.

pea axes above $0.07 \text{ g H}_2\text{O g dw}^{-1}$, activation energies below T_g were lower (7 – 11 kJ mol^{-1}) than above T_g (25 kJ mol^{-1}), as generally expected (Levine and Slade, 1988; Karmas et al., 1992; Nelson and Labuza, 1994). This indicates that the rotational motion below T_g changes at a slower rate than above T_g . Below $0.07 \text{ g H}_2\text{O g dw}^{-1}$, activation energies above T_g decreased substantially (Fig. 5.5). The activation energy values of rotational motion of CP in pea axes below T_g are in the same range as those found for glassy sugar systems (Roosen et al., 1991).

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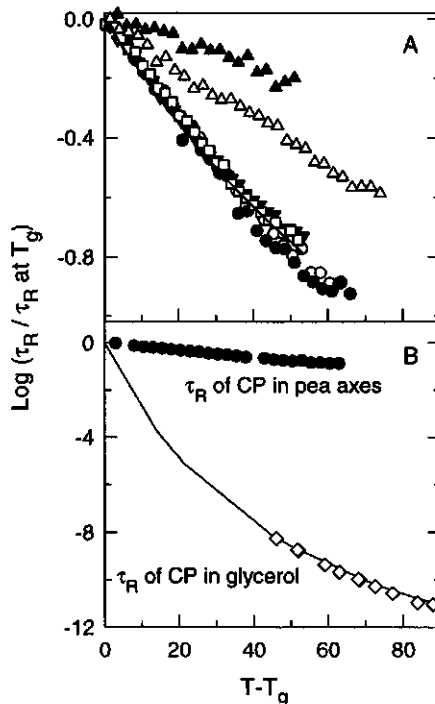
For model glasses, such as sugar-water systems and polymers, it has been shown that the temperature dependence of viscosity or mobility above T_g can not be described by an Arrhenius-like relationship (Williams et al., 1955; Ferry, 1980; Soesanto and Williams, 1981; Chan et al., 1986; Roos and Karel., 1991; Steffen et al., 1992; Champion et al., 1997). Instead, it can be described by the WLF equation (Williams et al., 1955; Ferry, 1980):

$$\log a_T = -C_1 (T - T_{ref}) / \{ C_2 + (T - T_{ref}) \} \quad (5.4)$$

where C_1 and C_2 are system-dependent coefficients (Ferry, 1980), and a_T is defined as the ratio of the relaxation phenomenon at T to the relaxation at the reference temperature T_{ref} . Average values for the WLF coefficients ($C_1=17.44$ and $C_2=51.6$) were calculated by Williams et al. (1955) using the available values for many synthetic polymers. The universal constants have been shown to also apply to molten glucose (Williams et al., 1955), amorphous glucose-water systems (Chan et al., 1986), amorphous sucrose and lactose powders at low moisture (Roos and Karel., 1991), and concentrated solutions of mixed sugars (Soesanto and Williams, 1981). However, several problems are associated with the use of the average coefficients in the WLF equation (Peleg, 1992), and other values have been used to obtain a better fit (Ferry, 1980; Slade and Levine, 1991a; Champion et al., 1997).

The τ_R values for CP in pea at different water contents were fitted to the WLF equation (Fig. 5.6A). The curves of τ_R from CP in pea axes with water

Figure 5.6. (A) WLF-plot of the temperature dependence of τ_R of CP in pea axes. The different curves represent pea axes with different water contents ($\text{g H}_2\text{O g dw}^{-1}$); 0.01 (solid triangle), 0.048 (open triangle), 0.07 (closed inverse triangle), 0.085 (open diamond), 0.12 (closed circle), 0.16 (open circle). The solid line is a WLF fit with $C_1 = 3.4$ and $C_2 = 150$. (B) WLF-plot of the temperature dependence of τ_R of CP in anhydrous glycerol and in pea axes of $0.12 \text{ g H}_2\text{O g dw}^{-1}$. The solid line is a WLF fit with the universal constants ($C_1=17.44$ and $C_2=51.6$).



contents higher than $0.07 \text{ g H}_2\text{O g dw}^{-1}$ followed approximately the same relationship (Fig. 5.6A). To obtain a reasonable fit, the WLF constants had to be changed considerably by decreasing C_1 and increasing C_2 compared to the universal constants. This becomes apparent from Fig. 5.6B, in which the τ_R of CP in pea axes is compared to the τ_R of CP in glycerol, fitted with the universal constants. These data were also obtained using ST-EPR spectroscopy (Van den Dries et al., 1998a, Buitink et al., 1998a). The curve fitted to the data in Fig. 5.6A was calculated using $C_1=3.4$ and $C_2=150$. In samples with water contents below $0.07 \text{ g H}_2\text{O g dw}^{-1}$, there was a further decrease in C_1 or increase of C_2 needed to obtain a good fit.

Although WLF behaviour is expected for glass-forming substances above T_g , we found that the Arrhenius equation can well describe the temperature dependence above T_g for the first 50°C (Fig. 5.4). Deterioration kinetics in complex food systems (Karmas et al., 1992; Nelson and Labuza, 1994) and ageing kinetics in pollen (Buitink et al., 1998b) were also found to follow Arrhenius behaviour both above and below the T_g . Apparently, kinetics in complex systems such as food materials and seeds can be described by the Arrhenius equation for the first 50°C above T_g . The activation energy of relaxation kinetics increases by a factor of ~ 3 when the pea axes are brought to conditions in which the intracellular glass melts. Interestingly, a comparable change in activation energy for ageing kinetics around T_g has been shown for cattail pollen (Buitink et al., 1998b).

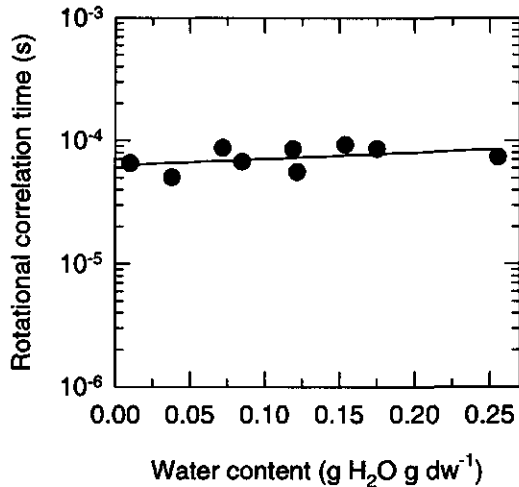
Mobility at any temperature depends primarily on the free volume present (Ferry, 1980). Some information regarding the free volume of the system can be derived from the constants of the WLF equation. C_1 is proportional to the inverse of the free volume of the system at T_g , and C_2 is proportional to the ratio of free volume at T_g over the increase in free volume due to thermal expansion above T_g (i.e. the ratio of free volume at T_g to the difference between the volumes of the rubbery liquid and glassy solid states, as a function of temperature above T_g) (Williams et al., 1955). A lower C_1 would indicate that the free volume associated with the intracellular glass is larger than that found for glycerol. Free volume is related to the packing irregularities caused by the side chains of the glass-forming molecules. This finding reinforces the notion that intracellular glasses are composed of many different molecules, such as ions, amino acids, sugars and proteins, that are responsible for the increase in free volume because of imperfect packing. The implication of a higher C_2 constant is that the difference in thermal expansion coefficient between the glassy and liquid states would be smaller than that for other glass-forming substances.

When spin probe techniques are used, the rotational mobility of probes in glassy systems is not only determined by the free volume of the host system, but also by the specific interaction of the spin probe with the chain molecules. Hydrogen bonds are often the most important interaction (Roozen and Hemminga, 1990). The deviating behaviour of rotational mobility of the spin probe in intracellular glasses compared to sugar glasses can therefore also be mediated by the spin probe's interaction with the surroundings. To learn which

5

of the two factors is determining the rotational mobility, the τ_r values at the T_g (measured by DSC) of CP in pea axes with different water contents were plotted (Fig. 5.7). With decreasing water content, the onset T_g from pea axes increases from -50°C at $0.26 \text{ g H}_2\text{O g dw}^{-1}$ to 90°C when they are completely dry, as measured by DSC. The τ_r is constant at T_g , on the order of 10^{-4} s (Fig. 5.7). These results would imply that it is indeed the free volume that is the major

Figure 5.7. τ_r of CP in pea axes of different water contents at the T_g . T_g was determined as the onset of the second-order transition measured by DSC at a scanning rate of $10^\circ\text{C min}^{-1}$. T_g for the different water contents ranges from -50°C at $0.26 \text{ g H}_2\text{O g dw}^{-1}$ to 90°C at $0.01 \text{ g H}_2\text{O g dw}^{-1}$.



factor in influencing the rotational mobility. If hydrogen bonding would affect the rotational mobility, it would be unlikely that the τ_r at the T_g would remain constant, considering the wide range of water contents (from 0.01 to $0.20 \text{ g H}_2\text{O g dw}^{-1}$) and temperatures (-50°C to 90°C) studied (Fig. 5.7).

The use of the WLF and other models in predicting temperature dependence of viscosity allows the establishment of state diagrams that show iso-viscosity states above T_g as a function of water content. Such diagrams may be used in the evaluation of changes in water content on relaxation times at constant temperature in establishing critical temperatures for the stability of amorphous materials (Roos, 1995) or may possibly aid in predictions of seed longevity. Slade and Levine (1991a) demonstrated that parallel to the T_g curve of sucrose, iso-viscosity lines can be found. Fig. 5.8 shows a state diagram in which iso-mobility lines are drawn above the T_g curve. Iso-mobility lines were found to be parallel to the T_g curve.

Implications for seed storage

The presence of intracellular glasses in seeds is thought to be of considerable significance for the storage longevity of the seeds (Leopold et al., 1994; Sun, 1997b; Buitink et al., 1998b). The role of intracellular glasses in longevity is derived from the dramatic increase in viscosity or decrease in the molecular mobility of molecules in other glass-forming substances, thus decreasing the

rate of detrimental reactions (Soesanto and Williams, 1981; Roozen et al., 1991; Steffen et al., 1992; Blackburn et al., 1996; Deppe et al., 1996; Champion et al., 1997; Hemminga and Van den Dries, 1998; Van den Dries et al., 1998a). Detrimental processes associated with ageing that take place in the cytoplasm of seeds are likely to be restricted by slow molecular motion of molecules in the cytoplasm. The low rotational mobility of CP in dry pea axes, when the cytoplasm is in a glassy state, indicates that formation of a glassy matrix is indeed beneficial to the preservation of molecules during storage in the dry state.

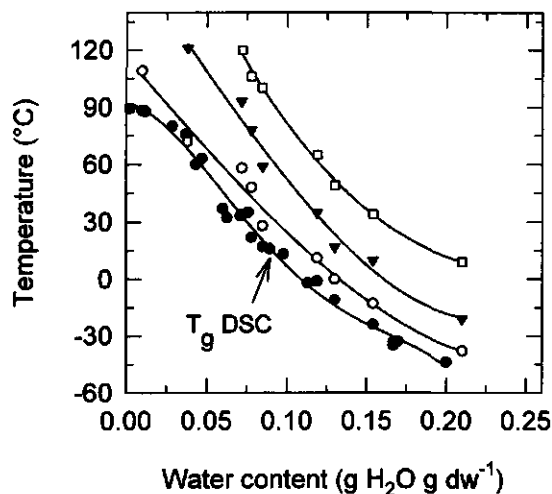


Figure 5.8. State diagram of pea axes. The solid circles represent the onset T_g measured by DSC at a scanning rate of $10^\circ\text{C min}^{-1}$. Lines represent the temperature/water content combinations at which τ_R is equal. $\tau_R = 5 \times 10^{-5}$ s (open circles), $\tau_R = 2 \times 10^{-5}$ s (closed triangles), $\tau_R = 1 \times 10^{-5}$ s (open squares). Lines are fourth order polynomial regressions.

The significance of glasses in seeds can be assessed by comparing the changes in mobility of molecules as a function of temperature and relating them to those found previously for other glass-forming substances. In particular, comparison with sugar glasses will ascertain whether the formation of intracellular glasses can be attributed to the soluble sugar present in the cytoplasm, as previously suggested (Williams and Leopold, 1989; Leopold et al., 1994). Intracellular glasses behave in a manner somewhat similar to that of other glass forming substances in that the temperature dependence of the rotational motion follows WLF behaviour above $0.07 \text{ g H}_2\text{O g dw}^{-1}$, which gives rise to iso-mobility lines parallel to the T_g . However, the rate of change of the rotational motion with temperature is not as large as seen in sugar glasses. Thus, although glasses are present in seeds, the effect of melting the glass by raising the temperature will not have a tremendous effect on changes in mobility of molecules in the cytoplasm. Nonetheless, although the difference in activation energy is relatively small below and above T_g , the change in activation energy warns us not to simply extrapolate kinetic data obtained by ageing seeds or pollen above T_g (brought about by high humidity and temperature) to ageing conditions below T_g (Franks, 1994b; Buitink et al., 1998b, Duddu and Dal Monte, 1997). Considering the different kinetics

5

between sugar glasses and intracellular glasses, sugars may aid in the formation of the intracellular glass in seeds, but other molecules will also participate in the formation of intracellular glasses (Leopold et al., 1994; Leprince and Walters-Vertucci, 1995).

Below $0.07 \text{ g H}_2\text{O g dw}^{-1}$, mobility starts to deviate from the general behaviour. The activation energy above T_g decreases with decreasing water content, and the temperature dependence of τ_R of CP in pea axes changes for each water content. It is interesting to note that below this water content, the storage behaviour of pea seeds also changes (Vertucci et al., 1994b). Instead of an increase in shelf life with decreasing water contents, the shelf life decreases. The factors that cause the change in kinetics of mobility and ageing rates are unknown. It has been suggested that the shelf life of foods and seeds or pollen is associated with the Brunauer, Emmet and Teller (BET) monolayer, derived from isotherms (Labuza et al, 1970; Labuza, 1980; Buitink et al., 1998b). Removal of this structural water may create holes in the cytoplasm, resulting in increased mobility (Buitink et al., 1998a) and increased ageing kinetics. Alternatively, removal of the last water changes the hydrogen bonding properties of molecules, thereby changing the behaviour of the spin probe, which could account for the increase in activation energy at these low water contents (Rozen et al., 1991).

Concluding remarks

A spectroscopic method was successfully employed to characterize the rotational motion of small spin probes incorporated into cells of biological materials. The results indicate that intracellular glasses can be formed in dry seeds, but that soluble sugars are not the only determining factor in the glass formation. The complex composition of the intracellular glass is suggested to be responsible for the moderate increase in mobility when the glass is melted, compared to other glass-forming substances. Our work shows that the use of a physical approach in obtaining detailed molecular information will be crucial in predicting the stability of food and biological materials.

Section III

Ageing Kinetics and Molecular Mobility

Overview

The rate of reactions is dependent on the local viscosity of the environment in which the reaction takes place. Detrimental reactions leading to ageing of seeds and pollens are therefore likely to be dependent on the molecular mobility in the cytoplasm. With the use of spin probe EPR spectroscopy we established a link between the molecular mobility of a polar spin probe that was incorporated into the cytoplasm and the rate of ageing or longevity of seed and pollen of different species. Elevation of the temperature or water content resulted in an increase in the molecular mobility as well as an increase in ageing rate. There appeared to be a linear relationship between the logarithms of molecular mobility and ageing rate. This suggests that molecular mobility is one of the controlling factors of ageing rate and thus influences the longevity of dry biological tissues.

Using the linear relationship between molecular mobility and ageing rate, it was possible to extrapolate the ageing rate or longevity of seeds to lower temperatures for which experimental determination of longevity is impossible due to their long life span. Predictions of longevity at -18°C showed an optimum water content of storage around $0.12 \text{ g H}_2\text{O g dw}^{-1}$, and it was predicted that further drying resulted in reduced longevity. These findings urge for a re-examination of current storage protocols.

6

The effects of moisture and temperature on the ageing kinetics of pollen: interpretation in terms of cytoplasmic mobility

Julia Buitink, Olivier Leprince, Marcus A. Hemminga, Folkert A. Hoekstra

This study shows that characterization of the molecular mobility in the cytoplasm of pollen provides a new understanding of the effects of moisture and temperature on ageing rates. Using saturation transfer EPR spectroscopy, we determined the rotational motion of the polar spin probe, 3-carboxy-proxyl, in the cytoplasm of *Typha latifolia* (cattail) pollen, under different temperature and water content conditions. Increasing the temperature resulted in faster rotational motion, analogous to faster ageing rates. With decreasing water content, rotational motion first decreased until a minimum was reached, after which rotational motion slightly increased again. The water content at which this minimal rotational motion was observed increased with decreasing temperature, comparable to the pattern of ageing rate. A significant linear relationship was found between ageing rates and rotational motion in the cytoplasm. This suggests that these parameters are causally linked. Upon melting of the intracellular glass, a two-fold increase in activation energy of rotational motion and ageing rate was observed. In contrast, melting of the sucrose glass resulted in an increase in rotational motion of 5 orders of magnitude. The difference in rotational motion upon melting glasses of pollen or sucrose suggests that other molecules beside sugars play a role in intracellular glass formation in pollen.

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6

Conservation of the biodiversity of plants can be achieved by preserving seed and pollen in genebanks. Because the effective preservation of seed and pollen depends on their water content and on the storage temperature, the effect of these two parameters on storage longevity has received much attention (Roberts, 1972; Ellis and Roberts, 1980a,b; Ellis et al., 1989, 1992; Roberts and Ellis, 1989; Vertucci and Roos, 1990, 1993b; Vertucci et al., 1994b; Buitink et al., 1998b). Several models have been developed to describe the effects of these two parameters. The viability equation, designed by Ellis and Roberts (1980a), is very valuable in describing and quantifying the effects of temperature and moisture on storage stability. Because of its empirical nature, however, the equation does not provide an enhanced understanding of the ageing kinetics. Another model, based on thermodynamic considerations (Vertucci and Roos, 1993b), suggests that the availability of water for chemical reactions could be the underlying explanation for the ageing kinetics. Using this thermodynamic approach, the authors investigated the optimal conditions for seed storage (Vertucci and Roos, 1990, 1993b; Vertucci et al., 1994b). It was predicted that an optimum water content would exist which would increase with decreasing temperature. This prediction was substantiated by experimental data (Vertucci et al., 1994b).

Another plausible hypothesis to explain ageing behaviour is that ageing rates of seed and pollen are governed by the molecular mobility within the cells. Molecular mobility is increasingly considered as a key factor influencing storage stability of foods, pharmaceuticals and biological tissues, because it is thought to control the rate of detrimental reactions that reduce shelf life (Leopold et al., 1994; Hancock et al., 1995; Roos, 1995; Buitink et al., 1998a,b). Indeed, higher temperatures and higher water contents are known to induce faster ageing rates and were also found to increase the molecular mobility of molecules present in the cytoplasm (Buitink et al., 1998a). Hence, characterization of the molecular mobility in relation to water content and temperature might shed some light on the ageing kinetics of seed and pollen.

A phenomenon associated with molecular mobility is the formation of glasses, which has been demonstrated to occur in dry biological tissues such as seed and pollen (Williams and Leopold, 1989; Bruni and Leopold, 1992; Leopold et al., 1994; Leprince and Walters-Vertucci, 1995; Buitink et al., 1996). The formation of intracellular glasses is thought to result in a considerably increased cytoplasmic viscosity (Burke, 1986; Leopold et al., 1994; Sun, 1997b). Consequently, the high viscosity has been suggested to be responsible for the decrease in the rates of detrimental reactions associated with ageing (Leopold et al., 1994; Leprince and Walters-Vertucci, 1995; Sun, 1997b; Buitink et al., 1998b).

Carbohydrates are excellent glass-formers (Levine and Slade, 1988; Roos, 1995) and are present in considerable amounts in seed and pollen (Amuti and Pollard, 1977; Hoekstra et al., 1992b). Thus, it has been suggested that carbohydrates are responsible for glass formation in biological tissues (Hirsh, 1987; Koster, 1991). One of the characteristics of carbohydrate glasses is that upon melting there is an abrupt decrease of viscosity, which does not

follow the conventional Arrhenius behaviour (Soesanto and Williams, 1981; Roozen and Hemminga, 1990; Roos, 1995; Champion et al., 1997; Hemminga and Van den Dries, 1998). Recent research suggested that molecules other than carbohydrates also might be involved in intracellular glass formation (Sun and Leopold, 1993; Leopold et al., 1994; Leprince and Walters-Vertucci, 1995; Sun and Leopold, 1997; Wolkers et al., 1998a; Buitink et al., 1999). For instance, addition of proteins substantially changed the properties of the glassy matrix (Wolkers et al., 1998a). It would be of interest to know whether intracellular glasses would exhibit a similar sharp decrease in viscosity or increase in molecular mobility upon melting, as found in model sugar glasses. If so, this would be expected to have a profound effect on ageing rates and storage behaviour.

In this study, cattail (*Typha latifolia*) pollen was used for two reasons. First, due to its relatively short lifespan, extensive data can be acquired on ageing rates of the pollen stored under a wide range of temperature and water conditions. Secondly, since the predominant soluble sugar present in the pollen is sucrose, the temperature dependence of the molecular mobility in the intracellular glass of the pollen can be compared with that of a model glass matrix made of sucrose.

The objective of this study was to investigate the relation between molecular mobility in the cytoplasm and ageing rates of pollen as a function of temperature and water content. The molecular mobility of molecules was assessed using saturation transfer EPR (ST-EPR) spectroscopy. With this technique, the rotational motion of a polar spin probe that is incorporated into the cytoplasm can be estimated (Hemminga and Van den Dries, 1998; Buitink et al., 1999). To assess the role of soluble sugars in intracellular glass formation, a comparison was made between changes in mobility of the spin probe in the pollen and in sucrose glasses upon melting of the glassy state.

Materials and methods

Plant material and storage experiments

Mature male inflorescences of *Typha latifolia* L. (cattail) were collected from field populations near Wageningen, The Netherlands, in 1996 and allowed to shed their pollen in the laboratory. Pollen (94% germination) was cleaned by sieving through a fine copper mesh, dried in dry air to 0.05 - 0.08 g H₂O g dry weight (dw)⁻¹ and stored at -20°C until use.

To determine the effects of storage conditions on ageing, viability was monitored in pollen that was kept in the dark at different RH and 10°C. The RH under which the pollen samples were stored was controlled and maintained by various saturated salt solutions [LiCl (14% RH), K(C₂H₃O₂) (25% RH), MgCl₂ (33% RH), CaCl₂ (37% RH), K₂CO₃ (43% RH), NaBr (58% RH), Ca(NO₃)₂ (51% RH), NaCl (75% RH), NH₄Cl (80% RH)] (Winston and Bates, 1960). Germination was determined by counting the percentage of empty, cytoplasm-less grains 24 h after placing the pollen in culture at 25°C. Non-germinated grains are still filled with cytoplasm. The culture medium was a 0.6% agar medium containing 0.2 M sucrose, 1.6 mM H₃BO₃, 1.3 mM Ca(NO₃)₂·4H₂O,

6

0.8 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 mM KNO_3 and 2 mM citric acid adjusted to a pH of 5.9 with KOH. To prevent imbibitional damage, pollen was prehydrated in saturated water vapour at room temperature for 4 h prior to the germination assay. This prehydration time was sufficient to prevent imbibitional damage. Each germination assay used 50 tetrads (200 grains) and was replicated four times.

The decrease in germination percentage over time of pollen stored at various RH at 10°C was plotted on a probability scale. A least squares linear regression was fitted to the data. Ageing rates (days^{-1}) were quantified as the slope of the different least squares regressions. Ageing rates of pollen stored at temperatures other than 10°C were taken from Buitink et al. (1998b).

Values of water content, expressed as $\text{g H}_2\text{O g dw}^{-1}$, were determined by comparing the fresh and dry weight of a sample for each RH/temperature treatment throughout the storage experiment. Dry weights were measured after heating the pollen for 36-48 h at 96°C.

ST-EPR study

Labelling of cattail pollen with the spin probe was performed according to Buitink et al. (1998a). Briefly, 3 g of pollen was prehydrated in water vapour for 16 h at 5°C to about $0.7 \text{ g H}_2\text{O g dw}^{-1}$ and then mixed at 25°C with 6 ml of liquid germination medium containing 2.5 mM 3-carboxy-proxyl (CP; Sigma). After a few min, an additional 20 ml of the germination medium was added, and the pollen was recovered by filtration. The pollen was then mixed with 20 ml of a solution of 1 mM CP and 120 mM potassium ferricyanide. After 5 more min, the pollen was recovered by filtration, spread out in a large petridish, and rapidly dried in a flow of dry air (3% RH) to water contents of less than $0.05 \text{ g H}_2\text{O g dw}^{-1}$. After drying, pollen was stored over several saturated salt solutions (Winston and Bates, 1960) to obtain various water contents. For each EPR measurement, approximately 20 mg pollen was sealed in a 2-mm diameter EPR capillary. After the measurements, the pollen was 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. The sucrose glass was prepared by rapidly drying 10 μl droplets of a solution containing 300 mM sucrose (Sigma) and 0.1 mM CP (Sigma) on a glass plate in an airflow of 3% RH for at least 72 h. After drying, the sucrose glass was removed from the glass plate after which the material was transferred into a 2-mm diameter EPR capillary. The residual water content was determined by sealing the sugar material into hermetically sealed DSC pans, and weighing before and after heating at 96°C for sufficient time to achieve a constant weight.

ST-EPR spectroscopy was applied to obtain the rotational correlation time (τ_r) of CP in cattail pollen (Buitink et al., 1998a, 1999), using an X-band EPR spectrometer (Bruker Analytik, Rheinstetten, Germany, model 300E). For random tumbling, τ_r roughly corresponds to the average time for a molecule to progress one radian. For ST-EPR measurements the second harmonic quadrature absorption signal was detected using field modulation amplitude 5 G, microwave power 100 mW, and field modulation frequency 50 kHz. Temperature was controlled using a temperature controller with liquid nitrogen vapour as the coolant. Scans were recorded during heating using a 2.5°C step increment followed by 5 min equilibration before recording the spectrum.

ST-EPR spectra can be well characterized by independent line shape parameters, such as the line-height ratio L'/L (see Fig. 6.1). 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. Because the viscosity for anhydrous glycerol is known over a broad temperature range, τ_R of CP in glycerol can be calculated (Hemminga and van den Dries, 1998; Buitink et al., 1999). Anhydrous glycerol, containing 1 mM CP, was cooled to -150°C , and after equilibration for 30 min, spectra were recorded at 3°C increments. For each temperature the value of the line shape parameter L^*/L was calculated. From the curves representing the line shape parameter of CP in glycerol against τ_R , the values of τ_R of CP in the pollen were obtained by interpolation. The variation of τ_R values within samples exposed to similar water content/temperature regimes was on average 5%.

Differential scanning calorimetry

Pollen of different water contents or the sucrose glass equilibrated to 3% RH was hermetically sealed into aluminium differential scanning calorimetry (DSC) pans. Second order transitions of the samples were detected using a Perkin-Elmer (Norwalk, CT) Pyris-1 DSC, calibrated for temperature with Indium (156.6°C) and methylene chloride (-95°C) standards, and for energy with Indium (28.54 J g^{-1}). Baselines were determined using an empty pan, and all thermograms were baseline-corrected. Scans were taken from -100°C to 120°C at a rate of $10^\circ\text{C min}^{-1}$. The T_g values were determined by the onset of the temperature range over which the change in specific heat occurred. All analyses were performed with Perkin-Elmer software.

Results

Temperature and water content influence rotational motion and ageing rate in a similar manner

The τ_R of the polar spin probe CP, incorporated in the cytoplasm of cattail pollen, was determined from ST-EPR spectra (Fig. 6.1). The τ_R of CP in pollen with different water contents was determined from such spectra for every 2.5°C temperature increment (Fig. 6.2). With increasing temperature τ_R decreased, which indicates that rotational motion increased. Arrhenius plots of the rotational motion revealed a break in the relationship at a definite temperature for all water contents tested (Fig. 6.2). Both below and above the break, the relationship between the logarithm of the rotational motion and the inverse of the temperature was linear. The break occurred at higher temperatures in pollen with lower water contents. The temperature at which the break occurred

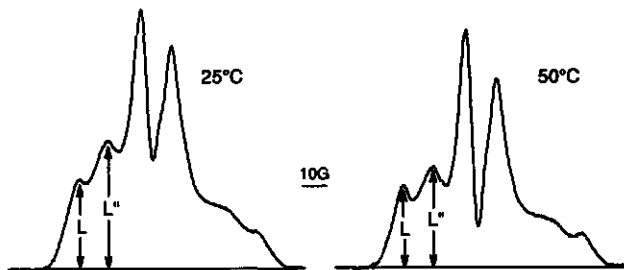
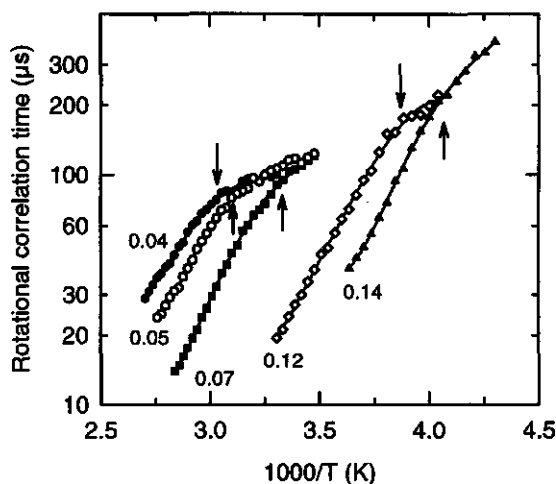


Figure 6.1. ST-EPR spectra of CP in cattail pollen ($0.07 \text{ g H}_2\text{O g dw}^{-1}$), recorded at 25 and 50°C . The line-height ratio L^*/L , used for the calculation of τ_R , is indicated.

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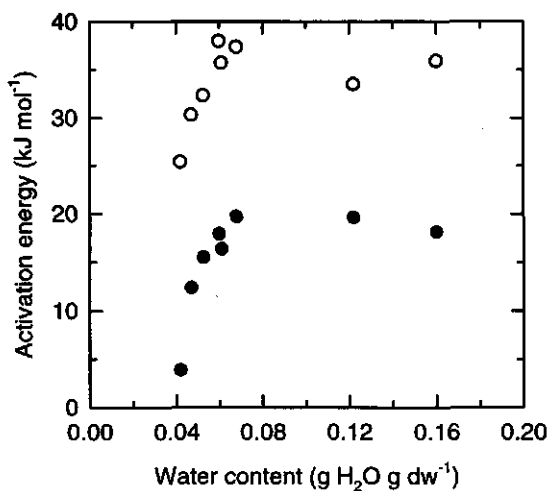
coincided with the onset of melting of the glassy state as measured by DSC (indicated by arrows). Thus, above the glass transition temperature (T_g), rotational motion changed more strongly with temperature than when the pollen was in a glassy state.

Figure 6.2. The effect of temperature on the τ_r of CP in the cytoplasm of cattail pollen equilibrated to different water contents, presented as Arrhenius plots. The values in the plot indicate water content in $\text{g H}_2\text{O g dw}^{-1}$. The arrows indicate the onset T_g as determined by DSC.



The slope of the Arrhenius plots (Fig. 6.2) gives information about the activation energy (E_a) of the rotational motion. A high activation energy indicates that there is a strong effect of temperature on the rotational motion. In Figure 6.3, the values of the E_a of the rotational motion below and above T_g were

Figure 6.3. Activation energies (kJ mol^{-1}) of τ_r of CP in cattail pollen below T_g (filled symbols) and above T_g (open symbols) at different water contents of the pollen.



plotted as a function of pollen water content. At water contents between 0.16 and 0.06 $\text{g H}_2\text{O g dw}^{-1}$, the E_a s were comparable, indicating that temperature had a similar effect on the rotational motion. The E_a above T_g was

approximately twice that below T_g . Below 0.06 g H₂O g dw⁻¹ the E_a decreased both below and above T_g as found previously for pea embryonic axes (Buitink et al., 1999).

Comparison of the temperature dependence of the ageing rates of the pollen at 0.07 g H₂O g dw⁻¹ (derived from Buitink et al., 1998b) with that of the rotational motion at the same water content revealed that both parameters responded similarly to temperature (Fig. 6.4). With increasing temperature,

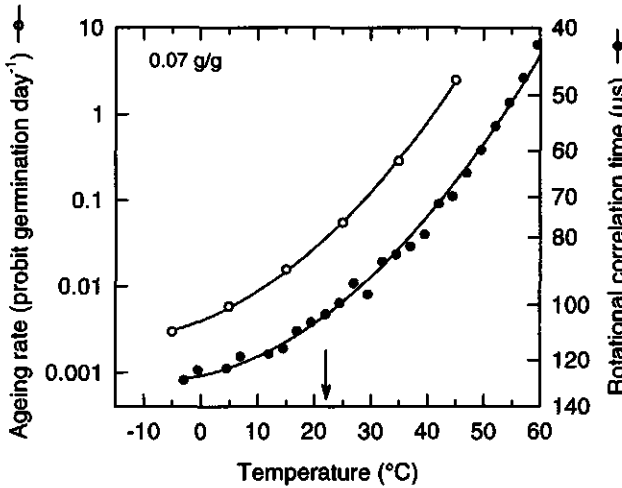


Figure 6.4. Temperature dependence of ageing rate (open symbols; days⁻¹) and τ_r (filled symbols; μ s) of CP cattail pollen containing 0.07 g H₂O g dw⁻¹. Ageing rates were derived from Buitink et al. (1998b). The arrow indicates the onset T_g as determined by DSC.

both ageing rates and rotational motion increased. Above T_g (indicated by the arrow), both parameters changed more steeply as a function of temperature. The comparable behaviour of ageing rate and rotational motion upon melting of the glassy state is further demonstrated by comparing the relative change in E_a of ageing rates and rotational motion below and above T_g (see Table 6.1). The shift in E_a was found to be between 2 and 2.5, indicating that melting of the glassy state in the pollen resulted in an approximate two-fold increase in activation energy of both rotational motion and ageing rate. The E_a of the ageing rates was approximately 3 to 5 times higher than that of rotational motion (Table 6.1).

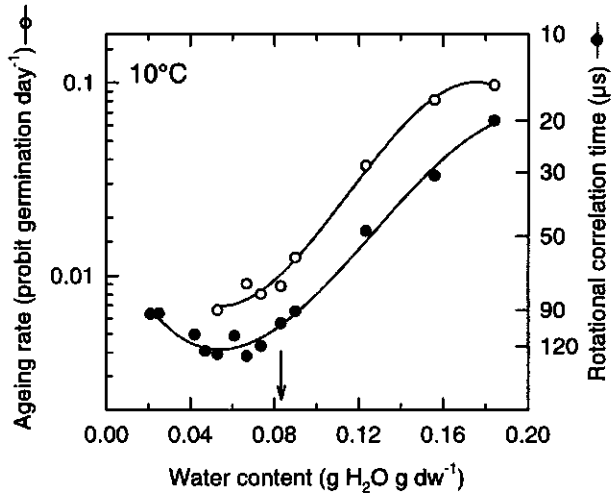
Table 6.1. Activation energies (E_a , kJ mol⁻¹) of ageing rate and rotational correlation time (τ_r) of CP in cattail pollen with different water contents (wc; g H₂O g dw⁻¹), determined above and below T_g . E_a 's were determined from Arrhenius plots of τ_r in Fig. 6.2 and ageing data derived from Buitink et al. (1998b).

wc g/g	E_a ageing rate (kJ mol ⁻¹)		E_a τ_r (kJ mol ⁻¹)	
	T < T_g	T > T_g	T < T_g	T > T_g
0.05	69	163	12	30
0.06	74	218	16	32
0.07	58	146	20	37
0.08	57	135	18	38

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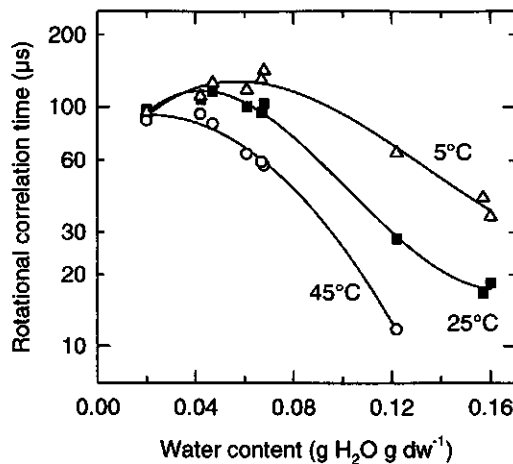
In addition to the temperature dependence of rotational motion and ageing rate, we investigated the effect of water content on the two parameters at 10°C (Fig. 6.5). The rotational motion and ageing rate followed a similar water content dependence. The rotational motion of the spin probe showed a minimum (i.e. a maximum in τ_R) at approximately 0.06 g H₂O g dw⁻¹. Below this water content, rotational motion appeared to increase again.

Figure 6.5. Water content dependence of ageing rate (open symbols; days⁻¹) and τ_R (filled symbols; μ s) of CP cattail pollen at 10°C. The arrow indicates the onset T_g as determined by DSC.



The increase in molecular motion of the spin probe at low water contents prompted us to have a closer look at how the molecular mobility in the cytoplasm was affected by water content at different temperatures. The relation between rotational motion and water content showed a minimum in rotational motion for each temperature between 5°C and 45°C (Fig. 6.6). The

Figure 6.6. Effect of water content on τ_R of CP in the cytoplasm of cattail pollen measured at different temperatures (5°C, 25°C and 45°C). Lines represent third-order polynomial regressions.



water content at which this minimum in rotational motion was observed appeared to increase with decreasing temperature.

6

Rotational motion correlates with ageing rates

The relationship between ageing rate and water content also has been found to exhibit a minimum in ageing rate at a definite water content that increased with decreasing temperature (Buitink et al., 1998b). This comparable effect of moisture on both rotational motion and ageing rate raised the possibility that the rate of ageing is causally linked with the rotational motion in the cytoplasm. Hence, ageing rates of pollen stored under different temperature and water content conditions were plotted as a function of τ_r values that had been determined on pollen equilibrated to the same conditions. Figure 6.7 shows that the relationship between the logarithms of ageing rate and rotational motion was linear for each temperature studied. The correlation coefficients of the linear regression were all above 0.91. The linear relationship between mobility and ageing rate strongly suggests that the detrimental ageing reactions leading to loss of survival are dependent on the molecular mobility in the cytoplasm. Moreover, the change in kinetics of the ageing rates around T_g can be explained by a similar change in the rotational motion around T_g .

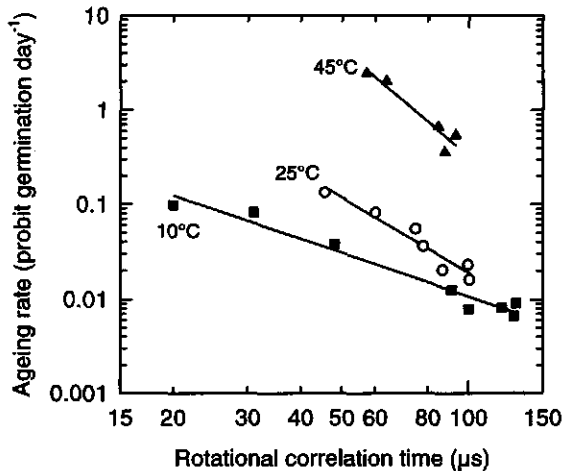


Figure 6.7. Relationship between the ageing rates of cattail pollen and the τ_r of CP in the cytoplasm. Equal symbols represent the same storage temperatures but different water contents. Filled triangles, 45°C ($r^2 = 0.914$); open circles, 25°C ($r^2 = 0.925$); filled squares, 10°C ($r^2 = 0.965$). Ageing rates at 45°C and 25°C were derived from Buitink et al. (1998b).

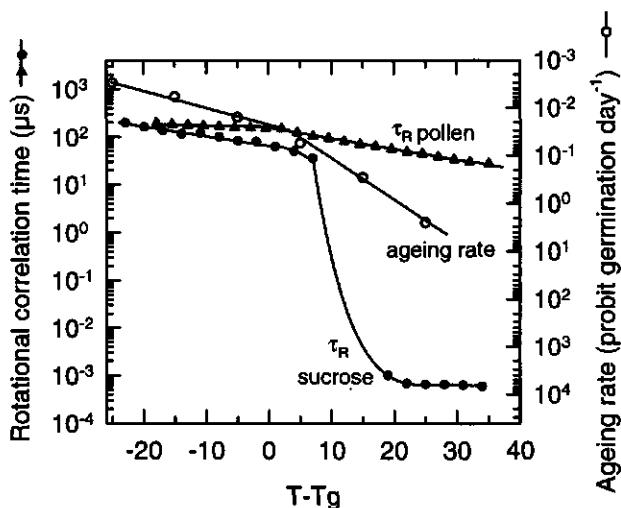
Magnitude of the increase in molecular mobility upon melting of intracellular and sucrose glasses

Because soluble sugars are excellent glass-formers and widely abundant in pollen and seed, it has been suggested that they play a pivotal role in the intracellular glass formation (Koster, 1991; Leopold et al., 1994). The

6

predominant soluble sugar present in cattail pollen is sucrose (about 25% of the dry weight, Hoekstra et al., 1992b). One characteristic of carbohydrate glasses is that upon melting, viscosity decreases (and molecular mobility increases) dramatically within the first 30°C above T_g (Soesanto and Williams, 1981; Roozen and Hemminga, 1990; Roos, 1995; Champion et al., 1997; Hemminga and Van den Dries, 1998). One way of describing this behaviour is to plot the viscosity or mobility as a function of $T-T_g$. Figure 6.8 shows the rotational motion of CP in a sucrose glass (filled circles), with a residual water content of 0.03 g H₂O g dw⁻¹. At temperatures below T_g ($T-T_g < 0$) the rotational motion increased linearly with increasing temperature. The rotational mobility increased sharply within the first 30°C above T_g by ca. 5 orders of magnitude. The rotational motion of CP in intracellular glasses of the pollen (0.07 g H₂O g dw⁻¹) also showed a linear relation with temperature below T_g (Fig. 6.8, filled triangles). Interestingly, above T_g the rotational motion did not show an abrupt increase as seen for the sucrose glass. Instead, it decreased linearly with increasing temperature above T_g , albeit with a steeper slope than below T_g . Also shown in Fig. 6.8 is the temperature dependence of ageing rates for pollen stored at 0.07 g H₂O g dw⁻¹ (open circles, derived from Buitink et al., 1998b). The ageing rate increased linearly with temperature, with a break in the

Figure 6.8. Changes in τ_R of CP in cattail pollen (0.07 g H₂O g dw⁻¹; filled triangles) and a sucrose glass (filled circles), or changes in ageing rate of cattail pollen (0.07 g H₂O g dw⁻¹; open circles) as a function of $T-T_g$. Ageing rates were derived from Buitink et al. (1998b). Onset T_g was determined by DSC.



relationship around T_g . Apparently, the dramatic effect of melting the glassy state on rotational motion, as seen for the sucrose glass, could not be found in cattail pollen (Fig. 6.8). Moreover, the temperature dependence of rotational motion of CP in pollen appears to be linear above T_g , implying Arrhenius behaviour at least for the first 30°C above T_g . The same is true for the temperature dependence of the ageing rates.

Discussion

6

Molecular mobility of the polar spin probe CP in the cytoplasm of cattail pollen changed as a function of water content and temperature. The temperature dependence of the rotational motion was similar for pollen with water contents above $0.06 \text{ g H}_2\text{O g dw}^{-1}$. Changes in rotational motion and ageing rates as a function of temperature were comparable, both showed a ca. two-fold increase in the E_a when pollen was brought above the T_g of its intracellular glass (see Table 6.1). However, the values of the E_a of rotational motion were 3 to 5 times lower than those of the ageing rates. The rotational motion of a spin probe and ageing reactions are two processes occurring at a different molecular level, thus, it is not surprising that different E_a s were found. It can be expected that the energy that is needed on a molecular scale for rotation of a molecule is less than the energy required for translation (i.e. diffusion) on a macroscale. Indeed, the temperature dependence of translational diffusion and rotational motion are not equal above T_g ; the translational motion changed approximately three times faster than the average rotational reorientation relaxation (Deppe et al., 1996). Nonetheless, measurements of rotational motion may still be used to describe reaction rates based on the diffusive properties of the cytoplasm. This is supported by Fig. 6.7, where a significant linear correlation was found between the logarithm of the ageing rate and the rotational motion. Our data show that molecular mobility in the cytoplasm of pollen correlates with the rate of detrimental reactions associated with ageing, indicating that ageing reactions might take place in the cytoplasm. Alternatively, considering that membranes are likely to be the most vulnerable target for detrimental reactions (Priestley, 1986; Wilson and McDonald, 1986; Van Bilsen and Hoekstra, 1993), the viscous cytoplasm might be responsible for restricting motion of membrane components (Lee et al., 1986), and thus influence ageing reactions indirectly.

For pollen containing less than $0.06 \text{ g H}_2\text{O g dw}^{-1}$, the E_a of the rotational motion decreased (Fig. 6.3), as found before for pea embryonic axes (Buitink et al., 1999). We reported previously that storing pollen below this water content resulted in an increase in ageing rates (Buitink et al., 1998b). The water content at which the minimum in ageing rate and rotational motion was observed corresponded to the Brunauer, Emmett and Teller (BET) monolayer value (Buitink et al., 1998b). It could be envisaged that removal of water from the cytoplasmic glass matrix below the BET monolayer value introduces cavities in which the spin probe can rotate more freely. The higher molecular mobility at these low water contents could be a reason for the increased ageing rates observed previously (Buitink et al., 1998b). Also, removal of the water below the monolayer value from tissues has been suggested to lead to loss of structural integrity of molecules, which could be an alternative explanation for the increased ageing rates at these low water contents (Vertucci and Leopold, 1987; Sun et al., 1997).

The sucrose glass was found to exhibit a dramatic increase in mobility upon melting (Fig. 6.8). This is clearly not the case for the mobility in cattail

6

pollen (Fig. 6.8). This is somewhat surprising since the major soluble sugar in the pollen is sucrose, present for about 25% of its dry weight (Hoekstra et al., 1992b). Other molecules, for instance proteins, aid in the formation of intracellular glasses, as suggested previously (Sun and Leopold, 1993, 1997; Leopold et al., 1994; Leprince and Walters-Vertucci, 1995; Wolkers et al., 1998a; Buitink et al., 1999). Thus, the effect of melting of the intracellular glass of pollen by raising the temperature will not abruptly increase the mobility of molecules in the cytoplasm, as would be expected when sucrose was the only component involved. This may have an ecological significance if survival of pollen in their natural habitat is considered. Fluctuations in environmental conditions that are likely to bring the cytoplasm out of the glassy state will not result in a large increase in the molecular mobility of the cytoplasm. Thus, the increase in ageing rates of the pollen due to adverse environmental factors is limited. The presence of a break in the relationship between ageing rate and temperature in the pollen around T_g , however, shows that the melting of glasses does alter the ageing kinetics of the pollen.

In conclusion, the rate of ageing of cattail pollen in relation to water content and temperature can be described by the rotational mobility in the cytoplasm. We suggest that the molecular mobility in the cytoplasm is a major controlling factor in the rate of detrimental reactions responsible for reducing lifespan.

Acknowledgements - We kindly thank I. van den Dries for helpful discussions.

7

Molecular mobility in the cytoplasm of lettuce radicles correlates with longevity

Julia Buitink, Folkert A. Hoekstra, Marcus A. Hemminga

Molecular mobility is hypothesised to be a key factor influencing storage stability, because it is thought to control the rate of deteriorative reactions responsible for reducing shelf life. We investigated the relationship between the longevity of lettuce seeds and the molecular mobility 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 measurements of rotational motion of a polar spin probe inserted into the cytoplasm, using saturation transfer electron paramagnetic resonance spectroscopy. Increasing the temperature resulted in faster rotational motion and shorter longevity. There was a linear relationship between the logarithms of rotational motion and estimated 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, we predicted the longevity at sub-zero temperatures 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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7

Seed longevity is, among others, determined by the environmental conditions during storage. In general, low temperatures and low water contents are beneficial to long storage life (Roberts, 1972). One of the hypotheses underlying the improved stability of germplasm at low temperatures and water 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, 1997b; Buitink et al., 1998b). The high viscosity of this glassy state has been suggested to be responsible for the delayed ageing and increased longevity. The lifespan of biological materials such as seeds and pollen was increased profoundly during storage under conditions in which the cytoplasm was brought in a glassy state (Sun and Leopold, 1994; Sun, 1997b; 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 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., 1998a). 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 degradation of the product (Hancock et al., 1995). Therefore, characterization 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 ($\sim 60^\circ$) 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, 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 during 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 obtaining longevity data is by the use of the viability equation from Ellis and Roberts (1980a). This empirical equation has been shown to give accurate predictions of longevity over a wide range of temperatures and water contents (Ellis and Roberts, 1980a,b; Kraak and Vos, 1987; Ellis et al., 1989, 1990b, 1992; Dickie et al., 1990; Mwashu 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, 1990b; Dickie et al., 1990; Vertucci and Roos, 1990; Vertucci et al., 1994b). Below a certain critical water content, the equation fails to adequately predict the viability of seeds (Ellis et al., 1989, 1990b; Vertucci and Roos, 1990; Vertucci et al., 1994b). 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 water 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 75°C to 5°C for water 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 spectroscopy. 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 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 a solution 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.

7

Subsequently, the lettuce radicles were dried 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 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 with 5°C increments in temperature 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 ($0.057 \text{ g H}_2\text{O g fw}^{-1}$).

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. 7.1A 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 characterized 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. 7.1B, 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, one can obtain the rotational motion of the spin probe in the immobile fraction (*i.e.* glassy cytoplasm) by determining the ratio H^*/H (see Fig. 7.1B). 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),

$$\tau_r = (\eta V/k_b T) k + \tau_0 \quad (7.1)$$

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 (Van den Dries and Hemminga, 1998). Anhydrous glycerol, containing 1 mM CP, was cooled to -150°C , and after equilibration for 30 min, spectra were recorded at 3°C increments. For each temperature the values of the line shape parameter H^*/H were 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.

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

$$\log \sigma = K_E - C_W \log m - C_H t - C_Q t^2 \tag{7.2}$$

where m is seed water content, t is temperature ($^{\circ}\text{C}$), 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 to 75 $^{\circ}\text{C}$ and 3.6 to 17.9 percent moisture on a fresh weight basis (Kraak and Vos, 1987).

Results

Using EPR spectroscopy, one can determine the rotational correlation time τ_R of a spin probe 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

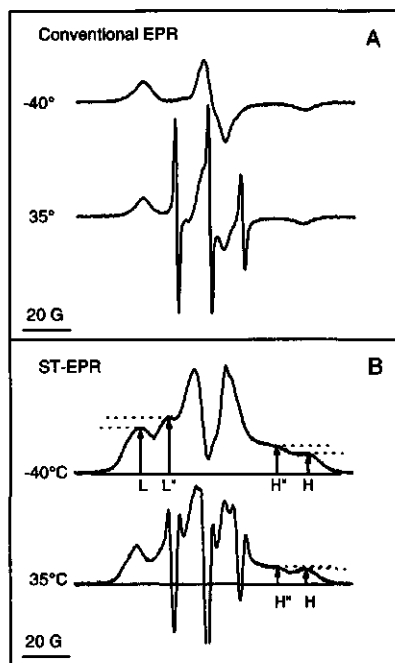


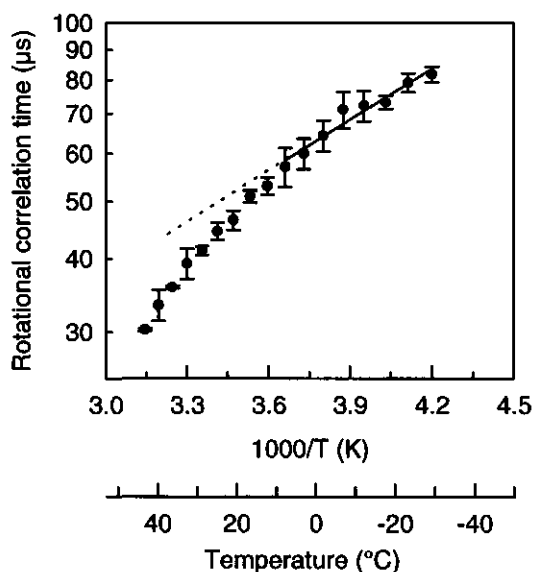
Figure 7.1. Conventional EPR (A) and saturation transfer EPR (B) spectra of CP in radicles of lettuce, recorded at two different temperatures. The lettuce radicles were equilibrated to 33% RH, and had a water content of 0.057 g H₂O g fw⁻¹. The line height ratios L'/L and H'/H are indicated; H'/H was used to calculate the τ_R .

7

probe has a τ_r longer than 10^{-8} s, the conventional EPR spectrum takes the shape of a so-called powder spectrum (Fig. 7.1A, top spectrum). In this range of mobility, conventional EPR spectroscopy can not be used anymore, because it becomes insensitive to rotational motion. However, one can use changes in the line shape of ST-EPR spectra 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 7.1B, 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 characterized by a sharp three-line conventional EPR spectrum (see Fig. 7.1A, 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 7.1A, top and bottom spectrum). From Fig. 7.1B it is clear that the ratio L'/L can not 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. 7.1B). Therefore, we calculated the τ_r of the polar spin probe CP inserted in lettuce radicles using the H'/H ratio.

Figure 7.2 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.

Figure 7.2. 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 ($0.057 \text{ g H}_2\text{O g fw}^{-1}$). Data are the average of three separate experiments, of which the standard errors are indicated.



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 7.3 shows the dependence of temperature on longevity of lettuce seeds with a water content of 0.057 g H₂O

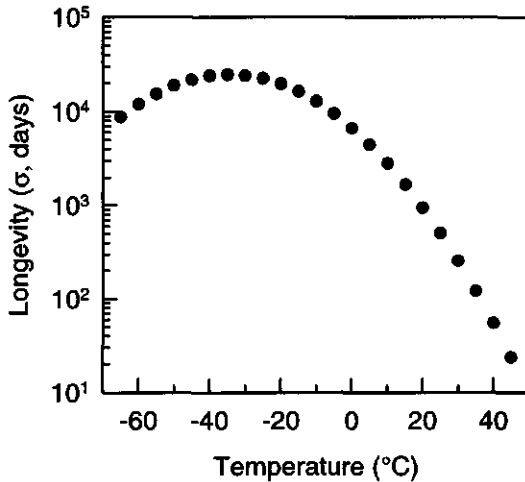


Figure 7.3. Estimated longevity (σ), derived from the viability equation, in relation to temperature. Viability constants were derived from Dickie et al. (1990).

g fw⁻¹. A decrease of temperature 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. 7.3) is the result of the quadratic term in the viability equation (eqn. 7.2), 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. 7.4). The estimated longevity increased linearly with longer τ_R (slower rotational motion). This log-log relationship extended from 5 and 45°C (Fig. 7.4) 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. 7.4), 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. Figure 7.5 shows the predicted longevities as a function of temperature, both determined using the viability equation (closed symbols), and by extrapolation using the linear regression between rotational motion and longevity (open symbols). It can be seen that predictions of longevity at low temperatures, based on rotational

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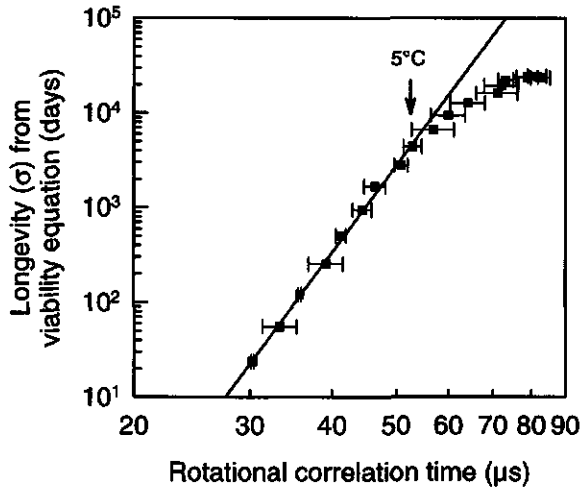
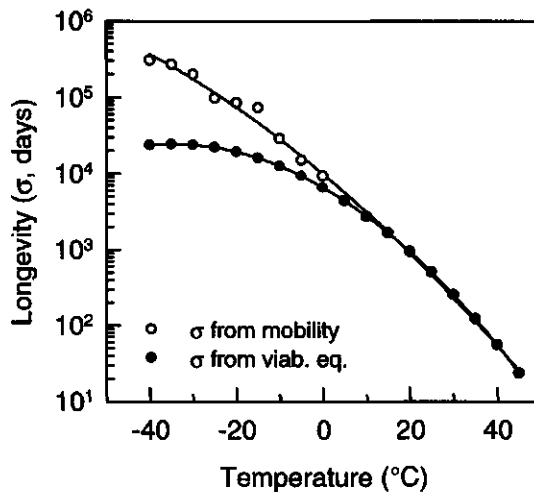


Figure 7.4. Relationship between rotational correlation time (τ_r) and estimated longevity at different temperatures. The estimated longevity was calculated using the viability equation with constants from Dickie et al. (1990). The τ_r and estimated longevity were determined every 5°C from -40°C to 45°C. 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 $a = -12.66$, $b = 9.42$.

motion, are greater than those based on the viability equation. At -20°C, a temperature commonly used by gene banks to store seeds, the longevity predicted through rotational motion was approximately four-fold higher than that estimated through the viability equation. At -10°C, this difference was about two-fold (Fig. 7.5).

Figure 7.5. Comparison of the relationships between predicted longevity and temperature. Longevity was predicted either using the viability equation (closed symbols, derived from Fig. 7.3) or by extrapolation of rotational mobility data using the linear regression of Fig. 7.4 (open symbols).



Discussion

7

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 H₂O g fw⁻¹) 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, 1997b). A steeper increase in rotational motion is expected when the glass has melted, and this would be manifest as an increase in 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., 1998a). An increase in activation energy upon melting of the glassy state has been found to be associated with an altered kinetics of ageing in cattail pollen, and in the rotational motion of CP in pea embryonic 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 water contents. Because the glass transition temperature increases with decreasing water 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 for the change in kinetics of motion, the change in kinetics is an important observation. If molecular mobility indeed 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 deviate 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).

7

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. 7.4). 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 ($-C_0t^2$). This is evident from the declining estimated longevity at temperatures below -36°C (Fig. 7.3). 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. 7.4) are accurate, longevity at sub-zero temperatures should be higher than estimated by the equation. Previously, Dickie et al. (1990) found a notable 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 eventually lead to improved predictions of longevity at low temperatures.

Acknowledgements - The authors thank Rijk Zwaan (De Lier, the Netherlands) for the gift of seeds.

8

Molecular mobility in the cytoplasm: an approach to describe and predict lifespan of dry germplasm

Julia Buitink, Olivier Leprince, Marcus A. Hemminga, Folkert A. Hoekstra

Molecular mobility is increasingly considered as a key factor influencing storage stability of biomolecular substances, because it is thought to control the rate of detrimental reactions responsible for reducing the shelf life of, for instance, pharmaceuticals, food and germplasm. We investigated the relationship between ageing rates of germplasm and the rotational motion of a polar spin probe in the cytoplasm under different storage conditions, using saturation transfer electron paramagnetic resonance spectroscopy. Rotational motion of the spin probe in the cytoplasm of seed and pollen of various plant species changed as a function of moisture content and temperature in a manner similar to ageing rates or longevity. A linear relationship was established between the logarithms of rotational motion and ageing rates or longevity. This linearity suggests that detrimental ageing rates are associated with molecular mobility in the cytoplasm. By measuring the rotational correlation times at low temperatures at which experimental determination of longevity is practically impossible, this linearity enabled us to predict vigour loss or longevity. At subzero temperatures, moisture contents for maximum lifespan were predicted to be higher than those hitherto used in genebanks, urging for a re-examination of seed storage protocols.

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Conservation of the genetic biodiversity of plants can be achieved by preserving seeds and pollens in genebanks. An important goal of germplasm facilities is the long-term preservation of viability. Because genebank operators must know when the loss of viability commences in order to timely regenerate samples, effective tools to predict longevity under given storage conditions and to minimise damage to the germplasm are required. A number of empirical equations to predict or describe longevity have been devised, with the most important one being the viability equation, developed by Ellis and Roberts (1980a). This viability equation has widely proven its validity (Ellis and Roberts, 1980a,b; Kraak and Vos, 1987; Ellis et al., 1989; Dickie et al., 1990; Mwashia et al., 1997; Usberti and Gomes, 1998; Hong et al., 1999). Nevertheless, it has been recognised that this empirical equation has some limitations regarding the validity of predictions, particularly at low temperatures and water contents (Dickie et al., 1990; Vertucci and Roos, 1993b, Vertucci et al., 1994b).

In recent years, a hypothesis to describe storage behaviour has been developed by Vertucci and co-workers, based on thermodynamic considerations (Vertucci and Roos, 1990, 1993b; Vertucci et al., 1994b). These authors suggested that the limiting factor in ageing of dry germplasm is the availability of water for chemical reactions, and that therefore the water potential would be a better way of predicting optimal storage conditions. Their approach resulted in the observation that the optimum water contents of storage shifted to higher values with lower temperatures (Vertucci and Roos, 1990, 1993b, Buitink et al., 1998b). Because the optimum storage conditions and predictions of germplasm longevity still remain a matter of debate (Ellis et al., 1995; Walters and Engels, 1998) we investigated alternative ways of describing longevity.

Over the past years, a new approach has been introduced to understand the effects of water content and temperature on longevity of germplasm. This kinetic model is based on the formation of glasses: highly viscous, semi-equilibrium solid liquids (Franks, 1994a). Low temperatures and low water contents drive the viscosity of the cytoplasm of tissues to such high values that it will form a glassy state (Williams and Leopold, 1989; Bruni and Leopold, 1992; Leopold et al., 1994; Buitink et al., 1998b). It is assumed that the high viscosity of intracellular glasses decreases molecular mobility and impedes diffusion within the cytoplasm, thus slowing deleterious reactions and changes in structure and chemical composition during ageing (Sun and Leopold, 1993, 1995, 1997; Leopold et al., 1994; Buitink et al., 1998b). Molecular mobility is increasingly considered a key factor influencing storage stability, because it is thought to control the rate of detrimental ageing reactions responsible for reducing shelf life (Sun and Leopold, 1993, 1995, 1997; Leopold et al., 1994; Hancock et al., 1995; Roos, 1995, Buitink et al., 1998a).

Saturation transfer electron paramagnetic resonance (ST-EPR) spectroscopy is a powerful technique that enables the determination of the rotational correlation time (τ_r) of a spin probe that is incorporated into the material under study. For random tumbling, τ_r roughly corresponds to the

average time for a molecule to progress one radian (Knowles et al., 1976). The time scale of the motion detected by this technique ranges from 10^{-7} to 10^{-3} s, encompassing an extremely wide range of temperatures and water contents (Hemminga, 1983; Buitink et al., 1998a, 1999). We have previously assessed the rotational motion of the polar spin probe 3-carboxy-2,2,5,5-tetramethylpyrrolidine-1-oxyl (CP) incorporated in the cytoplasm of seeds and pollen as a function of water content and temperature (Buitink et al., 1998a, 1999). It was shown that the temperature dependence of the rotational motion of the spin probe in seeds changed around the glass transition temperature (T_g), with the activation energy of rotational motion changing by a factor of three when tissues were stored below or above T_g (Buitink et al., 1999). Similar changes in activation energy were found in the ageing kinetics of cattail (*Typha latifolia*) pollen (Buitink et al., 1998b). The altered activation energy below T_g indicates that extrapolation of reaction rates above T_g to conditions below T_g can lead to incorrect estimates of shelf life (Franks, 1994b; Buitink et al., 1998b). Using molecular mobility as a potential means to describe ageing kinetics, the possible changes in kinetics resulting from the formation of glasses in the biological systems will be accounted for.

Here, we determined the rotational motion of the spin probe CP in the cytoplasm of seeds and pollen at different temperatures and water contents, using ST-EPR spectroscopy. For impatiens seeds and cattail pollen, the rotational motion was correlated to their ageing rates or half-viability times, derived from our own experiments, both as a function of temperature and water content. Similarly, rotational motion of CP in pea axes was compared with the ageing rates of pea seeds, based on vigour loss (Vertucci et al., 1994b) or loss of germination capacity (Ellis et al., 1989), derived from data in the literature. Finally, longevity of pea seeds was estimated by the viability equation of Ellis and Roberts (Ellis and Roberts, 1980a; Ellis et al., 1989) and correlated to the rotational motion in the axes. The established linear relationship enabled extrapolations of vigour loss and longevity to subzero temperatures. The importance of these estimates in relation to germplasm storage protocols is discussed.

Methods

Plant material, sample preparation and storage experiments.

Seeds of *Impatiens walleriana* L. cv impulse lila [initial water content of $0.05 \text{ g H}_2\text{O g dw}^{-1}$], were a gift from Novartis (Enkhuizen, The Netherlands). Initial percentage germination was 93%. Pea (*Pisum sativum* L.) seeds were obtained from Nunhems Zaden (Haalen, The Netherlands). Initial water content and percentage germination were $0.07 \text{ g H}_2\text{O g dw}^{-1}$ and 99%, respectively. Mature male inflorescences of *Typha latifolia* L. (cattail) were collected near Wageningen, The Netherlands and allowed to shed their pollen in the laboratory. Pollen (94% germination) was cleaned, dried in dry air to $0.05 - 0.08 \text{ g H}_2\text{O g dw}^{-1}$ and stored at -20°C until use.

For labelling of the tissues with CP (Sigma), impatiens seeds were soaked in water for 2 h at 20°C , after which the seed coat was removed. Pea seeds were soaked



in water for 16 h at 15°C, after which the axes were excised. Visible germination was not detected until 72 h. Decoated seeds of impatiens and isolated axes of pea were incubated for 45 min in 1 mM CP solution at 20°C. Potassium ferricyanide was then added to a final concentration of 200 mM, and the seeds were incubated for another 15 min. Ferricyanide broadens spin probe signals in the solution surrounding the cells to invisibility (Golovina et al., 1997). Subsequently, the labelled material was dried in dry air (3% relative humidity [RH]) for 24 h to a water content of 0.04 g H₂O g dw⁻¹. Germination experiments were performed to ensure that incubation in the spin probe/ferricyanide solution and subsequent drying back did not affect the percentage of germination. Labelling of cattail pollen was performed according to Buitink et al. (1998a). Briefly, pollen was prehydrated in water vapour for 16 h at 5°C to a water content of 0.7 g H₂O g dw⁻¹ and then mixed at 25°C with liquid germination medium containing 2.5 mM CP, after which the pollen was recovered by filtration. The pollen was then mixed with a solution of 1 mM CP and 120 mM potassium ferricyanide. After 5 more min, the pollen was recovered by filtration, and rapidly dried in a flow of dry air (3% RH) at room temperature to water contents < 0.05 g H₂O g dw⁻¹.

Immediately after drying, all tissues were exposed to various saturated salt solutions (Winston and Bates, 1960) at 25°C for 3-10 days to obtain different water contents. Approximately 20 seeds of impatiens, three axes of pea, or 20 mg cattail pollen were sealed in a 2-mm diameter capillary for EPR measurements. After the measurements, the tissues 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.

Longevity of impatiens seeds was monitored during 2 years under controlled storage conditions by using saturated salt solutions of various RHs in the dark at 25°C. At various intervals during storage, approximately 100 seeds were soaked on filter paper in the dark at 20°C. Germinated seeds were counted until final percentage germination was achieved (after approximately 3 wk). Ageing rates were calculated as the absolute value of the slope of the linear regression between germination percentage (expressed on probability scale) and storage time.

Ageing rates for pea seeds were derived from Vertucci et al. (1994b) and represent the absolute value of the slope of the loss in vigour (radicle length multiplied by percentage germination) over storage time. Ageing rates for cattail pollen were derived from our previously published results (Buitink et al., 1998b) and represent the absolute value of the slope of the linear regression between germination percentage (plotted on a probability scale) over storage time. The half-viability times (P_{50}) were also derived from Buitink et al. (1998b) and represent the time until germination had decreased to 50%.

ST-EPR measurements

ST-EPR spectroscopy was employed to obtain the τ_r of CP (Buitink et al., 1998a, 1999), using an X-band EPR spectrometer (Bruker Analytik, Rheinstetten, Germany; model 300E). 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. The phase was set with the self-null method (Thomas et al., 1976). In ST-EPR spectroscopy, τ_r values are obtained in an empirical way by using reference material of known viscosity. Here, spectra of CP in anhydrous glycerol were used to construct a calibration curve according to Van den Dries et al. (1998a). Because the viscosity data for glycerol are only published above -35°C, and viscosities at lower temperatures were needed to

construct the calibration curve, the known viscosity data for glycerol were fitted to the empirical Williams-Landel-Ferry (WLF) equation to provide the viscosities at lower temperatures (see Van den Dries et al., 1998a for details). The calculated T_g , obtained from the fit of the WLF equation with the universal constants, was found to be identical to the literature value, validating the use of the universal constants (Van den Dries et al., 1998a). The line shape parameter L^*/L , derived from the ST-EPR spectra, was used for the determination of τ_r (Buitink et al., 1999). It is possible that anisotropic motion of the spin probe, resulting from the interaction with its environment, might affect ST-EPR spectra. However, based on ST-EPR on anisotropic systems (Marsh, 1980), one can expect that this effect will be mainly exerted on the central part of the spectra, and that the effect on the L^*/L ratio will be small. From the calibration curve, representing the L^*/L ratio of CP in glycerol against τ_r , the τ_r values of CP in the seeds and pollen were obtained by interpolation. The τ_r values of CP in the different tissues were determined at different temperatures and water contents, corresponding to the storage conditions under which longevity or ageing rates were determined. The variation of τ_r values within samples exposed to similar water content/temperature regimes was on average 5%.

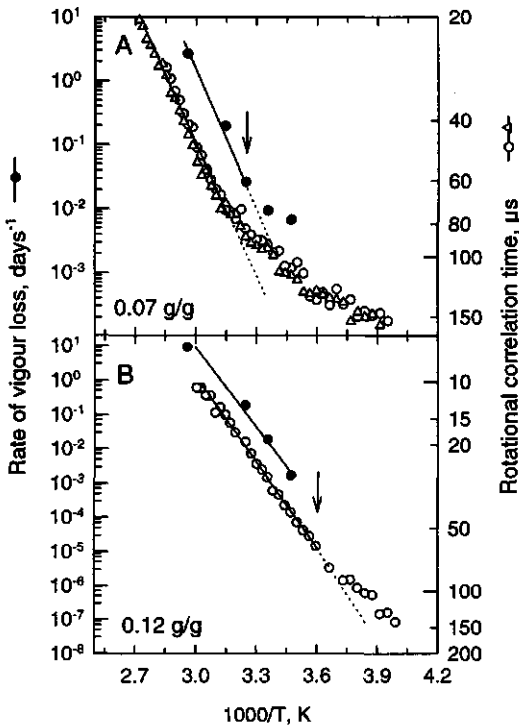


Figure 8.1. Dependence of ageing rate (expressed as rate of vigour loss, day⁻¹) of pea seeds (filled symbols) and τ_r (open symbols) on temperature of pea embryonic axes at a water content of 0.07 g H₂O g dw⁻¹ (A) and 0.12 g H₂O g dw⁻¹ (B). Two separate measurements of the τ_r of CP in pea axes at 0.07 g H₂O g dw⁻¹ are shown in A to indicate the reproducibility of the ST-EPR measurements. Ageing rates were taken from (Vertucci et al., 1994b). The arrows indicate the onset T_g as measured by differential scanning calorimetry, obtained from (Buitink et al., 1998a), using a scanning rate of 10°C min⁻¹.

8

Results

Temperature-dependent changes in ageing rates are related to rotational motion

Changes in ageing rate as a function of temperature were compared with changes in rotational motion of the spin probe CP for various seeds and pollen. A common way to plot the temperature dependence of an event is by Arrhenius plots, where the logarithm of the molecular mobility is plotted as a function of the reversed temperature (Fig 8.1). In Fig. 8.1, the ageing rate of pea seeds was expressed as the rate of vigour loss over time, where vigour is measured as hypocotyl length multiplied by percentage germination (Vertucci et al., 1994b). A slower rotational motion, i.e. an increased τ_r of the spin probe in the cytoplasm of axes of pea, corresponded to a lower ageing rate of the seed (Fig. 8.1). With decreasing temperature, the rotational motion and the ageing rate both decreased in a similar manner, as shown for pea embryonic axes at two water contents (Fig. 8.1). The arrow indicates the onset T_g for the pea axes as measured by differential scanning calorimetry (obtained from Butink et al., 1998a). When the cytoplasm of the axes was brought into a glassy state by decreasing the temperature below 34°C for axes with 0.07 g H₂O g dw⁻¹ (indicated by the arrow), the temperature dependence of both ageing rate and

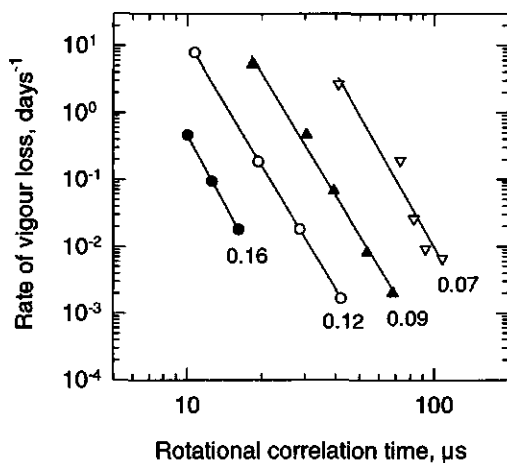


Figure 8.2. Relationship between ageing rate (expressed as rate of vigour loss, day⁻¹) of pea seeds and τ_r of pea axes. Ageing rates were determined at different temperatures (15, 25, 35, 45 and 65°C) and water contents (indicated values in g/g [g H₂O g dw⁻¹]). Data were plotted on a double logarithmic scale ($\log y = a + b \log x$). Water contents, constants and correlation coefficients of the linear regressions are: filled circles, 0.16 g/g ($a = 6.53$, $b = -6.86$, $r^2 = 0.9995$); open circles, 0.12 g/g ($a = 7.21$, $b = -6.15$, $r^2 = 0.99993$); filled triangles, 0.09 g/g ($a = 8.53$, $b = -6.10$, $r^2 = 0.991$); inverted open triangles, 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., 1994b).

rotational motion deviated from Arrhenius behaviour, as indicated by the deviation of a linear regression (Fig. 8.1A, dotted line). When the tissues were more hydrated (0.12 g H₂O g dw⁻¹), all data points for the ageing rates were above T_g (arrow, Fig. 8.1B), and parallel to the rotational motion. The rotational motion deviated from Arrhenius behavior around T_g. The observation that both parameters changed parallel to one another prompted us to plot the ageing rates obtained at different water contents and temperatures as a function of the τ_r .

Ageing rates of pea seeds stored at five different temperatures (15, 25, 35, 45 and 65°C) and four water contents (0.07, 0.09, 0.12 and 0.16 g H₂O g dw⁻¹) were plotted versus the rotational motion of CP in pea axes, determined under similar conditions of water content and temperature (Fig. 8.2). For each water content, a linear relationship was observed between the logarithm of the ageing rate and the logarithm of τ_r of CP, determined at the different temperatures (Fig. 8.2).

In addition to the established relationship between rotational motion and ageing rates for pea seeds, we also investigated this relationship in pollen. In this case, half-viability times (P₅₀, time until 50% germination is reached) were used as an estimate of viability loss. The advantage of studying the storage behaviour of pollen is that true reductions in loss of viability can be obtained within a realistic time scale over a wide temperature range. Analogous linear relationships were found when the logarithm of the half-viability times of cattail

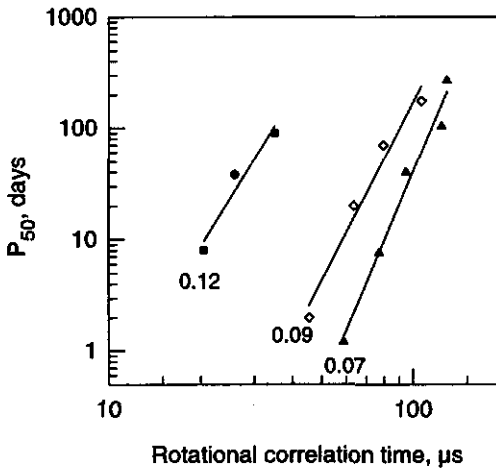


Figure 8.3. Relationship between half-viability time (P_{50}) and τ_r for cattail pollen. Half-viability times were determined under conditions of different temperatures (5, 15, 25, 35 and 45°C) and various water contents (indicated values in g/g [g H₂O g dw⁻¹]). Data were plotted on a double logarithmic scale ($\log y = a + b \log x$). Water contents, constants and correlation coefficients of the linear regressions are: circles, 0.12 g/g ($a = -4.80$, $b = 4.41$, $r^2 = 0.943$); diamonds, 0.09 g/g ($a = -8.39$, $b = 5.31$, $r^2 = 0.971$); triangles, 0.07 g/g ($a = -11.32$, $b = 6.47$, $r^2 = 0.980$). Half-viability times were obtained by interpolation of previously determined data (Buitink et al., 1998b).

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pollen was plotted against the logarithm of τ_r at three different water contents (Fig. 8.3). An increased rotational motion corresponded to decreased half-visibility times (Fig. 8.3). Also, linear regressions were obtained (data not shown) when rotational motion was plotted versus the ageing rates of the pollen (derived from Buitink et al., 1998b).

Water content-dependent changes in ageing rates are related to rotational motion

Apart from changing ageing rates by varying temperature at constant water content, one can also change ageing rates by varying water content at a constant temperature. Thus, the relationship between moisture-dependent changes in ageing rate and rotational motion was determined for pea seeds aged at 65°C (Ellis et al., 1989), impatiens seeds aged at 25°C, and cattail pollen aged at 35°C (Buitink et al., 1998b). For all species, the ageing rates represented the loss of germination capacity plotted on a probability scale over time (Ellis et al., 1989; Buitink et al., 1998b). A significant linear relationship was obtained between the logarithm of the ageing rate and τ_r for these species when the water content was varied from 0.04 to 0.16 g H₂O g dw⁻¹ (Fig. 8.4).

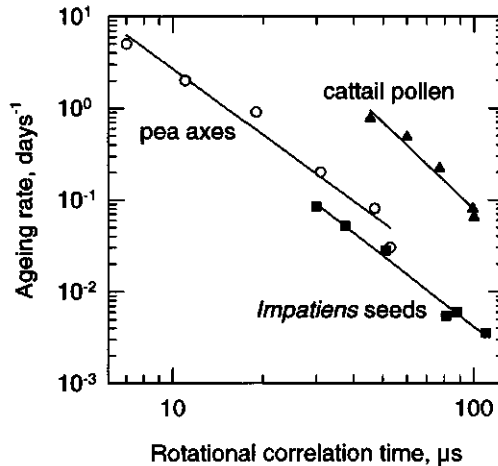


Figure 8.4. Relationship between ageing rate and τ_r for impatiens seeds (squares), pea seeds (circles) and cattail pollen (triangles). Ageing rates were determined at different water contents and one temperature (pea seeds, 65°C; cattail pollen, 35°C; Impatiens seeds, 25°C). Ageing rates from cattail pollen were taken from Buitink et al. (1998b), and those of pea seeds from Ellis et al. (1989). Ageing rates for all species represent the absolute value of the slope of the linear regression fitted to percentage germination plotted on a probability scale over storage time. Data were plotted on a double logarithmic scale ($\log y = a + b \log x$). Equation constants and correlation coefficients of the linear regressions are: pea, $a = 2.82$, $b = -2.40$, $r^2 = 0.972$; cattail, $a = 5.16$, $b = -3.13$, $r^2 = 0.965$; impatiens, $a = 2.73$, $b = -2.56$, $r^2 = 0.987$.

Estimated longevity from viability equation correlates with rotational motion

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To test the general validity of the linear relationship between ageing rate and rotational motion (Figs. 8.2-4), we estimated the longevity using the viability equation from Ellis and Roberts (Ellis and Roberts, 1980a) that has been demonstrated to adequately describe longevity of seeds and pollen (Ellis and Roberts, 1980a,b; Kraak and Vos, 1987; Ellis et al., 1989; Dickie et al., 1990; Mwashia et al., 1997; Usberti and Gomes, 1998; Hong et al., 1999). 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 which are specific for each species; t is temperature; and m is water content. The viability constants for most species with water contents below 17% (fresh weight basis) that have been published so far, have been experimentally determined from storage data between 40 and 90°C. In this study, the viability constants for pea seeds were obtained from (Ellis et al., 1989) and used to calculate the σ for a range of temperatures (t) at four different water contents (m) at which the respective τ_R values of pea axes were also obtained (0.16, 0.12, 0.09, and 0.07 g H₂O g dw⁻¹). The 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,

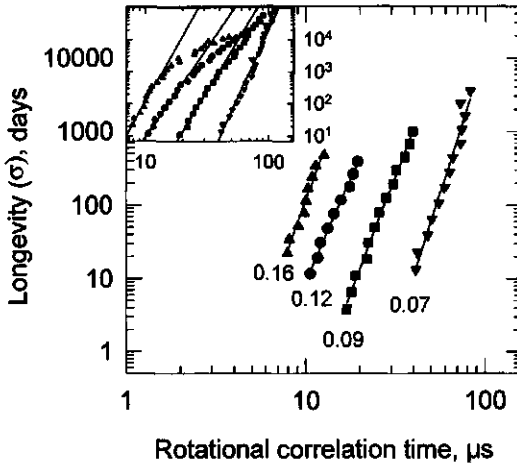


Figure 8.5. Relationship between the longevity (σ) of pea seeds and τ_R of pea axes. The σ was calculated for every 2.5°C at different water contents using the viability equation with experimental constants obtained from (Ellis et al., 1989). Data were plotted on a double logarithmic scale ($\log y = a + b \log x$). Water contents and temperature range for which σ was determined, together with constants and correlation coefficients are respectively: triangles, 0.16 g/g, 25°C to 45°C ($a = -4.73$, $b = 6.79$, $r^2 = 0.964$); circles, 0.12 g/g, 35°C to 55°C ($a = -4.58$, $b = 5.546$, $r^2 = 0.992$); squares, 0.09 g/g, 35°C to 65°C ($a = -7.14$, $b = 6.355$, $r^2 = 0.994$); inverted triangles, 0.07 g/g, 35°C to 65°C ($a = -11.2$, $b = 7.64$; $r^2 = 0.966$).

8

because τ_r in pea axes at these high water contents could not be determined at higher temperatures because of partitioning of the spin probe into the lipid phase (Buitink et al., 1998a, 1999). The relationship between the logarithms of σ and rotational motion of CP in the cytoplasm of pea axes was linear (Fig. 8.5). This indicates that the estimated viability data for pea seeds, derived from the viability equation, show a similar linear relationship with rotational motion as did the actual data on vigour loss of pea (Fig. 8.5 and 8.2).

When the σ values were calculated for temperatures below 25°C, the relationship between σ and τ_r deviated from a straight line (Fig. 8.5 inset). It is noteworthy that the range of temperatures for which the viability equation has been proven to be valid is the range for which σ was found to linearly correlate with rotational motion (Fig. 8.5).

Predictions of lifespan using cytoplasmic molecular motion

Predictions of lifespan are necessary to adequately preserve genetic resources. The established linear relationship between the logarithms of rotational motion and ageing rate enabled us to extrapolate ageing rates or longevity (σ) to sub-zero temperatures by simply measuring the τ_r values, assuming that the relationship between ageing rate and rotational motion remains linear. In this way, we estimated the loss of vigour (Fig 8.6A; extrapolated from the linear regressions in Fig. 8.2) or the longevity (σ) (Fig 8.6B; extrapolated from the linear regressions in Fig. 8.5) of pea seeds stored at different water contents at 35, 20, 0, and -18°C. The longevity at these temperatures was also calculated using the viability equation (Fig. 8.6C).

The loss of vigour (1/ageing rate; expressed as hypocotyl length day⁻¹) was estimated by extrapolating the linear regressions in Fig. 8.2 for the different water contents (Fig. 8.6A). At 35°C, the time to vigour loss increased with decreasing water content (Fig. 8.6A). At 20°C, a lowering of the water content from 0.16 to 0.09 g H₂O g dw⁻¹ increased the time to vigour loss. A further decrease of the water content resulted in a subsequent decrease in time to vigour loss. At 0°C, optimum storage behaviour was found at 0.12 g H₂O g dw⁻¹. Estimation of the rate of vigour loss at -18°C showed an optimum storage behaviour of 1,660 years at 0.12 g H₂O g dw⁻¹. Storage of seeds at -18°C and 0.16 g H₂O g dw⁻¹ reduced the time to loss of vigour to 1,224 years. However, decreasing the water content to 0.09 or 0.07 g H₂O g dw⁻¹ also decreased the time to vigour loss to 74 and 7 years, respectively.

Similarly, when the longevity data (σ , calculated from the viability equation) were estimated at the same temperatures (Fig. 8.6B) by extrapolation of the linear regressions in Fig. 8.5, the effect of water content on longevity was analogous to that on vigour loss (compare Fig. 8.6A with B). To validate the predictions, and to demonstrate that the predictions of vigour loss are comparable to loss of percentage germination, we plotted the linear regression of Fig. 8.2 against the linear regression of Fig. 8.5 for each water content. For all water contents, the regression coefficients of the relation between longevity



and rate of vigour loss were close to unity: -0.97 for 0.07 g H₂O g dw⁻¹, -1.00 for 0.09 g H₂O g dw⁻¹, -1.10 for 0.12 g H₂O g dw⁻¹, and -1.00 for 0.16 g H₂O g dw⁻¹. At 20°C, the optimum longevity was found around 0.09 g H₂O g dw⁻¹, while at 0°C, it was estimated to occur around 0.12 g H₂O g dw⁻¹. At these optimum water contents, longevity was estimated to be 44 and 2,466 years when the tissues would be stored at 20 and 0°C, respectively. At -18°C,

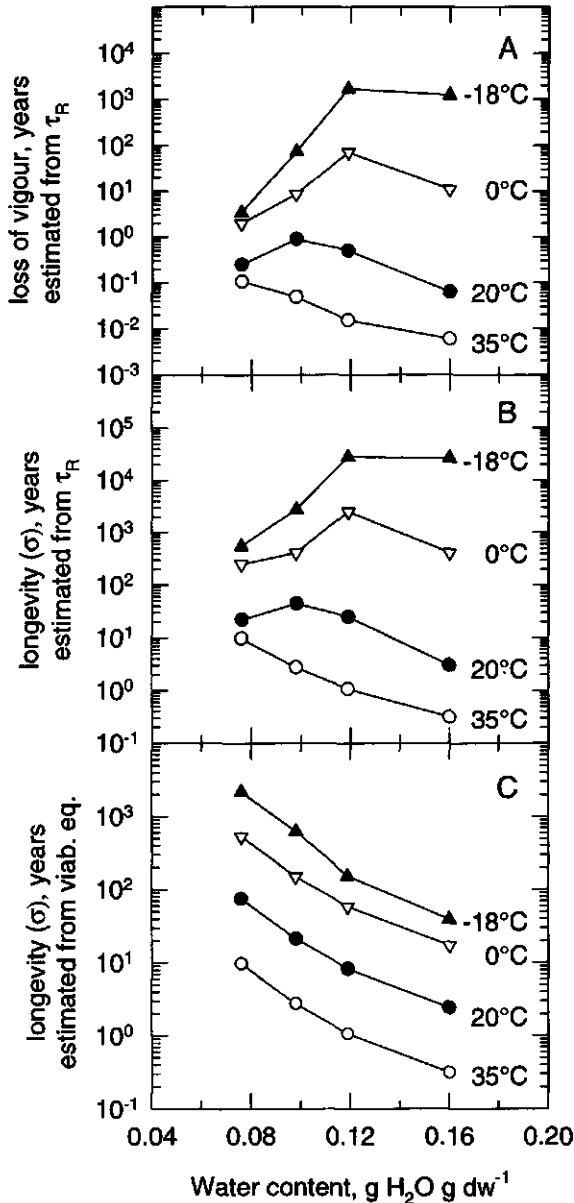


Figure 8.6. Relationship between rate of vigour loss or longevity of pea seeds and water content, determined for different temperatures. (A) Estimation of the loss of vigour, extrapolated from Fig. 8.2. (B) Estimation of the longevity (σ), extrapolated from the linear regressions in Fig. 8.5. (C) Estimation of the longevity (σ) calculated using the viability equation (Ellis et al., 1989).

8

estimated longevity was found to decrease when seeds were stored below a water content of $0.12 \text{ g H}_2\text{O g dw}^{-1}$ (Fig. 8.6B). In contrast, the calculations of longevity estimated from the viability equation showed a different effect of water content on longevity. At all temperatures, the calculated longevity increased with decreasing water contents (Fig. 8.6C). Unfortunately, no longevity predictions were possible for cattail pollen at sub-zero temperatures because of sensitivity limits of ST-EPR measurements in these samples.

Discussion

Our data show that the rotational mobility of the spin probe in the cytoplasm can adequately describe the ageing rates of germplasm over a wide range of temperatures and water contents. Therefore, it seems plausible that the molecular mobility is influencing the vigour and lifespan of germplasm. Many studies on ageing and glasses have suggested that molecular mobility will influence ageing rates of seed and pollen (Vertucci and Roos, 1990; Leopold et al., 1994; Sun and Leopold, 1993, 1995, 1997, Buitink et al., 1998a,b), or given evidence that certain detrimental reactions change in kinetics around T_g (Sun and Leopold, 1995, 1997). However, to our knowledge, this is the first experimental evidence that cytoplasmic molecular mobility in germplasm, measured by ST-EPR, is directly associated with ageing rate. Despite the often-reported damage on membranes that seems to be associated with the ageing of seeds and pollen (Priestley, 1986; Wilson and McDonald, 1986; Van Bilsen and Hoekstra, 1993), the determining factor in ageing appears to be associated with the molecular mobility in the cytoplasmic surroundings. Alternatively, the viscous cytoplasm might be responsible for restricting motion of membrane components, thereby slowing down deleterious reactions taking place in membranes (Lee et al., 1986).

It is known that the formation of the glassy state changes the activation energy of reactions (Karmas et al., 1992; Roos, 1995; Sun et al., 1996). It has been shown that formation of intracellular glasses in pollen also results in a change of the activation energy of ageing reactions (Buitink et al., 1998b). Similarly, the activation energy of the rotational motion in seeds was found to change around T_g (Buitink et al., 1999). Fig. 8.1 shows that there is a change in the kinetics of both ageing rate and molecular mobility in dependence of temperature, apparent from the deviation in Arrhenius behaviour. Nonetheless, we found a linear relationship when both parameters are plotted against each other on a logarithmic scale. Therefore, using the molecular mobility of molecules in the cytoplasm to describe ageing rates takes into account the formation of the glassy state and the concomitant change in kinetics.

The observation that the relationship between the logarithms of rotational motion and longevity of germplasm is linear enabled extrapolations to other storage conditions. Predictions of lifespan are especially necessary at low temperatures, since under these storage conditions, longevities can not practically be determined. It is these low temperatures that are chosen for seed storage protocols, because ultra-dry storage can not achieve the necessary

longevity needed by genebank conservers (Ellis and Roberts, 1998). The estimated longevity for pea seeds at 20°C ranged between 3 and 50 years, depending on the water content, while the estimated longevity at 0°C ranged from 250 to 2,466 years (Fig. 8.6B). It is not surprising to find these values for the predictions, because there are several reports showing that seeds can survive for over a hundred years (Kivilaan and Bandurski, 1981; Steiner and Ruckebauer, 1995) at ambient temperatures. The most remarkable discovery was that on ancient seeds of Sacred Lotus from China, where radiocarbon dating showed an age of the seed of $1,288 \pm 271$ years while it was still capable of germinating (Shen-Miller et al., 1995).

Predictions of longevity at 20, 0, and -18°C on the basis of molecular mobility in the cytoplasm showed that drying below an optimum water content resulted in decreased vigour and longevity (Fig. 8.6A and B). In general, viability constants for most seed species containing less than 17% water on a fresh weight basis have been determined using experimental storage temperatures between 40 and 90°C. In the case of pea seeds, the viability constants were determined at 65°C (Ellis et al., 1989). Therefore, it can be assumed that the calculations of the estimated longevity between 35 and 65°C for pea seeds as shown in Fig. 8.5 (filled symbols) are most likely accurate. The finding that the regression coefficients of the relation between the linear regressions of vigour loss (Fig. 8.2) and longevity (Fig. 8.5) were close to unity, and the observation that measurements of vigour loss and longevity, based on germination percentage, gave comparable viability predictions (Fig. 8.6A and B), suggests that both types of measurements can be applied to obtain estimates of viability under different storage conditions.

The prediction that longevity decreases when seeds are dried below the optimum water content is in marked contrast with the concept that seeds with a low water content survive longer than seeds with an elevated water content (Ellis and Roberts, 1980a,b; Priestley, 1986). 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, 1993b; Vertucci et al., 1994b; Buitink et al., 1998b). Currently, seed storage protocols recommend storage at 5% moisture and -18°C (Ellis and Roberts, 1998). Fig. 8.6 suggests that longevity at -18°C could be increased considerably by storing seeds at a water content higher than 7%. The consequences for germplasm preservation, therefore, are that when sub-zero temperatures for storage are considered, germplasm should preferably not be dried to too low water contents.

The observation that ageing rates change by an order of four whereas the τ_r changes less than one order of magnitude over the same temperature range (Fig. 8.2) raises the question of how rotational motion is linked with ageing rates. The rotational motion of a spin probe and ageing reactions are two processes occurring at a different molecular level. The rotational correlation time of the spin probe in its environment is determined by the size of the probe (Deppe et al., 1996). It was found that the temperature dependence of rotation increased with increasing size of the probe, which

8

could be interpreted as a qualitative indication that larger probes are more coupled to the macroscopic viscosity of the matrix than smaller ones (Deppe et al., 1996). Additionally, the size of the molecules responsible for detrimental reactions will determine the difference in temperature dependence between rotational motion and ageing rate. Nonetheless, rotational motion of CP was found to correlate with ageing, indicating that rotational motion can be used to describe reaction rates leading to loss of viability.

Section IV

Composition and Properties of Intracellular Glasses

Overview

For many years, it has been hypothesised that sugars play an important role in the formation of intracellular glasses in dry desiccation-tolerant organisms. In this section, the role of sugars in the formation of intracellular glasses is investigated.

Comparison of model systems of sugar glasses with intracellular glasses revealed that the temperature dependence of molecular mobility was completely different in the two systems. This, together with the finding that changes in sugar composition in vivo did not change the glass transition temperature (T_g) or cytoplasmic molecular mobility in the cells, suggests that sugars do not play an important role in intracellular glass formation.

Different techniques were applied for the investigation of the physical characteristics of glasses. Both $^1\text{H-NMR}$ as well as STEPR spectroscopy revealed that, apart from a change in the kinetics of molecular mobility around T_g , a second change in kinetics occurs at a temperature above T_g . This critical temperature T_c was interpreted as the crossover temperature, where the dynamics changes from solid-like to liquid-like. Above this temperature, the dynamics of the viscous matrix will be dominated by the diffusional motion. The temperature interval between T_g and T_c increased with increasing molecular weight of the sugars. The interval between T_g and T_c in biological tissues was over 50°C , implying that the stability remained high even at temperatures far above T_g . A comparably high T_c-T_g interval was found for the molecular mobility of poly-L-lysine, suggesting that proteins rather than sugars play an important role in the intracellular glass formation. The exceptionally high T_c of intracellular glasses is expected to provide excellent long-term stability to dry organisms, maintaining a slow molecular motion in the cytoplasm even at temperatures far above the glass transition temperature.

9

A critical assessment of the role of oligosaccharides in intracellular glass stability

Julia Buitink, Folkert A. Hoekstra, Marcus A. Hemminga

The role of oligosaccharides in enhancing the stability of intracellular glasses was investigated. Priming *Pisum sativum* seeds for 6 days in a polyethylene glycol solution of -1.0 MPa at 20°C resulted in a change in the sugar composition. In pea embryonic axes, total oligosaccharide content decreased from 168 to 27 mg g⁻¹ dry weight (DW), while sucrose content increased from 41 to 62 mg g⁻¹ DW. Despite the change in oligosaccharide:sucrose ratio, no difference in the glass transition temperature was measured in dry pea embryonic axes before and after priming as determined by DSC. Saturation transfer electron spin resonance spectroscopy was used to measure the rotational mobility of a polar spin probe, 3-carboxy-proxyl, which was inserted into the cytoplasm of axes. No difference was found between the rotational mobility in dry untreated axes and that of dry primed axes. There is no significant contribution of oligosaccharides to intracellular glass stability in terms of increasing the glass transition temperature or decreasing the mobility of molecules in the intracellular glass.

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9

Oligosaccharides often occur in considerable quantities in dry seeds of many plant species (Amuti and Pollard, 1977). The presence of these sugars appears to correlate with the longevity of seeds (Horbowicz and Obendorf, 1994; Bernal-Lugo and Leopold, 1995; Steadman et al., 1996). There are several hypotheses regarding the function of oligosaccharides in seeds. They may play a role in the protection of membranes and proteins, in a similar way as described for disaccharides (Crowe et al., 1992), or prevent crystallisation of sucrose (Caffrey et al., 1988). Moreover, they are thought to be involved in the formation of stable glasses by increasing the glass transition temperature (T_g) and thereby increasing the viscosity of the glassy cytoplasm (Leopold et al., 1994; Bernal-Lugo and Leopold, 1995). The underlying concept for the role of glasses in storage stability is that the high viscosity of intracellular glasses will slow down ageing reactions (Leopold et al., 1994; Sun, 1997b; Buitink et al., 1998a,b). Indeed, using saturation transfer electron paramagnetic resonance spectroscopy (ST-EPR), a relation between mobility of molecules in the glassy cytoplasm and longevity was found for *Typha latifolia* pollen and pea seeds (Buitink et al., 1998a).

Seed priming, *i.e.* the pre-imbibition of seeds in osmotic solution, is known to considerably improve seed quality by enhancing rates of germination and seedling uniformity. However, a drawback of this treatment is the often reduced longevity of primed seeds (Tarquis and Bradford, 1992; Saracco et al., 1995). The causes for this reduced longevity are not well understood. Hoekstra et al. (1994) found that priming of cauliflower seeds resulted in a decrease in oligosaccharide content. Considering the proposed role of oligosaccharides in increasing glass stability, it is possible that the reduced longevity of primed seeds may be attributed to the decrease in oligosaccharide content resulting in a decrease in cytoplasmic viscosity.

In this study, we investigated whether changes in sugar composition after priming lead to changes in the properties of intracellular glasses, such as a decrease in T_g (DSC) and an increase in the mobility of molecules in the cytoplasm (ST-EPR).

Material and methods

Seeds of pea (*Pisum sativum* cv. Karina) were primed for 6 days in a polyethylene glycol solution of -1.0 MPa at 20°C. After priming, the seeds were rinsed with demineralised water and dried at 3% RH for 2 days at room temperature. Subsequently, seeds were used for sugar content determination, differential scanning calorimetry (DSC) measurements and ST-EPR experiments.

Soluble sugars of isolated axes were extracted in a solution of 80% methanol (v:v) containing lactose as internal standard according to Hoekstra et al. (1994). Sugar concentrations were determined by HPLC using pulsed amperometric detection on a Carpac PA-1 column (Dionex Corp., Sunnyvale, CA, USA). Data are averages of duplicated extractions.

Glass transition temperatures (T_g) of pea embryonic axes were determined using a Perkin-Elmer (Norwalk, CT) Pyris-1 DSC. The T_g values were determined as the onset of

the temperature range over which the change in specific heat occurred in heating scans recorded at a scanning rate of $10^{\circ}\text{C min}^{-1}$. Different sample water contents were achieved by equilibrating the tissues over different saturated salt solutions for at least 7 days.

Labelling of isolated axes with the spin probe 3-carboxy-proxyl (CP) was performed according to Buitink et al. (1998a). After labelling, axes were dried in an airflow of 3% RH for 24 hours, then stored over saturated salt solutions for 6 days to obtain various water contents. For each ST-EPR measurement, 20 mg of tissue was sealed in a glass capillary. Spectra were recorded on a Bruker X-band EPR spectrometer (Bruker Analytik, Rheinstetten, Germany, model 300E). Instrument settings were according to Buitink et al. (1998a). After the measurements, tissues were removed from the capillary and the water contents were determined. Water contents were determined gravimetrically by weighing the samples before and after heating at 96°C for 36-48 h.

Results and Discussion

The longevity of seeds often has been related to their oligosaccharide contents (Horbowicz and Obendorf, 1994; Bernal-Lugo and Leopold, 1995). To establish whether priming of pea seeds results in changes in sugar composition, sucrose and oligosaccharide contents were determined in axes before and after priming (Table 9.1). Priming for 6 days at -1.0 MPa resulted in an increase in sucrose and a decrease in oligosaccharide content. The ratio between the oligosaccharide and the sucrose content in pea embryonic axes decreased from 4.2 in the untreated axes to 0.4 in the primed axes. Similar changes in soluble sugar contents after priming were found previously in cauliflower seeds (Hoekstra et al., 1994).

Table 9.1. Soluble sugar contents in untreated and redried primed pea embryonic axes. Seeds were incubated for 6 days in a solution of polyethylene glycol (-1.0 MPa) at 20°C then dried. Data are expressed as mg sugar g dw^{-1} and average of duplicate extractions.

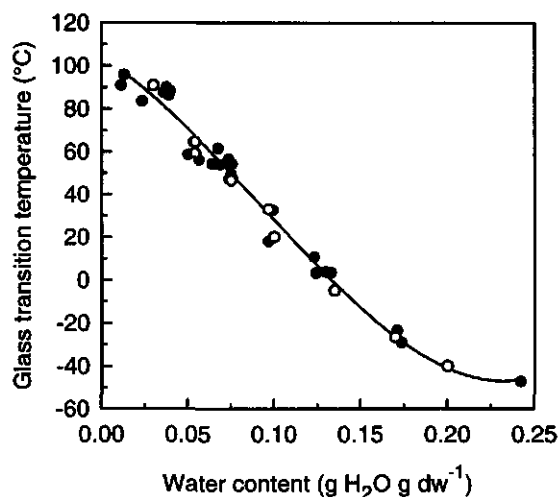
Treatment	Sucrose	Raffinose	Stachyose	Verbascose
Untreated	40.5	14.5	73.6	80.0
Primed	62.5	15.1	6.5	5.7

In model systems made of sugar mixtures, changes in the composition result in changes in glass properties (Levine and Slade, 1988). It has been shown that the T_g is dependent on the molecular weight of the components forming the glass. For example, the T_g of dry sucrose glasses is approximately 63°C , whereas the T_g of dry raffinose glasses is around 100°C (Levine and Slade, 1988; Wolkers et al., 1998b). In untreated and redried primed pea embryonic axes, the T_g was measured as a function of water content using DSC (Fig. 9.1). No difference could be found between the T_g values of untreated and primed axes, despite the differences in sugar composition (Table 9.1).

9

Furthermore, no change was observed in the apparent heat capacity that is associated with the glass transition (data not shown).

Figure 9.1. State diagram of untreated (●) or primed pea (○) axes. Priming was achieved by incubating seeds for 6 days at -1.0 MPa at 20°C. The T_g values were determined as the onset of the temperature range over which the change in specific heat occurred in heating scans recorded at a scanning rate of 10°C min⁻¹.



The viscosity of a raffinose glass is higher than that of a sucrose glass, under the same conditions of water content and temperature (Levine and Slade, 1988). Although no difference was found in T_g between untreated and primed pea embryonic axes, the viscosity or molecular mobility in the cytoplasm may still be different. Using ST-EPR, changes in molecular mobility can be estimated by determining the rotational correlation time (τ_R) of a nitroxide spin probe that is inserted into the cytoplasm of seed tissues (Buitink et al., 1998a). The τ_R corresponds to the lifetime of the probe in a given orientation. ST-EPR spectroscopy has been applied previously to determine rotational motions of nitroxide spin probes in glassy sugar systems (Hemminga and Van den Dries, 1998). The τ_R of a polar spin probe (CP) in untreated or primed pea embryonic axes was determined at 30°C at two water contents (Table 9.2).

Table 9.2. Rotational correlation time (τ_R) of the spin probe 3-carboxy-proxyl in the cytoplasm of untreated and primed pea embryonic axes at different water contents at 30°C. The water contents were chosen so that τ_R is determined within and out of the glassy state (see Fig. 9.1). Priming was performed as explained in Table 9.1. Data (\pm S.D.) are average of 3 experiments. Water contents are expressed as g H₂O g⁻¹ DW and τ_R in μ s.

Treatment	Water content	Rotational correlation time
Untreated	0.07 \pm 0.00	88 \pm 1.8
	0.12 \pm 0.01	24 \pm 2.1
Primed	0.07 \pm 0.01	91 \pm 3.0
	0.12 \pm 0.01	22 \pm 2.9

These water contents represent conditions under which the cytoplasm is in or out of the glassy state (Table 9.2, compare with Fig. 9.1). At both water contents, no significant difference was measured in τ_R between untreated and primed seeds, implying that the priming and redrying treatment does not affect the molecular mobility in the cytoplasm.

The above observations do not support the hypothesis that oligosaccharides stabilise the intracellular glass by decreasing the molecular mobility. Apparently, other molecules besides soluble sugars play an important role in intracellular glass formation. Another role proposed for oligosaccharides in seeds is prevention of crystallisation of sucrose *in vivo* (Caffrey et al., 1988). However, attempts to detect crystallisation *in vivo* within the cytoplasm have failed (Sun and Leopold, 1993). Most likely, the complex mixture of cytoplasmic solutes will prevent crystallisation of sucrose, regardless of the presence of oligosaccharides. Furthermore, oligosaccharides might contribute to the protection of membranes according to the water replacement hypothesis (Crowe et al., 1992). However, this hypothesis was put forward to explain the role of soluble sugars in desiccation tolerance. Yet, after priming, pea seeds are still desiccation tolerant. Further investigations will reveal whether the membrane structure and stability is affected by priming. However, it might be that there is no specific role for oligosaccharides in longevity. Oligosaccharides could simply be an indicator of seed maturity and could serve as a storage reserve (Kuo et al., 1988; Hoekstra et al., 1994).

Conclusions

We investigated the role of oligosaccharides in the stability of intracellular glasses using primed pea seeds as a model system. During priming, the oligosaccharide content decreased, but this change did not affect the intracellular glass properties in terms of glass transition temperature and mobility of a spin probe in the cytoplasm. A role for oligosaccharides in longevity, if any, does not appear to be mediated by changing the characteristics of the intracellular glass.

10

Is there a role for oligosaccharides in seed longevity? An assessment of intracellular glass stability

Julia Buitink, Marcus A. Hemminga, Folkert A. Hoekstra

We examined whether oligosaccharides extend seed longevity by increasing the intracellular glass stability. For that purpose we used a spin probe technique to measure the molecular mobility and the glass transition temperature of the cytoplasm of impatiens (*Impatiens walleriana*) and bell pepper (*Capsicum annuum*) seeds that were osmo-primed to change oligosaccharide contents and longevity. Using saturation transfer electron paramagnetic resonance spectroscopy, we found that the rotational correlation time of the polar spin probe 3-carboxy-proxyl in the cytoplasm decreased together with longevity as a function of increasing seed water content, suggesting that longevity may indeed be regulated by cytoplasmic mobility. Osmo-priming of the seeds resulted in considerable decreases in longevity and oligosaccharide contents, while sucrose contents increased. No difference in the glass transition temperature was found between control and primed impatiens seeds at the same temperature and water contents. Similarly, there was no difference in rotational motion of the spin probe in the cytoplasm between control and primed impatiens seeds and bell pepper seeds. We therefore conclude that oligosaccharides in seeds do not seem to affect the stability of the intracellular glassy state and that the reduced longevity after priming is not the result of increased molecular mobility in the cytoplasm.

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Since the glassy state has been detected in dry biological tissues, it has been put forward as a prominent factor in the control of deterioration rates during storage (Burke, 1986; Williams and Leopold, 1989; Leopold et al., 1994; Leprince and Walters-Vertucci, 1995; Buitink et al., 1998b). A glass is a thermodynamically unstable solid state with an extremely high viscosity (Franks et al., 1991), and its formation is promoted by low tissue water contents and low temperatures. The presence of glasses has been associated with improved storage stability (Sun and Leopold, 1993; Sun, 1997b; Buitink et al., 1998b). It is assumed that the high viscosity of intracellular glasses decreases molecular mobility and impedes diffusion, thus slowing down degradative processes during ageing (Sun and Leopold, 1993; Sun, 1997b). A relationship between longevity and the mobility of molecules in the glassy cytoplasm has been found in *Typha latifolia* pollen and pea seeds (Buitink et al., 1998a).

Tri- and tetra-saccharides such as raffinose and stachyose often occur in considerable quantities in dry seeds of many plant species (Amuti and Pollard, 1977). The presence and amounts of these oligosaccharides have been found to correlate with longevity (Horbowicz and Obendorf, 1994; Lin and Huang, 1994; Bernal-Lugo and Leopold, 1995; Steadman et al., 1996). Oligosaccharides are thought to contribute to the stabilisation of intracellular glasses by increasing the glass-to-liquid transition (T_g) and viscosity (Leopold et al., 1994; Bernal-Lugo and Leopold, 1995; Sun, 1997b). Indeed, the addition of oligosaccharides to sucrose glasses in a model system will increase the T_g considerably (Levine and Slade, 1988; Koster, 1991; Wolkers et al., 1998b). In this study, the suggested role of oligosaccharides in seed storage via increased glass stability will be examined.

The translational and rotational motion of molecules has been studied extensively in many glass-forming substances (Soesanto and Williams, 1981; Blackburn et al., 1996; Deppe et al., 1996; Champion et al., 1997; Hemminga and Van den Dries, 1998; Van den Dries et al., 1998a). ST-EPR spectroscopy is a suitable technique with which to study the rotational motion of spin probes incorporated into glasses (Hemminga and Van den Dries, 1998). Using this technique, the rotational correlation time (τ_r), which roughly corresponds to the lifetime of the probe in a given orientation, has been studied previously in sugar glasses (see Hemminga and Van den Dries, 1998, and references therein), organic liquids at low temperatures (Ito, 1983), and biological systems such as seeds and pollen (Buitink et al., 1998a, 1999).

Seed priming, (the pre-imbibition of seeds in osmotic solution), is known to considerably improve seed quality by enhancing germination rates and seedling uniformity (Heydecker et al., 1973; Bradford, 1986). However, a drawback of such a treatment is the reduced longevity of the primed seeds (Tarquis and Bradford, 1992; Saracco et al., 1995), the cause of which is unclear. Nonetheless, one of the processes known to occur during priming is a decrease in oligosaccharide content, as demonstrated for cauliflower seeds (Hoekstra et al., 1994). This decrease could be responsible for the reduced longevity of the primed seed by decreasing the T_g and increasing the molecular mobility within the intracellular glass. In this study, we investigated whether this

reduced longevity in primed seeds is due to an increased molecular mobility in the cytoplasm, allowing faster ageing rates. In particular, emphasis is placed on the role of oligosaccharides in relation to glass formation and molecular mobility in seeds.

Materials and methods

Storage and germination assays

Seeds of impatiens (*Impatiens walleriana* L. cv Impulse Lila) and bell pepper (*Capsicum annum* L. cv Atol) were a gift from Novartis (Enkhuizen, The Netherlands) and Enza Zaden (Enkhuizen, The Netherlands) respectively. The initial viability of the seeds was 98.3% and 98.4%, respectively. Bell pepper and impatiens seeds were imbibed for up to 8 days in polyethylene glycol 8000 at a water potential of -1.0 MPa at 20°C (Michel and Kaufmann, 1973). After priming, the seeds were rinsed and dried in a flow of dry air (3% RH) for two days at room temperature. Subsequently, the seeds were kept over saturated salt solutions of various relative humidities (RHs) at 25°C or 30°C for storage experiments, or used for EPR experiments and determination of sugar contents. At intervals during storage, approximately 100 seeds were imbibed at 20°C to determine final percentage germination. The half-viability time (P_{50}) was determined as the time over which percentage germination decreased to 50%. Water contents were analysed by weighing the samples before and after heating at 96°C for 36 to 48 h.

Sugar determination

Axes and cotyledons were isolated from dry, primed impatiens seeds. For each sugar extraction, approximately 50 cotyledons or 100 axes were used. For bell pepper seeds, embryos were isolated from the endosperm directly after priming but before drying. Embryos and endosperm were then dried for two days in a flow of dry air (3% RH), after which sugar extraction was performed on approximately 50 embryos or endosperm from 15 seeds. Seed parts were ground in a mortar in the presence of 3 ml 80% (v/v) methanol containing lactose as the internal sugar standard. The suspension was removed from the mortar with 80% (v/v) methanol and heated in a water bath at 76°C for 15 min. The liquid was evaporated under vacuum (Speedvac AES1010, Savant Instruments). The residue was dissolved in distilled water, and after appropriate dilution, sugars were analysed by HPLC on a Carbowax PA-1 column (Dionex Corp., Sunnyvale, CA, USA) using pulsed amperometric detection, as described by Hoekstra et al. (1994). Data are the average of three extractions.

EPR and ST-EPR spectroscopy

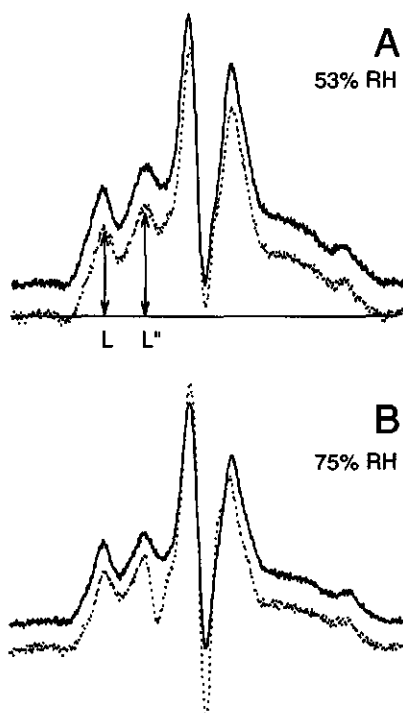
Dry impatiens seeds were allowed to imbibe for 2 h after which the seed coats were removed. Subsequently, seeds were incubated for 60 min in a 10 ml solution of 1 mM 3-carboxy-proxyl (CP) (Sigma, St. Louis). After 45 min, potassium ferricyanide was added to a final concentration of 200 mM, and the seeds were incubated for another 15 min. The potassium ferricyanide was added to broaden the signal of CP outside of the cells to invisibility (Buitink et al., 1998a). Because potassium ferricyanide cannot penetrate intact cells, the signal obtained is exclusively derived from CP in the cytoplasm. Dry, untreated bell pepper seeds were allowed to imbibe for 30 min, and then the axes and endosperm were separated. Labelling with the spin probe was done

as described above for impatiens seeds. After labelling, the seed tissues were dried in dry air (3% RH) for 24 h and subsequently stored over several saturated salt solutions for 7 d to obtain various water contents.

Conventional EPR spectra were recorded at increasing temperature with an X-band EPR spectrometer (Bruker Analytik, Rheinstetten, Germany, model 300E). Instrument settings were according to Buitink et al. (1999). For each EPR measurement, 20 mg tissue was sealed in a 2-mm diameter capillary. After the measurements, tissues were removed from the tube and water contents were determined. For samples that were heated above 50°C during the EPR measurements, similar samples equilibrated to the same RH were taken for water content determination. Water contents were analysed by weighing the samples before and after heating at 96°C for 36 to 48 h. From each spectrum, recorded at 10°C intervals, the distance between the outer extrema ($2A_{zz}$) was determined and plotted against temperature. The temperature dependence of $2A_{zz}$ was used to obtain an estimate of the T_g in our material (Buitink et al., 1998a).

For a quantitative assessment of the molecular mobility, ST-EPR spectroscopy was used to obtain the τ_R (Buitink et al., 1998a, 1999). 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. The phase was set with the self-null method (Thomas et al., 1976). In ST-EPR spectroscopy, τ_R values are obtained in an empirical way using reference material with known viscosity (Hemminga and Van den Dries, 1998). We used the spectra of CP in anhydrous glycerol to construct a calibration curve according to the method of Buitink et al. (1999). From the curve representing the line shape parameter L^*/L of CP in glycerol against τ_R , the τ_R values of CP in the seeds were

Figure 10.1. ST-EPR spectra of CP in untreated (solid line) or 7-d-primed at -1.0 MPa and 20°C (dashed lines) seeds of impatiens, equilibrated at 53% RH (A) or 75% RH (B). Spectra were recorded at 30°C. The parameters L^* and L are indicated.



obtained by interpolation of the calculated L^*/L ratio (see Fig. 10.1 for the parameters L^* and L in ST-EPR spectra). With this approach, the τ_R values are limited to the range from 10^{-7} to 10^3 s (Van den Dries et al., 1998), which is sufficient for the systems studied here. The τ_R values of CP inimpatiens and bell pepper seeds were determined at different temperatures and water contents corresponding to the storage conditions.

Results

Dependence of longevity on cytoplasmic molecular mobility

Before attempting to elucidate the role of oligosaccharides in intracellular glass stability, we investigated the relation between molecular motion of CP in the cytoplasm and longevity. The τ_R was determined from spectra as shown in Fig. 10.1. The ratio of L^*/L was calculated for each spectrum, and the τ_R was obtained from the calibration curve of CP in glycerol. A decrease in the ratio indicates an increase in the rotational motion of the spin probe (Van den Dries et al., 1998a). The spectra in Fig. 10.1A and B show that with increasing RH, the L^*/L ratio decreased, indicating increased rotational motion. The large dip in the center field seen in the spectrum of CP in primed seeds, equilibrated at 75% RH probably originated from partitioning of some CP into the lipid phase. This phenomenon is only seen at high water content and temperature. Similar partitioning of CP into the lipid phase was observed previously in pea embryonic axes at high temperatures (Buitink et al., 1999). The resulting small distortion in the central part of the spectrum did not influence the calculations of the L^*/L ratio from the ST-EPR spectra, allowing the rotational motion to be calculated.

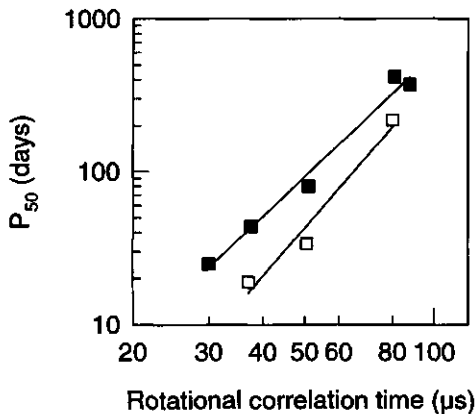


Figure 10.2. Relationship between rotational correlation time and half-viability times (P_{50}) forimpatiens seeds. Symbols represent samples of different water contents at 25°C (closed symbols) or at 30°C (open symbols).

The relationship between the τ_R of the polar spin probe CP in the cytoplasm and the half-viability times (P_{50}) ofimpatiens seeds with different water contents at both 25°C and 30°C is shown in Fig. 10.2. A long τ_R , i.e. reduced rotational motion of the spin probe in the cytoplasm, corresponded to a long half-viability time. There was a linear relationship between the logarithm of rotational motion of the spin probe in the cytoplasm and the logarithm of

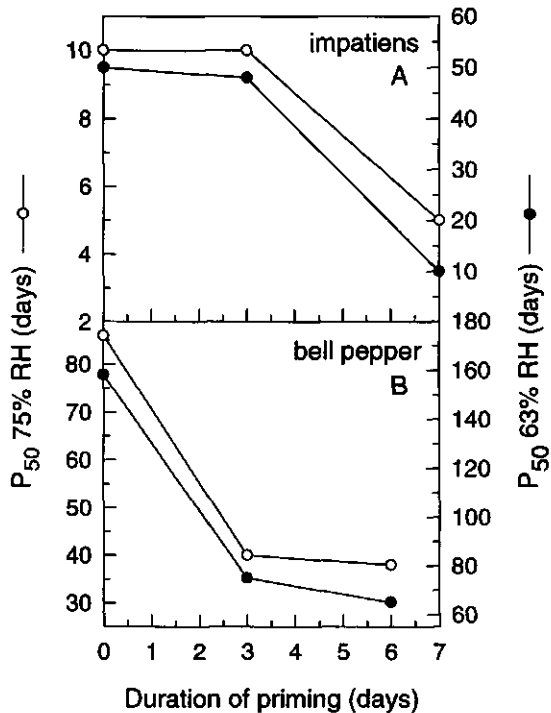
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longevity, indicating that longevity is possibly related with the molecular mobility in the cytoplasm, as suggested previously (Leopold et al., 1994; Sun, 1997b; Buitink et al., 1998a,b, 1999).

Osmo-priming-induced changes in longevity and sugar composition

To assess how longevity of *impatiens* seeds and bell pepper seeds was affected by osmo-priming, seeds were imbibed for different times at -1.0 MPa at 20°C , dried back, and subjected to storage conditions of 63% and 75% RH at 30°C . For *impatiens* seeds, no change in the P_{50} was observed after 3 d of priming compared with control seeds at both storage RHs (Fig. 10.3A). Priming seeds for 7 d decreased the P_{50} from 52 d for control seeds to 10 d at 63% RH, and from 10 to 5 d at 75% RH. The P_{50} of bell pepper seeds decreased after 3 d of priming at both storage RHs (Fig. 10.3B). At 63% RH the P_{50} for untreated bell pepper seeds was 158 d, and the P_{50} decreased to 75 or 65 d for 3- or 6-d primed seeds, respectively. At 75% RH the P_{50} for untreated bell pepper seeds was 86 d, and the P_{50} decreased to 40 or 38 d for 3- or 6-d primed seeds, respectively. The water contents of the untreated seeds compared to primed seeds were similar under the same conditions of storage. Differences in longevity were therefore not due to differences in water content during storage.

Figure 10.3. Relation between half-viability times (P_{50}) at 75% RH (open symbols) and 63% (closed symbols) at 30°C as a function of duration of priming at -1.0 MPa and 20°C . After priming, seeds were dried back and equilibrated over saturated salt solutions. (A) *impatiens* seeds, (B) bell pepper seeds.



To determine whether osmo-priming led to changes in the soluble sugar composition, *impatiens* and bell pepper seeds were primed for various times at -1.0 MPa at 20°C and then dried back, and then the sugar composition was determined. In *impatiens* seeds, sucrose contents increased from $1.1\ \mu\text{g}/\text{mg}$ for untreated seeds to $21\ \mu\text{g}/\text{mg}$ after 7 d of priming, whereas the concentration of an unknown trisaccharide decreased with priming from about $40.9\ \mu\text{g}/\text{mg}$ to $6.3\ \mu\text{g}/\text{mg}$ after 7 d of priming (Fig. 10.4). During priming, the

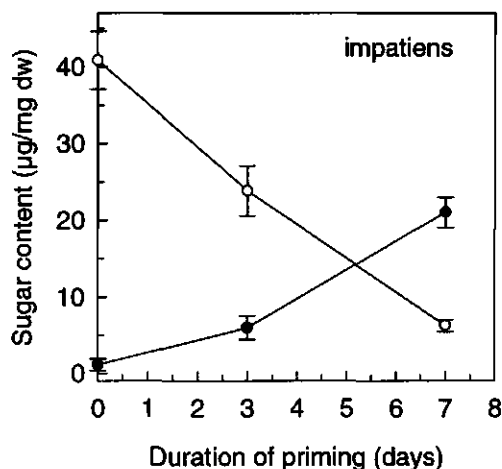
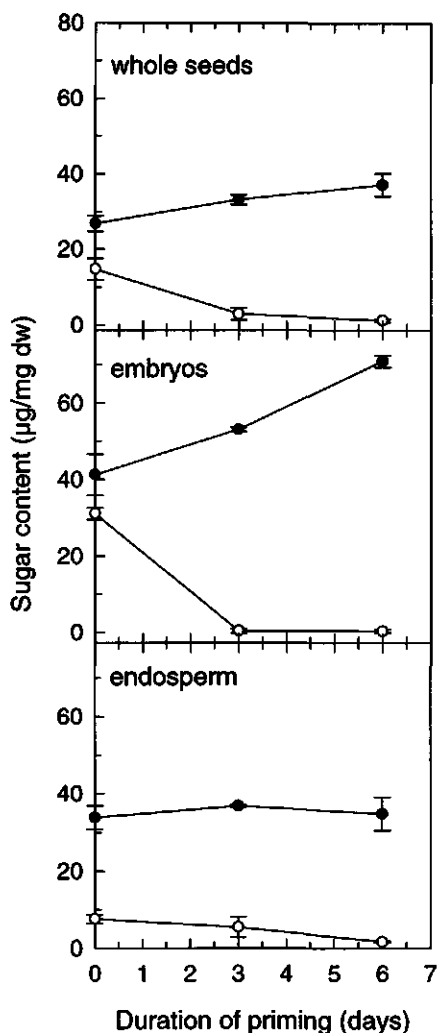


Figure 10.4. Changes in sucrose (closed symbols) and oligosaccharide (open symbols) content in whole seeds of *impatiens* seeds in dependence of the duration of priming. Error bars represent the standard deviation of three replicates.

changes in sugar composition between the axes or cotyledons of *impatiens* seeds were similar (data not shown). For bell pepper seeds, large differences in sugar composition were observed between the different seed parts during priming (Fig. 10.5). In untreated seeds, the embryo contained a higher amount of sucrose ($41.2\ \mu\text{g}/\text{mg}$) than the endosperm ($33.8\ \mu\text{g}/\text{mg}$). The sucrose concentration increased in the embryo up to $70.9\ \mu\text{g}/\text{mg}$ after 6 d of priming, but remained constant in the endosperm. Untreated embryos contained more of an unknown trisaccharide ($31.1\ \mu\text{g}/\text{mg}$) than the endosperm ($7.6\ \mu\text{g}/\text{mg}$). The decrease in oligosaccharide content upon priming was much faster in the embryos than in the endosperm. After 3 d of priming, the oligosaccharide content in the embryos decreased to almost undetectable levels ($0.4\ \mu\text{g}/\text{mg}$), whereas in the same time, the oligosaccharide content in the endosperm only slightly decreased to $5.5\ \mu\text{g}/\text{mg}$. After 6 d of priming, the oligosaccharide content in the endosperm decreased to $1.7\ \mu\text{g}/\text{mg}$.

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Figure 10.5. Changes in sucrose (closed symbols) and oligosaccharide (open symbols) content in various seed tissues of bell pepper seeds in dependence of the time of priming. Error bars represent the standard deviation of three replicates.



Changes in sugar composition do not change cytoplasmic glass properties

After establishing that there is a relation between cytoplasmic mobility and longevity (Fig. 10.2), it was possible to determine how the oligosaccharide content affects mobility and the resulting longevity. For that purpose, osmo-priming was used to induce changes in longevity and sugar composition. We investigated whether the change in sugar composition resulted in a change in the T_g of the intracellular glass. We also measured the molecular mobility of a spin probe inserted in the glassy cytoplasm, because systems with similar T_g values can still exhibit a different molecular mobility (Goff et al., 1993).

For oily seeds such as impatiens and bell pepper, the T_g is difficult to detect by differential scanning calorimetry (DSC) because of overlap with melting transitions of lipids. An alternative method of detecting melting of intracellular glasses in seeds is EPR spectroscopy (Buitink et al., 1998a). The shape of the conventional EPR spectrum of a spin probe inserted into the cytoplasm provides qualitative information about the mobility of the spin probe. A decrease in the distance between the outer extrema of the spectrum ($2A_{zz}$) is indicative of an increase in the mobility of the spin probe present in the cytoplasm (Buitink et al., 1998a). The relation between $2A_{zz}$ and temperature revealed a break (Fig. 10.6). Previously this break was found to coincide with

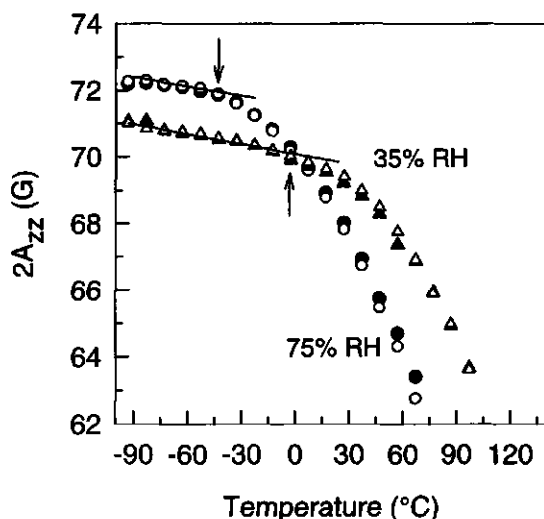


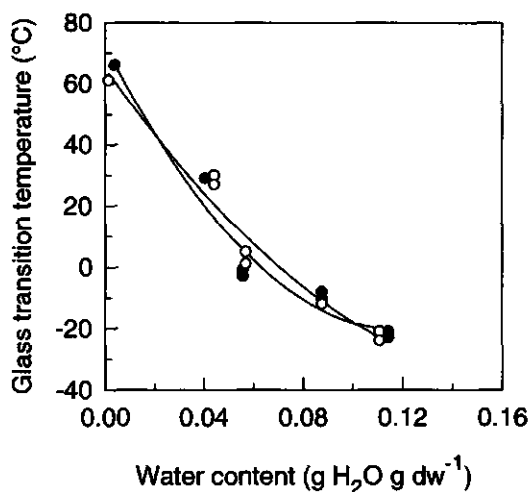
Figure 10.6. The distance between the outer extrema ($2A_{zz}$) derived from EPR spectra of CP in impatiens seeds as a function of temperature. Seeds containing CP were equilibrated to 75% RH (circles) or 35% RH (triangles) after which spectra were recorded. Seeds were untreated (closed symbols) or primed for 7 days at -1.0 MPa, 20°C (open symbols). The arrows indicate the point of deviation from a straight line, representing the onset of the melting of the glassy state (T_g).

the T_g as measured by DSC (Buitink et al., 1998a). Using this method, no difference could be found in the distance between the $2A_{zz}$ in relation to temperature for untreated impatiens seeds or seeds primed for 7 d and equilibrated to the same RH (Fig. 10.6). Using the break in the relationship between $2A_{zz}$ and temperature, a state diagram was established for both untreated and 7-d-primed seeds (Fig. 10.7), and showed no difference in T_g .

A more accurate and quantitative method to determine slow molecular mobility of spin probes is a technique referred to as ST-EPR spectroscopy (Hemminga, 1983). Using this method, one can obtain the τ_r of the spin probe under various conditions, related to the speed at which the spin probe rotates. Figure 10.8 shows the τ_r of CP present in the cytoplasm of impatiens seeds (A) and bell pepper seeds (B) as a function of duration of priming. The τ_r changed

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Figure 10.7. State diagram of untreated (closed symbols) or 7-d-primed (open symbols) *impatiens* seeds. The T_g was determined as the point of deviation from a straight line (shown in Fig. 10.6).



as a function of RH; a higher RH (or higher water content) of the seeds resulted in a faster rotational motion of the spin probe in the cytoplasm. No difference in the τ_r of CP could be found in whole *impatiens* seeds (Fig. 10.8A) or in bell pepper embryos (Fig. 10.8B) or endosperm (data not shown) after priming and redrying. Using the same technique on model glasses, large differences could be found in the temperature dependence of τ_r of CP in a dry sucrose glass compared to a dry raffinose glass. For example, at 70°C, the rotational motion of CP in sucrose was found to be more than 4 orders of magnitude higher than that in raffinose at the same temperature (J. Buitink, unpublished results), a temperature that resulted in melting of the amorphous sucrose but not of the amorphous raffinose (Levine and Slade, 1988; Wolkers et al., 1998b).

Discussion

The proposed role of oligosaccharides in seed longevity was derived from experiments performed on model systems (Koster, 1991; Levine and Slade, 1988). Oligosaccharides are known to increase T_g and viscosity in model sucrose glasses, and this increased viscosity is likely to slow down detrimental ageing reactions. So far, most studies concerning the relationship between oligosaccharides and longevity in seeds are based on correlative evidence (Horbowicz and Obendorf, 1994; Lin and Huang, 1994; Sun and Leopold, 1997). The ratio of oligosaccharide: total sugar between 0 and 0.7 was found to correlate with longevity for several species (Sun and Leopold, 1997). In the present study, we found that the oligosaccharides disappeared and longevity decreased upon priming (compare Figs. 10.3 with 10.4 and 10.5); however, this correlation was not perfect. For example, primed bell pepper seeds survived longer than *impatiens* seeds under the same storage conditions, yet they had a lower oligosaccharide: total sugar ratio (0.01) than *impatiens* seeds

(0.23). Because a correlation can not give a definite answer to the question of whether there is a role for oligosaccharides in longevity, we attempted to test the hypothesis that oligosaccharides increase T_g and decrease the cytoplasmic mobility using ST-EPR spectroscopy. The advantage of ST-EPR is that it provides a precise measurement of the rotational motion of spin probe molecules. Applying this technique to measure the rotational motion of CP in various model-sugar glasses, we indeed found that increasing the temperature above 70°C resulted in a much higher rotational motion of a dry sucrose glass compared to a dry raffinose glass (J. Buitink, unpublished results). Incorporation of CP in the cytoplasm of the seed tissues made it possible to directly compare the molecular mobility in the cytoplasm with longevity. A linear relationship was found between the logarithm of the rotational motion in the cytoplasm of the seeds and the half-viability time as a function of water content (Fig. 10.2). Evidently, longevity of seeds is related to the molecular mobility in the cytoplasm, as suggested previously (Leopold et al., 1994; Sun, 1997b; Buitink et al., 1998a,b).

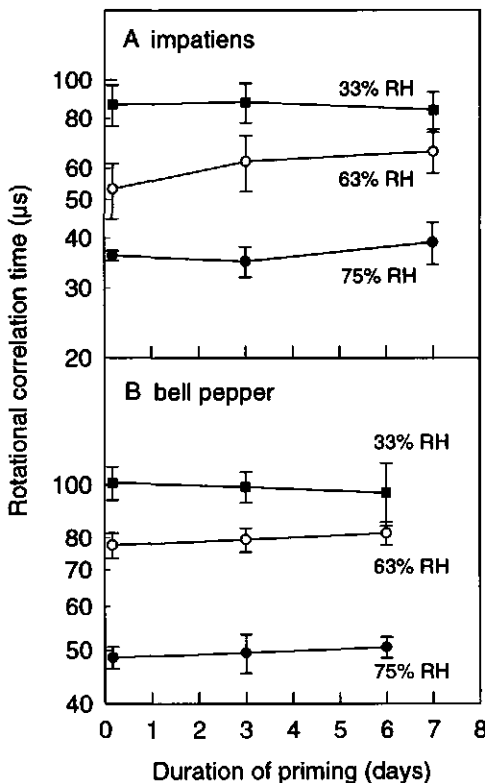


Figure 10.8. Rotational correlation times of CP in impatiens seeds (A) and bell pepper embryos (B) in dependence of time of priming at -1.0 MPa, 20°C . Symbols represent the rotational motion of CP in the tissues equilibrated to various RHs. Data (\pm SD) are the average of four replicates.

The osmo-priming treatment is a good model system to investigate whether a decrease in oligosaccharides (and loss of longevity) would result in increased rotational motion of CP in the cytoplasm of the seeds. Although the

10

ageing reactions involved in the deterioration of primed seeds may be different from those of untreated seeds, the decrease in oligosaccharides upon priming made it possible to study if this decrease resulted in an increase of the molecular mobility in the cytoplasm. However, our data show that no differences could be found in T_g or rotational motion in the cytoplasm of impatiens seeds, or bell pepper endosperm or embryos with different sugar compositions (Fig 10.7 and 10.8). Previously, we reported a similar result for pea embryonic axes in which the oligosaccharide content was reduced considerably after an osmo-treatment, but where no differences were found in the T_g measured by DSC (Buitink et al., 2000). Apparently, there is no measurable change in the mobility of the cytoplasm that can be held responsible for the faster ageing rates of the seeds after priming. A recent study on water sorption properties in osmotically-primed mung bean seeds suggested that priming might lead to a redistribution of water from strong binding sites to weak binding sites (Sun et al., 1997b). The authors argued that such water redistribution might lead to enhancement of molecular mobility in the primed seeds. However, although we find a strong effect of water on the mobility of CP in the cytoplasm of seeds (see Fig. 10.8), a possible water redistribution after priming does not appear to influence the mobility of the spin probe in the seeds.

The above observations do not support the hypothesis that oligosaccharides decrease the molecular mobility in intracellular glasses. Apparently, other molecules in addition to soluble sugars play an important role in the intracellular glass formation (Leopold et al., 1994; Leprince and Walters-Vertucci, 1995; Wolkers et al., 1998a; Buitink et al., 1999). Considering that oligosaccharides make up only 4% of the dry weight in the seeds, it is not surprising that no effect on intracellular glass properties could be measured. This is notwithstanding the fact that sugars still might participate in glass formation, for instance as network molecules.

If oligosaccharides do not decrease the molecular mobility of the intracellular glass, then do they have a role in increasing longevity? It has been suggested that oligosaccharides prevent crystallisation of sucrose during storage (Caffrey et al., 1988; Koster, 1991; Leopold et al., 1994). While model systems indicate that this crystallisation phenomenon can occur (Caffrey et al., 1988), to our knowledge, no studies exist in which crystallisation was found *in vivo* in seeds (Sun and Leopold, 1993). It is likely that the mixture of all the different components in the cytoplasm prevents crystallisation of sucrose, regardless of the presence of oligosaccharides. Another proposed role of oligosaccharides is in the protection of macromolecular structures, especially membranes (Crowe et al., 1992). Hydrogen bonding with sugar molecules will stabilise the macromolecules during drying; however, sucrose molecules show stronger hydrogen bonding properties than do oligosaccharides (Wolkers et al., 1998a, 1998b). The observation that during priming oligosaccharides disappear and sucrose content increases would suggest a better stabilisation of macromolecules after priming, an observation in apparent contrast with the reduced longevity after priming. It might be that there is no specific role for

oligosaccharides in longevity. The oligosaccharides could simply be an indicator of seed maturity and could serve as a storage reserve (Kuo et al., 1988; Hoekstra et al., 1994). Although it is unclear whether there is a role of oligosaccharides in longevity, if any, the results of the present study suggest that they are not involved in the stabilisation of the cytoplasmic matrix in seeds.

The discovery that the reduced longevity of seeds after priming can be partially restored by a combined heat shock and dehydration treatment (Bruggink et al., 1999) could point to a loss of protective chaperones during priming that are re-induced during the stress treatment. It is possible that the disappearance of these molecules is responsible for the increased rate of damage during storage, or the absence of these molecules could lead to damage during the later stages of priming or during the re-drying treatment.

11

High critical temperature above T_g may contribute to the stability of biological systems

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Marcus A. Hemminga

In this study, we characterized the molecular mobility around T_g in sugars, poly-L-lysine and dry desiccation-tolerant biological systems, using ST-EPR, $^1\text{H-NMR}$ and FTIR spectroscopy, to understand the nature and composition of biological glasses. Two distinct changes in the temperature dependence of the rotational correlation time (τ_R) or second moment (M_2) were measured in sugars and poly-L-lysine. With heating, the first change was associated with the melting of the glassy state (T_g). The second change (T_c), at which τ_R abruptly decreased over several orders of magnitude, was found to correspond with the so-called crossover temperature, where the dynamics changed from solid-like to liquid-like. The temperature interval between T_g and T_c increased in the order of sucrose < trehalose < raffinose \leq staychose < poly-L-lysine < biological tissues, from 17°C to >50°C, implying that the stability above T_g improved in the same order. These differences in temperature dependent mobilities above T_g suggest that proteins rather than sugars play an important role in the intracellular glass formation. The exceptionally high T_c of intracellular glasses is expected to provide excellent long-term stability to dry organisms, maintaining a slow molecular motion in the cytoplasm even at temperatures far above T_g .

11

The ability of sugars to preserve biomolecules has been recognised for years in food sciences, pharmaceutical sciences and biological sciences (Burke, 1986; Leopold et al., 1994; Roos, 1995; Hancock et al., 1995; Hancock and Zografi, 1997; Crowe et al., 1996, 1998). Apart from a direct interaction of the sugars with the biomolecules, the ability of these sugars to form glasses is thought to be a major factor in long-term preservation (Levine and Slade, 1988; Roos, 1995; Leopold et al., 1994; Crowe et al., 1998). The high viscosity of the glass and its concomitant low molecular mobility are known to preserve stability of proteins (Chang et al., 1996), to prevent fusion of liposomes (Sun et al., 1996), or to slow down reaction rates, such as browning reactions (Karmas et al., 1992; Roos, 1995).

Nature, too, makes use of glasses to preserve biological tissues in the dehydrated state. Some well-known examples are seeds (Williams and Leopold, 1989; Leopold et al., 1994, LePrince and Walters-Vertucci, 1995), pollen (Buitink et al., 1996), prokaryotes (Potts, 1994), and the so-called resurrection plants (Ghasempour et al., 1998). These tissues are capable of resisting extreme dehydration and are able to resume metabolic activities after the addition of water. All these tissues accumulate to a large extent soluble sugars, predominantly sucrose, trehalose, and, in the case of seeds, oligosaccharides such as raffinose and stachyose (Amuti and Pollard, 1977; Hoekstra et al., 1992b; Ghasempour et al., 1998). Because of the reputation of these sugars to be excellent glass formers, it has been suggested that these sugars play an important role in the glass formation in these biological tissues (Hirsh, 1987; Koster, 1991).

The glass transition temperature (T_g) varies with molecular weight (M_w) of the polyols in a characteristic and predictable manner, almost increasing linearly with the M_w (Slade and Levine, 1991a). The higher the T_g of the polyol, the more advantageous it will be in preserving biomolecules (Slade and Levine, 1991a). For example, trehalose, a sugar with a high T_g , has often been reported to be remarkably effective in stabilising dry or frozen biomolecules, cells, and tissues (Crowe et al., 1996, 1998). Oligosaccharides, such as raffinose and stachyose, have high T_g values, rendering them effective protectants (Levine and Slade, 1988). These sugars are also capable of forming crystalline hydrates (Aldous et al., 1995). It has been suggested that formation of some crystalline hydrate results in keeping the remaining amorphous sugar dry, thereby maintaining a high T_g (Aldous et al., 1995; Crowe et al., 1998).

Despite the frequent use of sugars as protecting agents, little is known about the molecular mobility in sugar glasses around their T_g . The melting of the glassy state of sugars has been implied to result in a dramatic decrease in viscosity and increase in molecular mobility (Williams et al., 1955; Soesanto and Williams, 1981; Roos, 1995). An understanding of how molecular mobility changes upon melting of the glassy state in the different sugars might give an alternative clue as to why trehalose and oligosaccharides are such effective stabilisers.

Biological organisms can survive in the dry state for extremely long periods, even at ambient temperatures (Priestley et al., 1985; Shen-Miller et al., 1995; Steiner and Ruckebauer, 1995). Sucrose and oligosaccharides comprise over 10 to 20% of the dry weight of seeds, and mixtures of these sugars were found to inhibit crystallisation (Caffrey et al., 1988), which could explain the absence of crystallisation in seeds (Sun and Leopold, 1993). Nonetheless, it has been suggested that these sugars are not the only components that participate in the intracellular glass formation (Hirsh, 1987; Sun and Leopold, 1993; Leopold et al., 1994; Leprince and Walters-Vertucci, 1995; Sun and Leopold, 1997; Wolkers, 1998; Buitink et al., 1999). The change in molecular mobility in the cytoplasm of seeds with temperature, as measured by spin probe saturation transfer electron paramagnetic resonance (ST-EPR) spectroscopy, was found to be quite different compared with that of pure sugars (Buitink et al., 1999). Upon melting of a pure sucrose glass, a large increase in mobility was found within the first 30°C above T_g , encompassing four orders of magnitude (Rozen and Hemminga, 1990), whereas in biological tissues, molecular mobility increased only a factor 7 upon melting of the intracellular glass over the same temperature range (Buitink et al., 1999). Beside sugars, another major compound present in the cytoplasm is protein. Polypeptides have been shown to alter the glass properties of sugars significantly (Kalichevsky et al., 1993; Wolkers et al., 1998a). Characterization of the molecular mobility of compounds that could be involved in intracellular glass formation might lead to a better understanding of how these biological tissues can survive for such long periods. Ultimately, this might aid in the preservation of biomolecules in the pharmaceutical and food sciences.

EPR spectroscopy is a powerful technique to obtain information about the rotational mobility of spin-labelled molecules, because a very broad range of molecular motions can be covered by this technique (Hemminga and Van den Dries, 1998). Conventional EPR is sensitive in the motional region for τ_r values between 10^{-11} and 10^{-7} s, whereas ST-EPR is sensitive to molecular motions between 10^{-6} and 10^{-3} s (Hemminga and Van den Dries, 1998, Buitink et al., 1999). However, a drawback of the use of spin probe EPR spectroscopy is that one measures the molecular motion of a foreign molecule (spin probe) introduced into a glass, and not the molecular mobility of the molecules that form the glass themselves. A technique that avoids this is ^1H -nuclear magnetic resonance (NMR) spectroscopy, that can be used to determine the second moment of the NMR line shape, M_2 , which provides information on the mobility of the immobile proton fraction around T_g (Van den Dries et al., 1998). Alternatively, fourier transform infrared (FTIR) spectroscopy can be used to assess the properties of the hydrogen bonding network of the sugar glasses around T_g (Wolkers et al., 1998b).

In this study, we characterized the molecular mobility around T_g in sugars that are thought to be involved in biological glass formation, using ST-EPR, ^1H -NMR and FTIR. Also, the molecular mobility of a polar spin probe present in the cytoplasm of pollen and seed was measured and compared with sugar and protein glasses in order to understand the nature and composition of biological

11

glasses. The high stability of biological tissues is discussed in relation to the composition of the intracellular glass.

Material and methods

EPR spectroscopy

Glasses were prepared by rapidly drying 10 μl droplets of 0.1 g ml^{-1} sucrose (Pfanstiel), amorphous trehalose (Pfanstiel), raffinose-pentahydrate (Sigma), amorphous stachyose (Sigma) and poly-L-lysine (PolyLys, Sigma, M_w 36 kDa), containing 0.1 mM 3-carboxy-proxyl (CP, Sigma), on a glass plate in a box with an airflow of 3% relative humidity (RH) at room temperature. In a similar way, glasses were formed of a 1:1 (w/w) sucrose:stachyose mixture, and a 1:1 (w/w) sucrose:PolyLys mixture. Glasses were formed within 5 minutes (Wolkers et al., 1998a). After 3 d of drying, the material was removed from the glass plate and equilibrated at 3% RH in the box or over a saturated salt solution of MgCl_2 (33% RH) at 10°C for at least 7 days. Subsequently, the glasses were loaded into EPR capillaries under the respective RH conditions, sealed and measured directly.

Typha latifolia L. (cattail) pollen was harvested in the Netherlands in 1997. Labelling of the pollen with the spin probe was performed according to Buitink et al. (1998a). Briefly, 3 g of dry pollen was prehydrated in water vapour for 16 h at 5°C to about 0.7 $\text{g H}_2\text{O g dw}^{-1}$ and then mixed at 25°C with 6 ml of liquid germination medium containing 2.5 mM CP. After a few min, an additional 20 ml of the germination medium was added, and the pollen was recovered by filtration. The pollen was then mixed with 20 ml of a solution of 1 mM CP and 120 mM of potassium ferricyanide. The ferricyanide 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. After 5 more min, the pollen was recovered by filtration, spread out in a large Petri dish, and rapidly dried in a flow of dry air (3% RH) to water contents of less than 0.05 $\text{g H}_2\text{O g dw}^{-1}$. After drying, pollen was stored over a saturated solution of MgCl_2 (33% RH) for 7 days. For each EPR measurement, approximately 20 mg pollen was loaded at 33% RH and sealed in a 2-mm diameter EPR capillary. After the measurements, the pollen was 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.

Dry bean axes (*Phaseolus vulgaris* L.) were excised from the seeds, and allowed to imbibe in water for 2 h at 15°C, after which the axes were incubated in 10 ml of a solution of 1 mM CP. After 45 min, potassium ferricyanide was added to a final concentration of 200 mM, and the axes were incubated for another 15 min. Subsequently, the bean axes were blotted dry on filter paper, and dried in dry air (3% RH) for 24 hours. After drying, axes were stored over a saturated solution of MgCl_2 (33% RH) for two weeks. Four axes were loaded at 33% RH and sealed in a 2-mm diameter capillary for EPR measurements. After the measurements, the axes 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.

EPR spectra were recorded with a Bruker X-band EPR spectrometer (Bruker Analytik, Rheinstetten, Germany, model 300E). The τ_r in the fast motional region was determined from the line shapes of EPR spectra according to the method of Knowles et al. (1976) for isotropic tumbling:

$$\tau_R = 6.5 \times 10^{-10} \Delta B_0 \left(\frac{h_C}{h_H} \right)^{1/2} - 1 \quad (1)$$

11

where B_0 is the width of the center field component in Gauss, and h_C and h_H are the amplitudes of the central and high field components of the three-line nitroxide radical spectrum, respectively.

At low water contents and temperatures, when τ_R becomes longer than 10^{-8} s, τ_R cannot be calculated according to equation 1 because of the appearance of a powder spectrum. ST-EPR was used to determine τ_R between 10^{-6} s to 10^{-3} s. 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. The phase was set with the self-null method (Thomas et al., 1976).

ST-EPR spectra can be characterized by independent line shape parameters, such as the line-height ratio L^*/L . Using reference material with known viscosity, τ_R values can be obtained in an empirical way. Here we used spectra of CP in anhydrous glycerol to construct a calibration curve (Hemminga and Van den Dries, 1998) of the line shape parameter L^*/L versus τ_R values (for details see Buitink et al., 1999). From this calibration curve, the values of τ_R of CP in the sugar, polypeptide and biological material were obtained by interpolation of the corresponding line shape parameters. For all samples, EPR measurements were performed at least twice to check for reproducibility.

¹H-NMR spectroscopy

Sugar glasses were prepared by lyophilising 20% (w/w) trehalose-dihydrate (Sigma), amorphous stachyose (Sigma) and sucrose (Merck). Subsequently, powders were stored for 3 weeks over phosphorus pentoxide or a saturated solution of $MgCl_2$ (33% RH) at room temperature. Samples were loaded in a 5 mm NMR tube that was sealed to prevent water evaporation.

¹H-NMR measurements were performed on a Bruker AMX 300 spectrometer equipped with a Bruker 5 mm proton probe operating at a resonance frequency of 300.13 MHz. The temperature was regulated with a liquid nitrogen temperature control. In this way, stability was within 0.5°C. A spectral width of 500 kHz was used. The duration of the pulse was 6-7 μ s. The presented free induction decays are averages of 128 or 256 scans having 2048 data points. For the analysis of the NMR, the FIDs $F(t)$ were fitted to the following equation (Van den Dries et al., 1998b):

$$F(t) = A \exp \left\{ \frac{a^2 t^2}{2} \right\} (\sin bt) / bt + B \exp \{-t/T_{2m}\} \quad (2)$$

In this equation, the parameters A and B represent the contributions of the immobile and mobile protons and T_{2m} is the spin-spin relaxation time of the mobile proton fraction. The NMR spectrum of the immobile proton fraction is assumed to be a rectangular line shape with a total width $2b$, convoluted with a gaussian line shape with a standard deviation given by a (Van den Dries et al., 1998b). The second moment M_2 of the broad line shape, which is a measure of the strength of the dipolar interactions, is calculated from the fit parameters a and b by the following equation:

11

$$M_2 = a^2 + b^2/3 \quad (3)$$

A reduction of M_2 takes place if the proton density in the samples is lowered, because dipolar interactions decrease with the sixth power of the distance between protons. Furthermore, M_2 will be reduced by anisotropic mobility or slow isotropic rotations that partly average out the dipolar interactions (Van den Dries et al., 1998b).

FTIR spectroscopy

Glasses were prepared by rapid air drying (at 3% RH) of a droplet of 0.01 g ml⁻¹ sucrose (Pfanstiel), amorphous trehalose (Pfanstiel), raffinose-pentahydrate (Sigma), and amorphous stachyose (Sigma) on circular CaF₂ windows of 13 mm diameter, width 2 mm. Glasses were formed within 5 minutes (Wolkers et al., 1998a). The absence of crystallisation was confirmed by the absence of sharp bands in the FTIR spectra (Wolkers et al., 1998b). Residual water in the sugar glasses was removed by heating the sample for 5 minutes to 70°C in the case of sucrose, and to 100°C for the other sugars. The absence of water in the glasses was verified by the absence of the water band around 1650 cm⁻¹. FTIR spectra were recorded on a Perkin Elmer 1725 spectrometer (Perkin Elmer, Beaconsfield, Buckinghamshire, UK) equipped with a liquid nitrogen-cooled mercury/cadmium/telluride detector and a Perkin Elmer microscope interfaced to a personal computer as described previously (Wolkers et al., 1998a, 1998b). Each sample was hermetically sealed between two CaF₂ IR-windows, using a rubber O-ring, and mounted into a temperature-controlled brass cell that was cooled by a liquid nitrogen source. The temperature was regulated by a computer-controlled device that activated a liquid nitrogen pump, in conjunction with a power supply for heating of the cell. The temperature of the sample was recorded separately using a PT-100 element that was located very close to the sample windows. The optical bench was purged with dry CO₂-free air (Balston, Maidstone, Kent, UK) at a flow rate of 25 l min⁻¹. The acquisition parameters were 4 cm⁻¹ resolution, 32 co-added interferograms, 3600-900 cm⁻¹ wavenumber range.

Spectral analysis and display were carried out using the Infrared Manager Analytical Software, version 3.5 (Perkin Elmer). The melting of glasses during heating of the sample at 1.5°C min⁻¹ was monitored by observing the position of the band around 3300 cm⁻¹ (OH-stretching vibration, ν_{OH}). The band position was calculated as the average of the spectral positions at 80% of the total peak height. T_g was determined by the intersection of linear regressions of the ν_{OH} at low and high temperatures. Temperature scans for each sugar sample were performed in triplicate.

Differential scanning calorimetry

Dry glasses were prepared by rapidly drying 10 μ l droplets of 0.1 g ml⁻¹ of different sugars, PolyLys or sucrose:PolyLys and sucrose:stachyose mixtures. After drying for 3 days in an airflow of 3% RH, glasses were removed from the glass plate and equilibrated for 7 days at 3% or 33% RH, after which the material was loaded under the respective RH conditions and sealed in hermetically sealed DSC pans. Second order transitions of the samples were determined using a Perkin-Elmer (Norwalk, CT) Pyris-1 DSC, calibrated for temperature with indium (156.6°C) and methylene chloride (-95°C) standards and for energy with indium (28.54 J g⁻¹). Baselines were determined using an empty pan, and all thermograms were baseline-corrected. Heating thermograms were

obtained at a scanning rate of $10^{\circ}\text{C min}^{-1}$. The T_g values were determined as the onset of the temperature range over which the change in specific heat occurred.

Samples prepared for EPR and NMR measurements were checked for crystallisation. Sucrose glasses equilibrated to 33% RH were found to be completely crystallised. Therefore, EPR and NMR measurements of sucrose equilibrated to 33% RH were omitted from the analyses. For the other sugar samples, not more than 2% hydrate formation was observed, by comparison of the melting enthalpies of pure trehalose-dihydrate, raffinose-pentahydrate and stachyose-dihydrate (Sigma) to the melting enthalpies of the prepared amorphous sugars. All analyses were performed with Perkin-Elmer software.

Sugar determination

The soluble sugar content in bean axes was determined by DIONEX-HPLC (Tetteroo et al., 1994). Excised dry bean axes were ground in a mortar in the presence of 3 ml 80% methanol containing lactose as the internal sugar standard. The suspension was removed from the mortar with 80% methanol and heated in a water bath at 76°C for 15 min. The liquid was evaporated under vacuum in a Speedvac AES1010 (Savant Instruments). The residue was dissolved in distilled water, and after appropriate dilution, sugars were analysed by HPLC on a Carbowax PA-1 column (Dionex Corp., Sunnyvale, CA, USA) using pulsed amperometric detection, as described by Tetteroo et al. (1994). Data are the average of three extractions, each extraction containing 10 axes.

Results

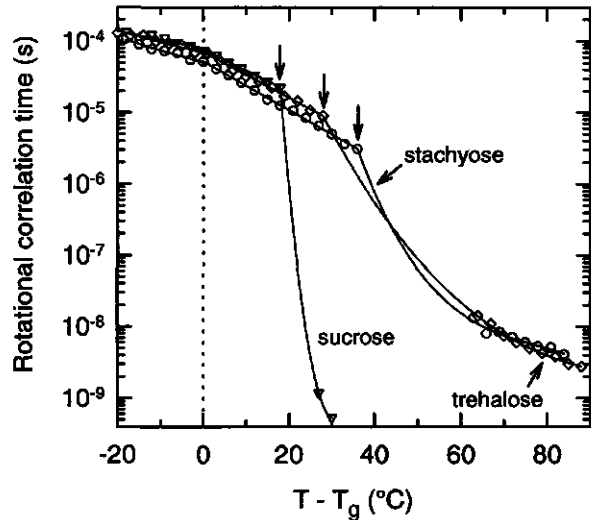
Molecular characterization of sucrose, trehalose and oligosaccharide glasses

We investigated the molecular mobility of polar nitroxide 3-carboxy-proxyl (CP) in glasses of the various biologically relevant sugars, sucrose, trehalose and oligosaccharides, using ST-EPR and conventional EPR spectroscopy. Figure 11.1 shows the rotational correlation time (τ_r) of CP in the sugars around T_g . For all sugar glasses, the τ_r became shorter below T_g with increasing temperature, indicating a faster rotational motion of the spin probe in the sugar glasses. Around T_g , a break in the linear relationship was observed (Fig. 11.1). Above T_g , the logarithm of the rotational motion increased linearly but more strongly with an increase in temperature compared with below T_g . Remarkably, a second break in the relationship between τ_r and temperature was observed, hereafter referred to as the critical temperature (T_c) (Fig. 11.1, indicated by arrows). For sucrose, this T_c occurred at 18°C above T_g , whereas for trehalose sample this T_c occurred at 28°C above T_g . For raffinose, T_c occurred 35°C above T_g (data not shown), and for stachyose T_c occurred 37°C above T_g (Fig. 11.1). Above T_c , rotational motion increased abruptly with an increase in temperature over several orders of magnitude for all sugars, until the environment of the spin probe had become completely fluid (indicated by the presence of a three line EPR spectrum). The τ_r values depicted in Fig. 11.1 were from trehalose and raffinose glasses that were equilibrated to 33% RH. The reason for using this elevated RH was the following: the high T_g for these sugars when equilibrated to 3% RH prevented the determination of the entire τ_r

11

curve because of a technical limitation of 127°C for the EPR measurements. Because sucrose crystallised at 33% RH, τ_r was only determined for sucrose samples equilibrated to 3% RH. The temperature interval between T_g and T_c in dry trehalose, raffinose and stachyose, all equilibrated to 3% RH, was 26, 34

Figure 11.1. Rotational correlation time (τ_r , s) of CP in different sugars as a function of $T - T_g$. Dashed line indicates T_g . Arrows indicate the critical temperature T_c . Inverted triangles, sucrose equilibrated to 3% RH; diamonds, trehalose equilibrated to 33% RH; circles, stachyose equilibrated to 33% RH.



and 34°C, respectively (data not shown). Comparison of these temperature intervals with those of sugars equilibrated to 33% RH indicated that a change in water content of the sugar glasses did not affect these temperature intervals significantly. When a second scan of the sugar samples was performed, τ_r had the same temperature dependence (data not shown), indicating that the measured τ_r was not dependent on the thermal history of the sugar samples.

The range of molecular motions for which τ_r could not be determined using our protocols is from about 10^{-6} and 10^{-8} s. For τ_r longer than 10^{-8} s, the EPR spectrum takes the shape of a powder spectrum, which makes it impossible to use equation 11.1. On the other hand, the L^*/L ratio of the ST-EPR spectra is insensitive when τ_r is shorter than 10^{-6} s. The curves drawn through the data points suggest that there is an abrupt increase in rotational motion at T_c (Fig. 11.1). This abrupt increase is derived from the shape of the EPR and ST-EPR spectra of CP recorded at the temperature just above the last data point that could be calculated by ST-EPR (3°C higher). The insensitivity of the line shape of the ST-EPR spectrum indicated that τ_r was shorter than 10^{-6} s. In addition, the conventional EPR spectrum showed that there was a decrease in the principal values of the hyperfine interaction of CP ($2A_{zz}$), indicating faster rotational motion (Buitink et al., 1999).

The τ_r at T_g for sugars equilibrated to 3% RH had the following order: sucrose > trehalose > raffinose > stachyose (Table 11.1). For sugars equilibrated to 33% RH, the τ_r at T_g changed in the same order. As can be seen

from Table 1, the T_g values obtained by FTIR measurements are higher than those obtained by EPR measurements. This can be explained by the different methods of sample preparation. With FTIR, samples were first heated to 70°C to remove the residual water. Therefore, the T_g values found with this technique were close to the literature values (Slade and Levine, 1991a; Roos, 1995). For the EPR measurements, samples were equilibrated to 3% RH for 7 days, which was not sufficient to remove all the residual water (see Table 11.1). A remarkable observation was that an increase in water content in the sugar glasses resulted in slower rotational motion at T_g (Table 11.1).

Table 11.1. Rotational correlation time (τ_r) and wavenumber position (ν_{OH}) of sugar glasses at T_g . Sugar glasses were equilibrated to 3% or 33% RH (ST-EPR) or first heated to remove the residual water (FTIR). The T_g values were taken as the break in the relationship of the τ_r or ν_{OH} as a function of temperature. WC, water content, g H₂O g dw⁻¹; nd, not determined.

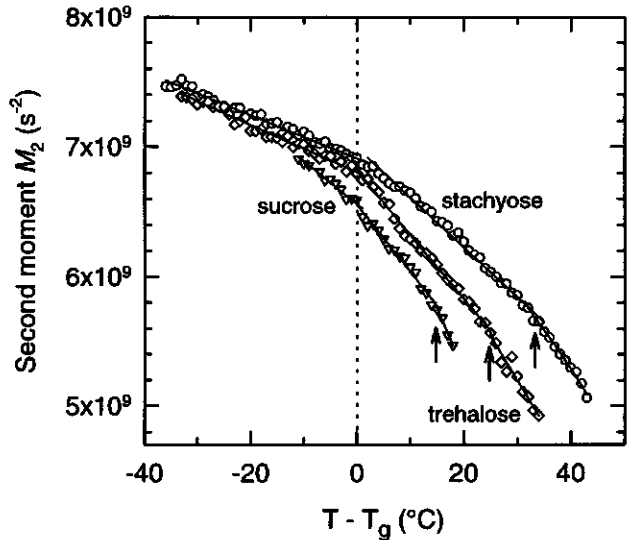
Sugar	EPR			FTIR		
	WC g/g dw	T_g °C	τ_r at T_g s	WC g/g dw	T_g °C	ν_{OH} at T_g cm ⁻¹
Sucrose	0.027	43	80.1	<0.01	69	3373
Trehalose	0.037	63	78.6	<0.01	107	3392
Raffinose	0.041	72	58.1	<0.01	106	3401
Stachyose	0.025	81	36.0	<0.01	123	3411
Trehalose 33% RH	0.072	23	85.0	nd	nd	nd
Raffinose 33% RH	0.077	29	80.0	nd	nd	nd
Stachyose 33% RH	0.060	39	52.0	nd	nd	nd

A comparable pattern of the temperature dependence of the mobility in the sugar glasses was observed with ¹H-NMR (Fig. 11.2). With this technique, one can measure the second moment (M_2), which provides information on the mobility of the immobile proton fraction around the T_g (Van den Dries et al., 1998b). For all sugars, M_2 decreased with increasing temperature below T_g , indicating an increase of the mobility of immobile proton fraction of the sugar glasses. Around T_g , a break in the linear relationship occurred, after which M_2 decreased more strongly with an increase in temperature. Also with ¹H-NMR a second break in the relationship between M_2 and temperature was observed for the various sugars (Fig. 11.2, indicated by arrows). At temperatures above this second transition the immobile proton fraction became so mobile that they

11

corresponded to the mobile fraction and could no longer be characterized by M_2 (Van den Dries et al., 1998b). This explains the occurrence of only a few data points above T_c (Fig. 11.2). Similar to τ_r measurements, the temperature interval between T_g and T_c was smallest for sucrose (17°C) and largest for the oligosaccharide stachyose (32°C) (Fig. 11.2).

Figure 11.2. The second moment (M_2 , s^{-2}) of immobile protons of different sugars as a function of $T - T_g$. Dashed line indicates T_g . Arrows indicate the critical temperature. Inverted triangles, sucrose equilibrated to 3% RH; diamonds, trehalose equilibrated to 33% RH; circles, stachyose equilibrated to 33% RH.



To understand why the τ_r of the spin probe at T_g became shorter with increasing size of the sugar molecules, information on the packing of the sugar molecules at T_g is necessary. FTIR spectroscopy was applied to obtain information on the hydrogen bonding properties and molecular packing of the sugars. The wave number position of OH-stretching vibrations (ν_{OH}) reveals information about the strength and length of hydrogen bonding (Jeffrey, 1997). Figure 11.3 shows the ν_{OH} of the sugar samples as a function of temperature. A break in the relationship corresponded to the T_g (Wolkers et al., 1998b), which is confirmed by molecular dynamics simulations, where it was found that the number and lifetime of hydrogen bonds increases below T_g (Caffarena and Grigera, 1996, 1999). The ν_{OH} over the whole temperature range had the following order: sucrose < trehalose < raffinose < stachyose. This indicates that for larger sugars, the hydrogen bond length increased (Jeffrey, 1997), implying a less dense hydrogen bonding network for larger sugars. Note that although the M_w of sucrose and trehalose is equal, the latter had a less dense hydrogen bonding network. The T_g values and ν_{OH} at T_g found with this technique are presented in Table 11.1.

Intracellular glass formation in seed and pollen in relation to sugars and proteins

11

Sugars, being present in high amounts in desiccation-tolerant tissues such as seeds and pollen, have been suggested to play a pivotal role in intracellular glass formation (Hirsh, 1987; Koster, 1991). To investigate the role of sugars in biological glass formation, the τ_r of CP in cattail pollen, equilibrated to 33% RH ($T_g = 38^\circ\text{C}$), was determined. The predominant sugar in the pollen is sucrose (23% of the dry weight), present for about 97% of the total sugars, the remaining few percent being mono-saccharides like glucose and fructose (Hoekstra et al., 1992b). The logarithm of τ_r of CP in the pollen showed a linear decrease with increasing temperature, with a break around T_g (squares, Fig. 11.4). The temperature dependence of τ_r in the pollen was quite different from τ_r of CP in the sucrose glass (triangles, Fig. 11.4). In the sucrose matrix, τ_r strongly decreased at about 18°C above T_g , corresponding to the T_c , while the τ_r in the pollen did not appear to exhibit a T_c within the range of the measurements. Instead, the logarithm of τ_r continued to decrease linearly with temperature for at least 45°C above T_g .

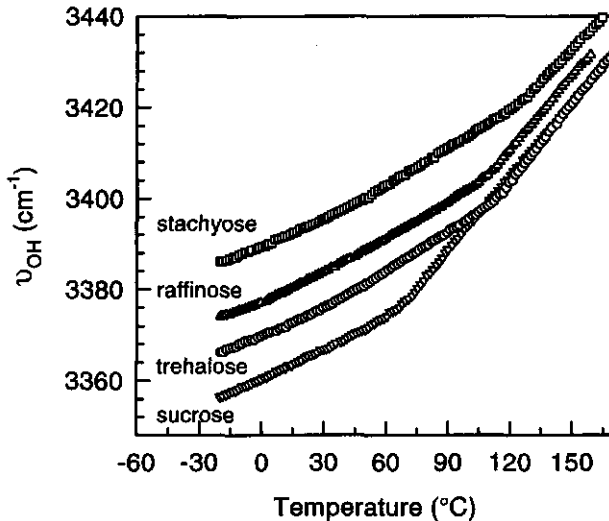


Figure 11.3. The wavenumber of the OH stretching vibration (ν_{OH} , cm^{-1}) for different sugars as a function of temperature. Sugar glasses were first heated to remove residual water. Inverted triangles, sucrose; circles, trehalose; triangles, raffinose; squares, stachyose.

Proteins are likely candidates to participate in intracellular glass formation because of their high abundance in the cytoplasm. Therefore, we also investigated the τ_r of CP in PolyLys. Although PolyLys is a synthetic polypeptide, it was used to obtain information of how a spin probe behaves in high M_w proteins. Also, the interaction of this polypeptide with sugars has been investigated previously using FTIR spectroscopy (Wolkers et al., 1998a), enabling comparison of these results to the molecular mobility of CP in the polypeptide. The T_g of PolyLys, equilibrated at 33% RH, was 29°C . The τ_r of CP

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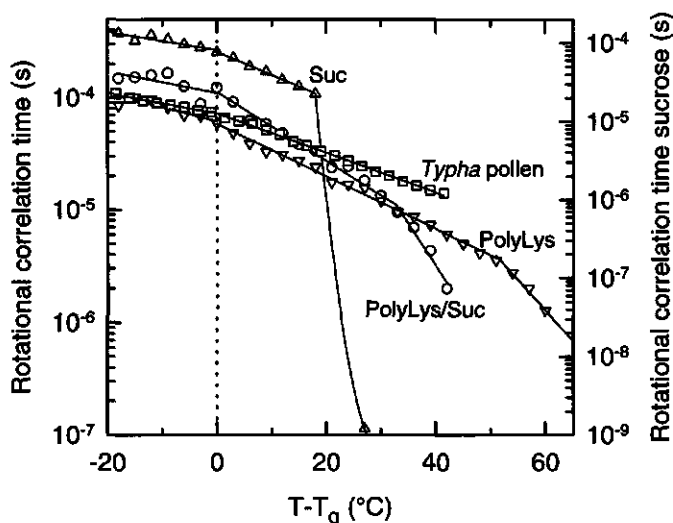


Figure 11.4. Rotational correlation time (τ_R , s) of CP in the cytoplasm of cattail pollen (squares), and in sucrose (triangles), PolyLys (inverted triangles) and a 1:1 (w/w) mixture of PolyLys and sucrose (circles) as a function of $T - T_g$. Dashed line indicates T_g . Samples were equilibrated to 33% RH, except for sucrose and the mixture of PolyLys and Suc (3% RH).

in PolyLys below T_g decreased linearly on a logarithmic scale with increasing temperature (inverted triangles, Fig. 11.4). Above T_g , τ_R decreased more steeply with increasing temperature until T_c was reached at approximately 50°C above T_g . At T_c , there was a sharp decrease in τ_R , reaching a value of 7×10^{-9} s at 50°C above T_c (Fig. 11.5). Also depicted in Fig. 11.4 is the τ_R of CP in a 1:1 (w/w) mixture of sucrose and PolyLys, equilibrated to 3% RH (open circles, $T_g = 69^\circ\text{C}$). For this mixture, T_c was observed at 33°C above T_g , intermediate to the T_c of sucrose and PolyLys alone.

Seeds contain a large amount of oligosaccharides such as raffinose and stachyose (Amuti and Pollard, 1977). Soluble sugar analysis using DIONEX-HPLC revealed that axes of bean seeds contain in total 20% of their dry weight in soluble sugars. The composition of the soluble sugars was 43% sucrose, 7% raffinose, 49% stachyose and 1% verbascose. To understand the role of these sugars in glass formation in the cytoplasm of seeds, we compared the rotational motion of CP in bean seed axes (inverted triangles, Fig. 11.5, $T_g = 25^\circ\text{C}$) with the glass mixture of 1:1 w/w sucrose and stachyose (circles, Fig. 11.5, $T_g = 33^\circ\text{C}$), both equilibrated to 33% RH. The slope of the relationship between the logarithm of rotational motion and temperature was steeper above T_g for the sugar mixture than for bean axes. Furthermore, the temperature interval between T_g and T_c for bean axes was at least $> 50^\circ\text{C}$, but only 33°C for the sugar mixture. The rotational motion of CP in the axes resembled that of CP in PolyLys glasses (squares, Fig. 11.5). A clear difference between the two glasses was the overall faster rotational motion of CP in the PolyLys as compared with the intracellular glass at similar temperatures.

Discussion

Characterization of molecular mobility around T_g

Sugars that are abundantly present in desiccation-tolerant tissues are trehalose, sucrose, and particularly in seeds, oligosaccharides such as raffinose (a trisaccharide) and stachyose (a tetrasaccharide) (Amuti and Pollard, 1977; Hoekstra et al., 1992b; Crowe et al., 1998; Ghasempour et al., 1998). These sugars have been suggested to play a pivotal role in the glass formation of desiccation-tolerant biological tissues (Hirsh, 1987; Koster, 1991). Determination of the molecular mobility in these sugar glasses and comparison with that in intracellular glasses was expected to reveal what role these sugars play in intracellular glass formation. Here, the molecular mobility around T_g in the sugar glasses was characterized by τ_R of the nitroxide spin probe CP, measured by EPR, and the second moment M_2 , measured by $^1\text{H-NMR}$. FTIR spectroscopy provided information on the hydrogen bonding network of the sugars around T_g .

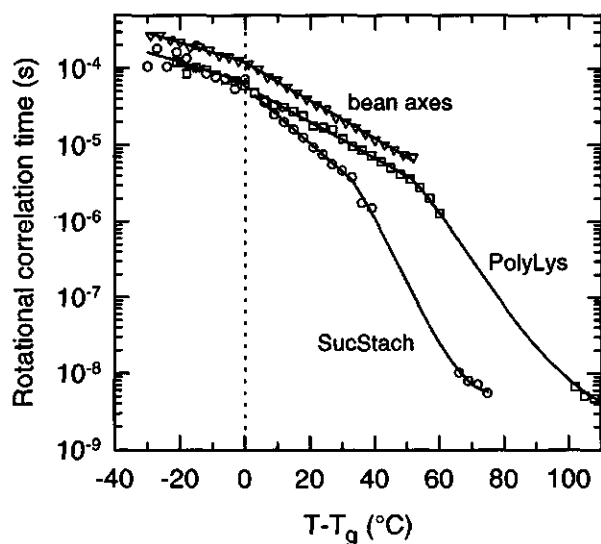


Figure 11.5. Rotational correlation time (τ_R , s) of CP in the cytoplasm of bean seed axes (*Phaseolus vulgaris*) (inverted triangles), and in a 1:1 (w/w) mixture of sucrose and stachyose (SucStach, circles), and PolyLys (squares) as a function of $T - T_g$. Dashed line indicates T_g . Samples were equilibrated to 33% RH.

The τ_R of CP in the different sugars at T_g was dependent on the size of the sugars; a higher M_w of the sugar gave rise to faster rotational motion. Comparison of the τ_R at T_g with the ν_{OH} at T_g revealed that in dry sugars, this faster rotational motion was accompanied by a less dense hydrogen bonding network (Table 11.1). It has been reported that the molecular free volume at T_g increases with increasing M_w for small polymers/oligomers (Fox and Loshaek, 1955). Also, the average radius of molecular free volume holes at T_g was found to increase with T_g (Bartos, 1996; Li et al., 1999). Thus, the tetrasaccharide stachyose will form a glass matrix with a higher molecular free volume, in which

11

the spin probe is less hindered in its rotation compared to a glass matrix formed by the disaccharide sucrose. PolyLys, with a M_w of 36 kDa, permitted an even faster rotational motion at T_g (Fig. 11.4), indicating an even larger molecular free volume in the PolyLys glass. Interestingly, although trehalose and sucrose have equal M_w , the faster rotational motion and higher ν_{OH} at T_g indicate that the trehalose glass is less densely packed than a sucrose glass.

Transition in molecular mobility above T_g : explanation of the critical temperature

The molecular mobility in the sugar glasses, measured by τ_R of a spin probe and M_2 by 1H -NMR, increased with increasing temperature (Figs. 11.1 and 11.2). The most characteristic feature that was observed in the temperature dependence of the molecular mobility was the presence of two transitions, the first one associated with melting of the glassy state, and the second one associated with the so-called critical temperature, T_c . Recently, this T_c was also found for glucose-water glasses, measured by 1H -NMR, and was interpreted as the so-called crossover temperature, where the dynamics changes from solid-like to liquid-like (Van den Dries, van Dusschoten and Hemminga, submitted for publication). This crossover temperature has been observed for many polymer glasses and can theoretically be predicted by the mode coupling theory (Sokolov, 1996, 1997; Rössler et al., 1994, 1998). Above the crossover temperature a viscous solution behaves as a normal liquid, where diffusional motion dominates the dynamics (α -relaxation). Below this temperature, a transition to a more and more pronounced solidlike behaviour with spatial and dynamic heterogeneities starts (Sokolov et al., 1999). At the crossover temperature the second relaxation, the fast relaxation process, becomes temperature dependent and can be visualised as rattling of a molecule inside a cage formed by its neighbours (Sokolov, 1997). In the temperature range between T_g and the crossover temperature these cages still undergo collective distortions leading to translational diffusion (α -relaxation). At temperatures below T_g only the motion of a molecule inside a cage persists. Additional evidence that this second transition can be explained by the concept of the crossover temperature comes from the similar time scale of the motion found at T_c and the crossover temperature (Van den Dries, et al., 1998b, Van den Dries, van Dusschoten and Hemminga, submitted for publication).

Relation between the critical temperature and stability: explanation for the stability of biological organisms?

The T_c or crossover temperature appears to be related to the stability of sugar glasses above their T_g . One of the characteristics of sugar glasses is that they exhibit a collapse at a temperature above the T_g , observed as a macroscopically visible change in physical properties. The collapse is generally attributed to a reduction in viscosity upon increasing temperature above the T_g such that flow on a practical time scale is observed, generally around 10^8 Pa s (for review see Roos, 1995). This is comparable to what happens at a

molecular scale above the crossover temperature (T_c), where the dynamics of the viscous matrix will be dominated by the diffusional motion. For various mono- and disaccharides, the temperature of structural collapse and glass softening occur 10-17°C above T_g (Levine and Slade, 1988; Roos, 1995; Sun, 1997a). These values are comparable with those corresponding to the critical temperature (T_c) found in our study for sucrose (Table 11.2). Apparently, the macroscopic collapse is a result of the change in molecular dynamics at T_c . The collapse phenomenon results in a dramatic change in the stability of the matrices, such as loss and oxidation of encapsulated lipids and flavours, loss of enzymatic activity, nonenzymatic browning, stickiness and caking or structural collapse (Levine and Slade, 1988; Slade and Levine, 1991a; Roos, 1995).

Table 11.2. The magnitude of the temperature interval between the glass transition temperature and critical temperature (T_g-T_c) for various materials, measured by τ_R using ST-EPR or M_2 using 1H NMR. Samples that were performed in duplicate are indicated. nd: not determined.

Material	RH (%)	T_c-T_g τ_R (°C)	T_c-T_g M_2 (°C)
Sucrose	0	16/18	17
Trehalose	0	25/26	26
	33	28/30	23/26
Raffinose	0	33/35	nd
	33	34/36	nd
Stachyose	0	33/35	nd
	33	36/38	32
Sucrose/Stachyose (1:1 w/w)	33	33	nd
PolyLys	0	34/37	nd
	33	50/52	nd
PolyLys/Sucrose (1:1 w/w)	0	33	nd
Cattail pollen	33	> 45	nd
Bean seed axes	33	> 60	nd
Pea seed axes	33	> 60	nd

The observation that T_c occurred at substantially higher temperatures above T_g in trehalose and oligosaccharides than in sucrose implies that these glasses might be more stable above T_g (Table 11.2). The superior stability of trehalose over sucrose glasses could be related to their differences in T_c (Fig. 11.1, 11.2 and Table 11.2). Some evidence for this conclusion comes from the observation that the protection of G6PDH was found to be better in glucose/trehalose than glucose/sucrose (1:4) systems during storage at

11

similar temperatures above T_g (Sun and Davidson, 1998). The system containing trehalose could be heated 8°C more above T_g than the sucrose containing system to preserve the protein to the same level. Crowe et al. (1998) also found that trehalose was better in protecting enzyme activity at high RH than sucrose. Another indication that the high T_c of polysaccharides might influence stability above T_g was the discovery that a frozen mixture of polysaccharides and sucrose resulted in a higher viscosity upon melting of the glass as compared with frozen sucrose alone (Goff et al., 1993). Apart from the beneficial property of trehalose, raffinose and stachyose to form hydrates, their high T_c might also be responsible for the improved stability these sugars confer above T_g .

In biological tissues, T_c could not be measured, indicating that it likely occurs at temperatures $> 50^\circ\text{C}$ above T_g (Table 11.2). Considering that above T_c there is a strong reduction in viscosity, the absence of T_c in biological tissues implies that the system is very stable, still exhibiting a high viscosity ($> 10^8$ Pa s) far above T_g . This extremely high temperature interval between T_g and T_c could be an explanation for the long lifespan of dry biological tissues. In a natural environment, changes in environmental conditions to which seed and pollen are exposed are likely to bring the tissues above their T_g . Although resulting in a slight increase in the temperature dependence of the cytoplasmic molecular mobility, an increase of the temperature above T_g does not lead to an abrupt decrease in viscosity as seen for the sugar systems, that could lead to, for instance, crystallisation or collapse. The observation that ageing kinetics of cattail pollen changes only a factor 3 around T_g , and remains constant for $> 30^\circ\text{C}$ above T_g (Buitink et al., 1998b) might be explained by the large temperature interval between T_g and T_c ($>45^\circ\text{C}$) (Fig. 12.4). Comparable high crossover temperatures were found for myoglobin or DNA (Sokolov, 1997; Sokolov et al., 1999). It was hypothesised (Sokolov, 1996) that the stabilisation of biological objects below T_c might be very important for their preservation and suppression of their degradation with time. Possibly, stabilisation of biological objects could be improved by changing the chemical composition such that T_c is optimally increased (Sokolov, 1996).

A well-known classification of glass forming liquids is that of "strong/fragile liquids" (Angell et al., 1994). For a strong liquid the temperature dependence above T_g corresponds to Arrhenius behaviour, whereas fragile glasses show a very exaggerated departure from Arrhenius behaviour (Angell et al., 1994). The position of a liquid in this classification can be interpreted in terms of the resistance of the short- and medium range order of the initially glassy structure to thermal disruption during increase in temperature above T_g . Liquids with structures that resist thermal degradation are called "strong" liquids (Angell et al., 1994). For fragile liquids, the crossover temperature was found to be around $1.15\text{--}1.2 T_g$, whereas for strong liquids, it is around $1.5\text{--}1.6 T_g$ (Sokolov, 1996, 1997). In our study, the T_c was $\sim 1.2 T_g$ for sucrose, $\sim 1.3 T_g$ for trehalose and raffinose, and stachyose, $\sim 1.5 T_g$ for PolyLys, and $>1.5 T_g$ for biological systems (Table 11.2). This indicates that intracellular glasses in biological systems are strong liquids, also evident from their moderate

temperature dependence in molecular mobility above T_g . It was speculated that liquids below the crossover temperature can be stable against crystallisation (Sokolov, 1996), which could explain the absence of crystallisation found in biological tissues (Sun and Leopold, 1993).

Composition of intracellular glasses: role for sugars and proteins

The exceptionally large temperature interval between T_g and T_c found in biological tissues could be beneficial for a prolonged lifespan. A better understanding of the composition of intracellular glasses might benefit the preservation of biomolecules. It is clear from Figs. 11.4 and 11.5 that sugars are not the only molecules participating in the intracellular glass formation. Apart from the difference in slopes between sugar and intracellular glasses, the undetected T_c in the latter suggest that other molecules are equally important in the glass formation. The large temperature interval between T_g and T_c observed for the polypeptide PolyLys indicates that proteins might fulfil a role in intracellular glass formation (Table 11.2). Proteins comprise over 20% of the dry weight of the cytoplasm of seed and pollen. Comparison of the temperature dependence of mobility in PolyLys glasses with intracellular glasses of bean (Fig. 11.5) showed that rotational motion in the two glasses increased parallel in both systems. A clear difference between the two glasses was the overall faster rotational motion of CP in the PolyLys as compared with intracellular glass at similar temperatures, probably due to the larger molecular free volume present in the PolyLys matrix. The slower rotational motion in the biological tissues indicates that, if proteins or other high M_w molecules form intracellular glasses, the free volume between those molecules is filled up with small molecules such as small sugars or amino acids. Indeed, addition of sucrose to PolyLys decreased the rotational motion at T_g (Fig. 11.4). This is confirmed by an FTIR study on the interaction of sucrose with PolyLys, where addition of sugar increased the molecular packing density of the total matrix (Wolkers et al., 1998a).

Apparently, sugars are not the predominant molecules that are associated with glass formation in dry biological systems. However, they are still present in large quantities in cells, suggesting that they have an important function in biological systems. They could play a role in filling the molecular free volume between the large molecules to increase the density of glasses, allowing less molecular mobility in the matrix. The high T_c of oligosaccharides might indicate that these large sugars might help to increase the T_c of the total systems to high values. Another role for sugars in dry anhydrobiotic systems comes from the "water replacement hypothesis" (for review see Crowe et al., 1998). In the present study, proteins were rapidly dried to form glasses, but sugars were found to be important in preventing changes in protein conformation during slow drying in PolyLys (Wolkers et al., 1998a). This prevention of conformational change was the result of direct hydrogen bonding interactions between the sugar and the protein. There is large body of evidence suggesting that direct interaction between sugars and membranes or proteins

11

prevent membrane fusion or changes of protein conformation (Prestrelski et al., 1993; Sun et al., 1996; Crowe et al., 1997b; 1998; Wolkers et al., 1998a). It was also demonstrated that small sugars are more capable of hydrogen bonding than large polymers such as hydroxyethyl starch (Crowe et al., 1997b).

This study indicates that proteins could fulfil a role in intracellular glass formation, as suggested previously (Sun and Leopold, 1997; Wolkers et al., 1998b, 1998c). It is likely that many different proteins participate in intracellular glass formation. Highly abundant proteins in the cytosol are those belonging to the family of the so-called LEA (late embryogenesis abundant) proteins (for review see Dure, 1997). Interestingly, these proteins have been associated with desiccation tolerance and longevity of seeds (Dure, 1997), and they have been suggested to participate in intracellular glass formation (Sun and Leopold, 1997).

Conclusions

Here, we demonstrated the occurrence of a kinetic change in molecular mobility at a critical temperature (T_c) above T_g in various glasses, measured by EPR and NMR spectroscopy. At T_c , the dynamics of the system changed from solid-like to liquid-like properties, accompanied by a large reduction in viscosity. The temperature interval between the T_g and T_c increased in the order of sucrose < trehalose < raffinose \leq staychose < PolyLys < biological tissues. The critical temperature in biological organisms was extremely high, at least 50°C above T_g . This large temperature interval is expected to give rise to very stable dry organisms, maintaining a slow cytoplasmic molecular motion even at temperatures far above T_g . Temperature dependent changes in molecular mobility in intracellular glasses resembled that of protein glasses, with T_c 50°C above T_g . Soluble sugars, present in large amounts in desiccation-tolerant organisms, exhibited a much smaller interval between T_g and T_c (between 17 and 35°C). Possibly, protein participates in the formation of intracellular glasses, in which small molecules are embedded that decrease the molecular free volume.

General Discussion

"Despite its ubiquity and long history of investigation, the nature of the glass still remains one of the most interesting and puzzling unsolved problems in the physics of condensed matter."

Alexei P. Sokolov (1997)

A better understanding of the storage behaviour of germplasm will advance the long-term preservation of genetic resources. From an applied perspective, insight in the factors contributing to seed longevity might lead to improved seed quality for commercial use. The main focus of our investigations was to understand the relations between intracellular glasses, cytoplasmic stability and storage longevity in seed and pollen. This study was based on the premise that a high cytoplasmic stability, i.e. low molecular mobility, will result in low rates of ageing reactions.

Seed companies use priming treatments (imbibition in an osmotic solution) to improve germination rate and uniformity. A disadvantage of this treatment, however, is that longevity can be impaired. Priming is also known to induce changes in the cytoplasmic composition (Hoekstra et al., 1994). Because the composition of the cytoplasm determines the cytoplasmic viscosity, a change in this composition is likely to affect viscosity, and hence the rate of ageing. Consequently, we reasoned that this compositional change will likely result in a decreased cytoplasmic viscosity and, thus, might explain the reduced longevity after priming.

We established that the composition of seeds indeed changed after priming, and that those components (oligosaccharides) that had disappeared were typically capable of increasing the stability of model glass systems. However, the attractive hypothesis mentioned above could not be confirmed, as the intracellular stability was not affected by the decline in the oligosaccharides. Nonetheless, the research performed in this thesis did lead to several discoveries, as listed below:

- i. The apparent absence of a role for oligosaccharides in the intracellular glass stability of seeds
- ii. The complexity of intracellular glass composition and behaviour
- iii. An exceptionally large temperature interval between T_g and the critical temperature T_c in biological tissues, which results in a high stability even far above T_g
- iv. The description of longevity through cytoplasmic molecular mobility as a function of temperature and water content
- v. The possibility to predict longevity by extrapolation to low temperatures at which no experimental data can practically be obtained

In the following part of this discussion, we will elaborate on the findings mentioned above, and comment on the current state of the art regarding characteristics and functions of intracellular glasses, including the methods applied. In the second part, we will highlight some of the implications of our findings.

Characteristics of intracellular glasses

Composition

sugars

When the concept of glasses was introduced in seed science, sugars were thought to take an important part in the composition of the glass. This assumption was based on the fact that sugars are present in large amounts in desiccation-tolerant tissues and are known to be excellent glass formers. Because models of sugar glasses imply that the molecular mobility increases dramatically above T_g (Williams et al., 1955; Slade and Levine, 1991a; Roos, 1995), it was assumed that this would also be true for intracellular glasses. The correlation between oligosaccharides and longevity (Horbowicz and Obendorf, 1994; Lin and Huang, 1994; Bernal-Lugo and Leopold, 1995; Sun and Leopold, 1997) and the knowledge that oligosaccharides increase T_g in model systems (Slade and Levine, 1991a; Roos, 1995) added to the notion that sugars are pivotal for *in vivo* glass formation. In the first chapters of this thesis, we suggested that the elevated state diagram of seeds compared to pollen could be explained by the exclusive presence of oligosaccharides in seeds. In the course of this thesis, we realised that this view was rather naïve, and that the composition of glasses was much more complex than initially assumed. We discovered that a difference in sugar composition *in vivo* did not change T_g (chapter 9) or the molecular mobility in the intracellular glass (chapters 9 and 10). Furthermore, state diagrams for seeds from different plant species are almost similar (Williams and Leopold, 1989; Bruni and Leopold, 1991; Leopold et al., 1994; Leprince and Walters-Vertucci; Sun, 1997b; Buitink et al., 1998a) despite large differences in sugar compositions. This suggests that, besides sugars, other molecules play a crucial role in intracellular glass formation, a conclusion that is supported by recent literature on intracellular glasses (Sun and Leopold, 1993, 1997; Sun et al., 1994; Leprince and Walters-Vertucci, 1995; Wolkers, 1998). It is remarkable that there is *not even a small* effect on intracellular glass properties when the sugar composition or content is changed (Sun et al., 1994; this thesis).

mobility
above T_g

The innovative approach of measuring rotational motion has given us a stunning insight into the differences between biological glasses and sugar- or polymer glasses. Comparison between rotational motion of a probe molecule in oligosaccharide and sucrose glasses confirmed the expected higher T_g and lower molecular mobility for oligosaccharides (chapter 11). Surprisingly, the temperature dependence of the molecular mobility above T_g was found to be much steeper for all these sugar glasses than for intracellular glasses. Over the first 10°C above T_g the activation energy for rotational motion in sucrose glasses increased by a factor 40. In contrast, this increase was only a factor 2 to 3 in intracellular glasses (chapter 5 and 11). In addition, Wolkers et al. (1998a, 1998c) found that the molecular density (i.e. hydrogen bonding strength) of dry seeds of *Arabidopsis thaliana* was quite different from that of a sugar glass, but much more comparable to that of a protein-sugar glass. Our findings also point to a role for proteins in intracellular glass formation (chapter

proteins

11). The temperature dependence of the rotational motion above T_g in intracellular glasses is much more comparable with protein glasses than sugar glasses, with the activation energy also changing a factor 2 to 3 (chapter 11). Although more research is needed to elucidate what type of proteins may play a role in the glass formation, one could speculate that late embryogenesis abundant (Lea) proteins are possible candidates (chapter 11; Sun and Leopold, 1997; Wolkers, 1998). Dure (1997) pointed out that it is the extent of the accumulation in late embryogenesis that seems to be the most distinctive feature of Lea proteins. This might suggest some role for Lea proteins in the overall protection of the cytoplasm during drying. Wolkers et al (1999) suggested that Lea proteins that are embedded in the glassy matrix might confer stability to slowly dried carrot somatic embryos. Indeed, Lea proteins changed the hydrogen bonding properties of model sugar systems in the direction of those of intracellular glasses, pointing to a possible participation of Lea proteins in intracellular glass formation (Wolkers, 1998).

Molecular mobility and stability

A remarkable observation originating from the ST-EPR measurements was the occurrence of a second kinetic change in mobility at a definite temperature above T_g , referred to as the critical temperature T_c . This temperature-dependent phenomenon was found to correlate with the so-called crossover temperature, at which the dynamics change from solid-like to liquid-like, known from polymer science (Sokolov, 1996, 1997; Rössler et al., 1994, 1998). It was not until recently that this critical temperature T_c was also detected in sugar glasses (Van den Dries et al., 2000a). The occurrence of T_c has been coupled to the collapse temperature of sugar glasses, a well-documented phenomenon that is attributed to a reduction in viscosity such that a flow on a practical time scale is observed. The collapse temperature has been associated with dramatic changes in matrix stability, leading to phenomena such as loss and oxidation of encapsulated lipids and flavours, loss of enzymatic activity, nonenzymatic browning, stickiness and caking, or structural collapse (Levine and Slade, 1988; Slade and Levine, 1991; Roos, 1995). Chapter 11 presents the first study on the existence or, rather, *absence* of T_c in biological tissues. This is because T_c could not be detected within the temperature range of 0 to 50°C above T_g . Considering that above T_c there is a strong reduction in viscosity, the absence of T_c in biological tissues implies that the system is very stable, still exhibiting a high viscosity ($> 10^8$ Pa s) far above T_g . Interestingly, we found that this T_c occurs at much higher temperatures above T_g in protein glasses than in sugar glasses, pointing once more to a role of proteins in intracellular glasses. The discovery that oligosaccharides have some intermediate T_c between sucrose and proteins suggests that they can partially elevate T_c in intracellular glasses, thereby contributing to the high temperature stability of biological tissues. Unfortunately, thus far it was impossible to investigate the effect of a changed oligosaccharide content on T_c of intracellular glasses because T_c was beyond detection in biological tissues.

critical
temp T_c

Further studies will be needed to investigate the role of oligosaccharides in the high temperature stability of desiccation-tolerant systems.

WLF
equation

One of the consequences of the existence of T_c is that models that describe viscosity or molecular mobility above T_g are valid only above T_c (Rössler et al., 1998). Since biological tissues do not exhibit a measurable T_c within the range of temperatures that are of biological significance, we can conclude that application of the Williams-Landel-Ferry (WLF) equation (such as performed in chapter 5; Sun, 1997b and Sun et al., 1998) should be considered with great caution. Again, this shows the importance of *actual measurements* when studying complex systems, instead of using simple extrapolations derived from model systems.

Evaluation of methods used to characterize glass properties

DSC

From the results presented in this thesis, it is clear that the use of only DSC to predict stability of a sample by simply measuring T_g is often not adequate enough. As emphasised in this work, measurements on a molecular level, such as mobility of molecules, rather than T_g measurements by DSC should be considered as a tool to investigate optimal storage conditions (chapters 2 and 3). Many conclusions in the literature have been made on the assumption that T_g reflects directly the mobility on the basis of the existence of iso-mobility lines (Roos, 1995) that run parallel to the T_g curve. These lines were indeed found for pea embryonic axes above T_g (chapter 5), but did not run parallel to T_g for pollen (J. Buitink, unpublished results). Also below the T_g curve of pea or cattail pollen iso-mobility lines are not parallel to the T_g curve. Another pivotal aspect that was completely ignored by the sole use of DSC was that samples with similar T_g values could exhibit a quite different behaviour in their mobility above T_g . As shown in chapter 11, biological tissues or even protein glasses show a much less dramatic increase in rotational motion above T_g than sugar glasses, despite an almost similar T_g value.

ST-EPR
spectroscopy

Although much insight into the characteristics of glasses can be gained by the use of ST-EPR spectroscopy, some caution has to be exerted when interpreting ST-EPR spectroscopy results. First of all, the spin probe has to be introduced, which requires hydration of the tissues. A short hydration treatment was previously found to have little effect on desiccation tolerance and storage stability of barley (Hong and Ellis, 1992), lettuce (Tarquis and Bradford, 1992), mung bean (Hong and Ellis, 1992), and wheat seeds (Nath et al., 1991). However, we found a slight increase in storage stability when *Impatiens* seeds received a short hydration treatment (J. Buitink, unpublished results). Therefore, proper controls should be included when performing physiological experiments. Furthermore, ST-EPR spectroscopy requires the use of a reference material to derive the rotational correlation time from the line shape parameters of the spin probe in the tissue. To do this, one needs to incorporate a dimensionless interaction parameter (λ), which is a measure of the coupling of the rotational motions of the spin probe to the shear modes of the fluid. This parameter has been found in many cases to be independent of temperature and viscosity

(Roosen and Hemminga, 1990), but it does depend on specific solvent-probe interactions and the geometry of the molecules considered. In our studies, this parameter was based on the behaviour of the spin probe in anhydrous glycerol. Possibly, interaction of the spin probe with the cytoplasmic environment might result in an interaction parameter different from that with glycerol. In addition, the assumption that the interaction parameter is constant as a function of water content and temperature cannot be verified for our complex systems. Therefore, the absolute value given to the rotation of the spin probe might not be completely accurate (see equation 5.2, chapter 5). It has to be noted that the temperature dependence will *not* be affected when the interaction parameter would have a value slightly different from that used in our studies (eqn 5.2). Despite possible small errors in the absolute value of the rotational correlation times, it is clear from the results presented in this thesis that measurements of molecular mobility do contribute to the understanding of intracellular glasses, and at present, EPR spectroscopy is the only applicable technique to gain access to viscosity or molecular mobility *in vivo*.

Function of intracellular glasses

Glass formation has been detected in seeds (Williams and Leopold, 1989; Bruni and Leopold, 1992; Leopold et al., 1994; Leprince and Walters-Vertucci, 1995), pollen (chapter 1) and the resurrection plant *Craterostigma plantagineum* (J. Buitink, unpublished results). Apparently, it is a characteristic typical of all desiccation-tolerant tissues. Glass formation is therefore an intrinsic property of any complex system that can dry out and survive (Walters, 1998). However, desiccation-intolerant systems can form glasses as well (Sun et al., 1994; chapter 1), and in recalcitrant seeds, desiccation results in loss of viability at water contents far above those water contents at which the tissues reach the glassy state (Pammenter et al., 1993; Pammenter and Berjak, 1999). Thus, glass formation is not a mechanism that initially confers the tolerance to desiccation during drying, but the formation of intracellular glasses is indispensable to survive the dry state, as will be explained below.

desiccation
tolerance

One of the most studied functions of glasses is maintaining the structural and functional integrity of macromolecules (for reviews see Slade and Levine, 1993; Roos, 1995). Many model systems have shown that glasses retain the activity of enzymes and conformation of proteins or retards crystallisation due to their high viscosity (Lim and Reid, 1991; Roos and Karel, 1991; Karmas et al., 1992; Karel et al., 1993; Slade and Levine, 1993; Bell and Hageman, 1996; Chang et al., 1996; Duddu and Dal Monte, 1997; Hancock and Zografis, 1997; Sun, 1997a; Crowe et al., 1998; Sun and Davidson, 1998). It has also been postulated that glasses are capable of maintaining stability of membranes. Indeed, glasses were able to inhibit membrane fusion, although the depression of the melting transition temperatures of the membranes appeared to be caused by direct hydrogen bonding of the sugar with the phospholipid headgroups (Sun et al., 1996; Crowe et al., 1997b, 1998).

structural
and
functional
integrity

One of the major functions of the glassy state in tissues is the avoidance of crystallisation (Leopold et al., 1994; Sun and Leopold, 1997). The complex cytoplasmic composition is most probably responsible for preventing crystallisation, but considering that the tissues are at all times below their critical temperature T_c (chapter 11), crystallisation is unlikely to occur due to the persistent high viscosity (Sokolov, 1996).

We showed that an extended longevity correlated with the presence of the glassy state, implying that the viscosity of the intracellular glass slows down detrimental ageing reactions. Evidence for the formation of a highly viscous cytoplasm comes from the measurements of rotational motion during drying (chapter 5). We found that the rotational motion in pea embryonic axes increased approximately 5 orders of magnitude during drying. An indication for a relation between ageing rate and molecular mobility originates from the similar shape of the temperature-dependence of ageing rate and of rotational motion. Also the activation energy of the ageing rate increased with the same order of magnitude as that of rotational motion upon melting of the glassy state, merely a factor 2 to 3 (section III). But probably the most compelling evidence to suggest that ageing rates are affected by the molecular mobility of the environment came from the linear relationship between ageing rate and molecular mobility found for many different tissues over a wide range of temperatures and water contents (section III). Recently, the storage stability of seeds of *Arabidopsis thaliana* was found to correlate with density of the molecular matrix (Wolkers et al. 1998c). All these arguments suggest that ageing rates and thus lifespan of germplasm will be influenced by the molecular stability of the cytoplasm, signifying an important function of intracellular glasses in conferring stability during storage.

A key discovery that was made as a result of the molecular mobility measurements (chapter 11) is the existence of a critical temperature T_c , which reflects the temperature at which the dynamics of the viscous matrix is dominated by diffusional motion (see previous section). In sugar glasses, this T_c was found to occur 10-30°C above T_g , whereas in biological tissues this T_c could not be detected at temperatures as high as 50°C above T_g . This finding explains the initially strange result (chapter 5) that melting of intracellular glasses did not result in a fast rotational motion ($\tau_r < 10^{-9}$ s) as found for sugar glasses. The absence of a T_c in biological tissues has important implications for the storage stability of germplasm in its natural environment. Under ambient conditions, say 20°C and 50% RH, seed tissues are around their T_g . This means that any environmental fluctuation that results in an increase in RH or temperature will bring the tissue above its T_g . The unique composition of the intracellular glass protects the tissue from dramatic changes caused by environmental fluctuations. If the intracellular glass were composed of sucrose alone, a small increase in RH or temperature would bring the sucrose glass above its T_c , resulting in collapse, crystallisation and loss of macromolecule function and integrity. Therefore, the characteristically high (or absence of) T_c of intracellular glasses serves as an eco-physiological advantage. This

extremely large temperature interval between T_g and T_c could be an explanation for the long lifespan of dry biological tissues under natural conditions.

Perspectives for seed storage physiology

Ageing kinetics can be described by cytoplasmic molecular mobility: opportunity to predict lifespan

The results of this thesis provided insight into the field of seed storage physiology. Foremost, the molecular mobility of small molecules in the cytoplasm of cells can be used to describe the rate of ageing reactions in relation to temperature and water content. The existence of this relationship has been hypothesised for several years (Leopold et al., 1994; Sun et al., 1994; Leprince and Walters-Vertucci, 1995; Sun, 1997). Yet, the results presented in section III for the first time show that there is a direct link between *actual* measurements of molecular mobility (not derived from a model) and the rate of ageing, measured as a loss of both percentage germination and vigour. The results show that there is a change in the activation energy of ageing kinetics around T_g , which has important implications when extrapolations are made from ageing rates obtained at high temperatures (above T_g) to low temperatures. Chapters 2, 3 and 6 to 8 clearly demonstrate that this extrapolation would result in predicted ageing rates that are lower than the actual experimental data. Incorporation of the concept of molecular mobility influencing ageing kinetics into an existing viability model such as the viability equation (see introduction) would result in an even more accurate viability model. This model, then, will also be accurate at low temperatures by taking into account the change in ageing kinetics below T_g .

Even though there is a perfect linear relationship between ageing rate and mobility as a function of temperature and water content (section III), the linear regressions do not overlap each other (figures 6.7, and 8.2 to 8.5). There appears to be an additional effect of these two parameters on the relationship between τ_r and ageing rate. It could be imagined that the amount of water present in the tissues would be used partially in reactions - the more water, the faster the reaction. Similarly, temperature will affect the rate of reactions. Therefore it is not unlikely that water and temperature will have a direct effect on reaction rates besides exerting an effect on the local viscosity of the intracellular glass.

It has to be stressed here that differences in longevity cannot *always* be explained by changes in molecular mobility. Examples are the reduced longevity after priming without a change in molecular mobility, and the intrinsic difference in longevity between pollen and seeds. Although cattail pollen has a comparable rotational motion as found for seeds, its lifespan is much smaller. Clearly other factors besides cytoplasmic mobility play a role in the control of ageing rates, such as number of cells, type of ageing reactions and level of repair and defence mechanisms.

One of the consequences of the apparent linear relationship between cytoplasmic molecular mobility and ageing rates is that this linear regression can be extrapolated to get access to ageing rates at low temperatures. Our calculated predictions of longevity (chapter 8) were different from those obtained using the viability equation. First of all, our predictions indicated that drying below a critical water content resulted in decreased longevity. When the predicted longevity was compared to previous predictions, made using the viability equation, it appeared that the viability at higher water contents was much *higher* than initially thought (fig 8.6). A question that remains is whether the predicted values using the molecular mobility are reasonable. For instance, at 12% moisture, the longevity of pea seeds is predicted to be around 2,500 years, and stored at -18°C it is predicted to be 30,000 years! There is no literature that records such longevities for seeds. It is likely that most old seeds in nature have been predated or damaged by fungus infections. The oldest viable seed for which the age was accurately determined using radiocarbon dating was around 1,250 years (Shen-Miller et al., 1995), but as with most studies on ancient seeds, the conditions of storage are imprecise, making comparisons with predictions impossible (Priestley, 1986). The only slight hint that certain seeds might indeed reach a great longevity comes from a study by Porsild et al. (1967), who reported successful germination of arctic lupine (*Lupinus arcticus*) seeds, recovered from frozen silt in the Canadian Yukon. Based on the remains of nearby lemming remains, it was suggested that the seeds were probably 10,000 years old. Unfortunately, no direct radiocarbon dating of the seeds was provided, and the assertion of the seeds' great longevity has in consequence been subjected to considerable criticism (Priestley, 1986).

Site of cellular damage during ageing: cytoplasm as controlling factor?

Based on the extensive literature pointing to membranes as the main target for detrimental reactions, it is a little surprising to find that ageing rates are linked to mobility in the cytoplasm rather than mobility in membranes (Priestley, 1986; Wilson and McDonald, 1986; Van Bilsen and Hoekstra, 1993). There are several explanations for this discrepancy. First, the initiation and/or propagation of ageing reactions may take place in the cytoplasm. Another explanation of the existing relationship between cytoplasmic mobility and lifespan is that the viscosity of the cytoplasm might influence the rigidity and mobility of the membranes (Lee et al., 1986). Moreover, we found that the mobility of the spin probe in lipids followed the temperature dependence found for spin probe in the cytoplasm, and the change in mobility around T_g was also visible with the mobility in the lipids (J. Buitink, unpublished results). Bruni and Leopold (1991) also showed that mobility in the lipids could be used to construct a state diagram, although at the time they were not aware that the apolar spin probe had completely partitioned into the lipids. These findings imply that the stability of the glassy state in which lipids and membranes are embedded affects their mobility. Because we have not measured molecular mobility in membranes, this possibility remains to be answered.

Existence of an optimum water content that shifts with temperature

Measurements of both survival of pollen and rotational motion in relation to temperature and water content (chapters 2, 3, 4 and 6) confirm the existence of an optimum water content of storage which shifts with temperature, in a manner similar as found by Vertucci and co-workers (Vertucci and Roos, 1990, 1993; Vertucci et al., 1994b). Also, the predictions of longevity or vigour loss suggest the existence of an optimum water content, even as high as 0.12 g H₂O g dw⁻¹ at 0°C (chapter 8). The implications of these findings are important in regard to the conservation of genetic material. Because of the increase in optimum water content with lower temperatures, too much drying should be avoided when cryogenic storage is planned, as the beneficial effects of the low temperature will then be lost partially. It has to be noted, though, that longevity still is remarkably high at storage temperatures below freezing, even when tissues are dried to 5% moisture.

Causes for reduced longevity after priming

As mentioned before, our research indicated that there is no change in intracellular glass properties after priming of seeds which could be responsible for the increased ageing rate (chapter 9 and 10). Because the question as to why priming leads to reduced longevity was still open, we performed some preliminary experiments, which suggest that priming of seed results in a change in membrane properties. Measurements of membrane permeability using spin probe EPR spectroscopy demonstrated that priming of seeds resulted in an increase of membrane permeability in the radicles after drying and rehydration, as compared with non-treated seeds (J. Buitink, unpublished results). The reason for this increased membrane permeability is unclear. Perhaps priming leads to a reduction in protective mechanisms or repair mechanisms. The discovery that the reduced longevity of seeds after priming can be partially restored by a combined heat shock and dehydration treatment (Bruggink et al., 1999) could indeed point to a loss of protective chaperones during priming that are re-induced during the stress treatment. Additionally, the respiration rate is known to increase during priming (Smith and Cobb, 1992; Dawidowicz-Grzegorzewska, 1997). High respiration rates are thought to promote the production of free radicals upon drying (Leprince et al., 1993, 1994). The observation that the increased membrane permeability was especially detectable in the radicles (where respiration rates are likely to be highest) could favour the latter explanation.

Role of oligosaccharides in storage stability

The question of whether and how sugars, and especially oligosaccharides, play a role in storage stability in seeds, is still actual. Considering that the oligosaccharides make up only 4% of the dry weight in the seeds (chapter 10), it is maybe not surprising that no effect on intracellular glass properties could

be measured. A likely role for sugars in seeds is the protection of macromolecular structures in the dry state, especially membranes (Crowe et al., 1992). Hydrogen bonding of sugar molecules with macromolecules will increase their stability (Crowe et al., 1998). However, this explanation does not provide a clue for the role of oligosaccharides in particular. Proposed roles for oligosaccharides in longevity included increased stabilisation of the glassy state, for which no evidence could be found in our investigations. Also, oligosaccharides in seeds have been suggested to be able to prevent crystallisation. Notwithstanding the argument that oligosaccharides are indeed capable of preventing crystallisation (Caffrey et al., 1988), this characteristic would most probably not be required *in vivo* due to the complex mixture of all the different components in the cytoplasm, as mentioned before. Because the maturation stage of seeds seems to correlate with storage stability (Demir and Ellis, 1992; Sinniah et al., 1998), and oligosaccharides accumulate during maturation (Leprince et al., 1990; Blackman et al., 1992; Brenac et al., 1997; Sinniah et al., 1998), it could be possible that oligosaccharides are linked *indirectly* to storage stability, being merely an indicator of seed maturity. This reasoning could indeed explain some of the discrepancies found in the correlation between oligosaccharide content and longevity (chapter 10).

Perspectives for food- and pharmaceutical sciences

The results found in this thesis do not only have an impact on preservation of biological tissues; there might also be some implications for food- and pharmaceutical sciences. First of all, the study of intracellular glasses has provided insight into the behaviour of complex, multi-component model systems compared with simple, two-component systems. It is clear that, apart from determination of the T_g value of a formulation, measurements of molecular mobility (or any other comparable characteristic) have to be included to understand the stability around T_g . The application of theoretical models to describe glass properties can be misleading, especially in complex systems. The discovery of the critical temperature T_c and its dependence on the composition of the sample improves the understanding of the physical properties and stability of complex systems. From a preservation point of view, it appears that optimal stability of a formulation will be obtained using a mixture of various compounds with specific characteristics: to provide hydrogen bonding (small M_w sugars), to increase the T_g and T_c of the formulation (for instance proteins), and to fill small voids. The resulting mixture having a high T_c is expected to evade crystallisation, even at temperatures far above T_g , guaranteeing a stable product.

Conclusion

This research demonstrates that biological glasses are complex systems with properties quite different from those of sugar glasses. Characterization of the temperature dependence of molecular mobility in sugar-, protein- and

General Discussion

intracellular glasses revealed the existence of a critical temperature (T_c) above T_g , where the dynamics change from solid-like to liquid-like. In sugar glasses, this T_c occurred at 15-35°C above T_g , whereas in intracellular glasses, it occurred at temperatures >50°C above T_g . This implies a high stability and low molecular mobility above T_g in biological systems, which could serve as an ecological advantage. Similarity between the temperature dependence of intracellular glasses and protein glasses suggests that proteins are a major component involved in intracellular glass formation.

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Summary

As a result of drying, the cytoplasm of desiccation-tolerant organisms, such as seed and pollen, enters into a highly viscous, solid-like, semi-equilibrium state: the glassy state. The work in this thesis is focussed on the function and characteristics of intracellular glasses in these organisms.

It was established that intracellular glasses are formed in both desiccation-tolerant and -intolerant pollen at around 10% moisture during drying at room temperature. (chapter 1). However, desiccation-intolerant pollen loses its viability during drying before intracellular glasses are formed. This indicates that the formation of glasses during drying is not reason for desiccation tolerance. Storage of cattail (*Typha latifolia*) pollen under different water contents and temperatures revealed the existence of an optimum water content for survival at a constant relative humidity (11-15%) (chapter 2). The water content corresponding to this relative humidity shifted to higher values with lower storage temperatures, and was found to be associated with the Brunauer, Emmet, and Teller monolayer value. Drying of the pollen below these water contents had detrimental effects on longevity. The water content-temperature combinations of optimal storage were found to be below the glass transition curve, indicating that optimum storage conditions are achieved when intracellular glasses are present. There was a change in ageing kinetics of cattail pollen associated with the melting of the intracellular glass. Above the glass transition temperature (T_g) the activation energy of the ageing rates increased two to three times. This suggests that the presence of glasses in the dry state improves storage stability by decreasing viscosity and, thus, ageing rate. It was concluded that T_g curves might be useful for predictions of storage longevity above optimum water contents. However, they cannot be used solely to predict the precise conditions of optimum storage. Subsequently, we sought for a more direct measurement to assess the viscosity of the cytoplasm of tissues.

For this purpose, we used electron paramagnetic resonance (EPR) spectroscopy to study the molecular mobility of the hydrophilic nitroxide spin probe 3-carboxy-proxyl (CP) that was incorporated into embryonic axes of pea seeds and cattail pollen. Using the distance between the outer extrema of the EPR spectrum ($2A_{zz}$) as a measure of molecular mobility, a sharp increase in mobility was observed at a definite temperature during heating (chapter 3). This temperature increased with decreasing water content of the samples, and was found to be associated with the melting of the glassy state as measured by differential scanning calorimetry (DSC). Molecular mobility was found to be inversely correlated with storage stability: the higher the molecular mobility, the shorter the longevity: with decreasing water content, the molecular mobility reached a minimum, in concert with ageing rates. At very low water contents, both molecular mobility and ageing rates increased again. Minimum mobility and maximum storage stability occurred at similar water contents, suggesting

that storage stability might be partially controlled by molecular mobility. To understand the nature of the changes in $2A_{zz}$ in spectra of CP in the tissues, echo detected (ED) EPR spectroscopy was employed (chapter 4). The shape of the ED EPR spectrum revealed the presence of librational motion of the spin probe, a motion typically present in glassy materials. The change in $2A_{zz}$ appeared to be the result of librational motion of the spin probe.

With the use of saturation transfer (ST) EPR spectroscopy, a more quantitative measure of molecular mobility was acquired: the rotational correlation time (τ_r), which corresponds to the time it takes for the spin probe to rotate a radian around its axis (chapter 5). At room temperature, τ_r of CP in pea embryonic axes increased during drying from 10^{11} s in dehydrated state to 10^4 s in the dry state. At T_g , τ_r was constant at $\sim 10^4$ s for all water contents studied. The temperature dependence of τ_r at all water contents studied followed Arrhenius behaviour with a break at T_g . The temperature effect on τ_r above T_g was much smaller in pea axes as found previously for sugar and polymer glasses. Thus, the melting of the intracellular glass by raising the temperature caused only a moderate increase in molecular mobility in the cytoplasm as compared to a huge increase in amorphous sugars.

The application of saturation transfer EPR spectroscopy to biological tissues enabled a quantitative comparison between storage stability and molecular mobility in different tissues (section III). The temperature and moisture dependence of ageing rates of seeds and pollen was found to correlate with the rotational motion of CP in the cytoplasm (chapter 6-8). An increase in the temperature resulted in a faster rotational motion in the cytoplasm of cattail pollen, analogous to faster ageing rates (chapter 6). Decreasing the water content of the pollen resulted in a decrease in rotational motion until a minimum was reached, after which rotational motion slightly increased again. The water content at which this minimal rotational motion was observed increased with decreasing temperature, comparable to the pattern of ageing rate. A significant linear relationship was found between ageing rates and rotational motion in the cytoplasm of the pollen.

We also investigated the relationship between the longevity of lettuce seeds and the molecular mobility in the cytoplasm of their radicles (chapter 7). Longevity of lettuce seeds was predicted using the viability equation of Ellis and Roberts. Increasing the temperature resulted in faster rotational motion and shorter longevity. There was a linear relationship between the logarithms of rotational motion and estimated longevity for temperatures above 5°C , which is the same temperature range for which experimental data were used to obtain the viability constants of the viability equation. Below 5°C , there was a deviation from linearity, which might stem from inaccurate predictions by the viability equation at low temperatures.

Chapter 8 further demonstrates that there is a linear relationship between the logarithms of rotational motion in the cytoplasm of seed and pollen of several plant species and their ageing rates or longevities. This linearity suggests that cytoplasmic mobility might be an important controlling factor of

ageing rates. The linear relationship between the two parameters could be used to predict lifespan of germplasm at low temperatures (at which experimental determination of longevity is practically impossible) by simply measuring the τ_R values at these low temperatures (chapter 7 and 8). Based on the predictions using the linear regression between ageing rate and rotational motion of CP in pea embryonic axes, an optimum water content of storage was found. This optimum water content shifted to higher values with lowering the storage temperature, as was found previously for cattail pollen based on experimental data (chapter 2). It was predicted that the longevity of seeds at high (0.12 to 0.16 g/g) water content is much higher than previously suggested on the basis of the viability equation. The predictions show that drying germplasm too far leads to decreased longevity compared to storage of germplasm at higher water contents, suggesting that current storage protocols might have to be re-examined.

Desiccation-tolerant organisms contain large amounts of soluble sugars. This, and the fact that sugars are excellent glass-formers has led to the suggestion that sugars play an important role in intracellular glass formation. The presence and amounts of oligosaccharides have been found to correlate with longevity. Furthermore, oligosaccharide glasses are known to increase the T_g and viscosity of model sucrose glasses. This suggests that oligosaccharides might enhance the stability of intracellular glasses (chapter 9 and 10). Osmo-priming, i.e. pre-imbibition of seeds in an osmotic solution, can result in a decrease in oligosaccharide content and longevity. Priming pea seeds decreased the total oligosaccharide content in the embryonic axes (chapter 9). Despite the change in oligosaccharide:sucrose ratio, no differences in T_g values were detected in the dry axes before and after priming as determined by DSC. Also no difference was found between the rotational mobility of CP in dry untreated axes and that of dry primed axes. Chapter 10 demonstrates that osmo-priming of impatiens and bell pepper seeds resulted in considerable decreases in longevity and oligosaccharide contents, while sucrose contents increased. Again, no differences in the T_g curves were found between control and primed impatiens seeds. Similarly, there was no difference in rotational motion of CP in the cytoplasm between control and primed impatiens seeds and between control and primed bell pepper embryonic axes. It was concluded that oligosaccharides in seeds do not appear to affect the stability of the intracellular glassy state, and that the reduced longevity after priming is not the result of increased molecular mobility in the cytoplasm.

To understand the nature and composition of biological glasses we investigated the molecular mobility around T_g in sugars, poly-L-lysine and dry desiccation-tolerant biological systems, using ST-EPR, $^1\text{H-NMR}$ and FTIR spectroscopy. Two distinct changes in the temperature dependence of molecular mobility were detected in sugars and poly-L-lysine. With heating, the first change was associated with the melting of the glassy state (T_g). The second change, at which the molecular mobility abruptly increased over several

Summary

orders of magnitude, was found to correspond with a critical temperature (T_c) where the dynamics of the system changed from solid-like to liquid-like. The temperature interval between T_g and T_c increased with increasing molecular weight of the sugars. The interval between T_g and T_c in biological tissues was over 50°C, implying that the stability remained high even at temperatures far above T_g . A comparably high T_c-T_g interval was found for the molecular mobility of poly-L-lysine, suggesting that proteins rather than sugars play an important role in the intracellular glass formation. The exceptionally high T_c of intracellular glasses is expected to provide excellent long-term stability to dry organisms, maintaining a slow molecular motion in the cytoplasm even at temperatures far above T_g .

Samenvatting

Uitdroogtolerante organismen zoals zaden en stuifmeel kunnen het overgrote deel van hun cellulaire water kwijt raken zonder hun levensvatbaarheid te verliezen. Het cytoplasma van droge zaden en stuifmeel bevindt zich in een zogeheten "glas-toestand". Een glas is een semi-equilibrium matrix met een enorme hoge viscositeit, zonder geordende kristalstructuur – anders gezegd: een "vaste vloeistof". Het werk in dit proefschrift betreft de functies en eigenschappen van intracellulaire glazen die gevormd worden in uitdroogtolerante organismen.

In Sectie I is de rol van glazen in relatie tot uitdroogtolerantie en overlevingsduur bestudeerd. Intracellulaire glazen worden gevormd in zowel uitdroogtolerant stuifmeel als -intolerant stuifmeel tijdens drogen, bij ongeveer 10% vochtgehalte (hoofdstuk 1). Het intolerante stuifmeel bleek de vitaliteit te verliezen voordat het cytoplasma een glasachtige structuur kon vormen, waaruit blijkt dat de vorming van glazen niet de verklaring voor de uitdroogtolerantie kan zijn.

Verouderingsexperimenten bij verschillende temperaturen lieten zien dat de langste overlevingsduur optrad onder opslagcondities van 11 tot 15 % relatieve luchtvochtigheid (RV) (hoofdstuk 2). Opslag van stuifmeel bij lagere RV had een negatief effect op de levensduur. Het watergehalte dat overeenkwam met deze optimale RV nam toe met een lagere bewaringstemperatuur. De optimale condities van opslag vielen samen met de condities waaronder het cytoplasma van het stuifmeel in een glas-toestand was. Het smelten van het intracellulaire glas in stuifmeel door verhoging van de temperatuur resulteerde in een toename van de activeringsenergie van verouderingsprocessen. Dit impliceert dat de aanwezigheid van intracellulaire glazen in stuifmeel de overlevingsduur aanzienlijk verbetert, mogelijk door de hoge viscositeit van het glas.

Omdat metingen van de glastransitietemperatuur (T_g) alleen niet genoeg informatie kunnen verschaffen over de viscositeit van het cytoplasma van uitdroogtolerante organismen werd gezocht naar additionele technieken die een meer directe meting van de viscositeit van het cytoplasma mogelijk maken. Sectie II bespreekt verschillende toepassingen van electron paramagnetische resonantie (EPR) spectroscopie. Met behulp van deze techniek kan de moleculaire beweeglijkheid van een gastmolecuul (een "spin probe") bepaald worden. Het blijkt dat de probe een zeer hydrofiel molecuul moet zijn om er zeker van te zijn dat de probe zich in het polaire cytoplasma bevindt tijdens drogen. De beweeglijkheid van de spin probe 3-carboxyproxyl (CP) in droge zaden van erwten en stuifmeel van lisdodde nam toe met een verhoging van het watergehalte en de temperatuur (hoofdstuk 3 en 4). Het smelten van het intracellulaire glas resulteerde in een abrupte toename van de beweeglijkheid van de probe. Maximale overleving van het stuifmeel bij verschillende bewaartemperaturen ging gepaard met minimale beweeglijkheid. Met behulp van "saturation transfer" EPR spectroscopie werd het mogelijk een meer

kwantitatieve benadering te verkrijgen van de beweeglijkheid van de spin probe: de rotatiecorrelatietijd (τ_r) (hoofdstuk 5). Deze τ_r komt overeen met de tijd voor de probe om ongeveer 60° rond zijn as te draaien. Des te langer de rotatiecorrelatietijd, hoe langzamer de spin probe roteert. Metingen van de τ_r in worteltjes van erwtenzaden lieten zien dat de toename in beweeglijkheid boven T_g veel minder sterk was dan verwacht volgens de theorie van suiker-glazen, die een toename van twaalf orden in grootte voorspelt.

In Sectie III werd de hypothese getest dat de levensduur van droge uitdroogtolerante organismen gerelateerd is aan de moleculaire beweeglijkheid van CP in het cytoplasma van de organismen. Het effect van temperatuur en watergehalte op snelheid van verouderingsreacties in zaden en pollen bleek te correleren met de rotatietijd van CP in het cytoplasma. Een vermindering in deze rotatiecorrelatietijd van de probe in het cytoplasma van lisdodde stuifmeel (dus toegenomen beweeglijkheid) correleerde met een verhoging van de verouderingssnelheid van het stuifmeel (hoofdstuk 6). Wanneer beide parameters uitgezet werden op een logaritmische schaal bleek er een lineair verband te bestaan tussen de moleculaire beweeglijkheid en de snelheid van veroudering. Een vergelijkbare lineaire relatie bleek te bestaan tussen de levensduur van slazaden, voorspeld met behulp van de "viability equation" van Ellis en Roberts, en de cytoplasmatische moleculaire beweeglijkheid van CP in de embryo-as van de zaden (hoofdstuk 7). Beneden 5°C wijkt de relatie af van het lineaire verband, wat het resultaat zou kunnen zijn van de inaccuraten voorspellingen door de viability equation bij lage temperaturen. In hoofdstuk 8 wordt de lineaire relatie tussen levensduur of verouderingssnelheid en moleculaire beweeglijkheid in het cytoplasma nogmaals aangetoond bij verschillende zaden en stuifmeel, zowel als functie van veranderd watergehalte als veranderde temperaturen. Het lineaire verband suggereert dat de cytoplasmatische beweeglijkheid een belangrijke factor is in de regulatie van de snelheid van verouderingsreacties.

De lineaire relatie kan gebruikt worden om de levensduur van droge organismen bij lage temperaturen te voorspellen door de rotatiesnelheid van de probe te meten bij deze lage temperaturen en vervolgens de levensduur te voorspellen via extrapolatie. Omdat de levensduur van zaden bij lage temperatuur te lang is om experimenteel te bepalen, is deze extrapolatie de enige mogelijkheid om inzicht te krijgen in de lengte van de overleving. Voorspellingen op basis van deze extrapolatie lieten zien dat er een optimum watergehalte voor overleving bestaat die naar hogere waarden verschuift met een verlaging van de temperatuur. Deze verschuiving van het optimale watergehalte naar hogere waarden met lagere temperatuur was eerder gevonden voor lisdodde stuifmeel (hoofdstuk 2). Bij -18°C, een temperatuur die vaak gebruikt voor opslag van zaden door zaadbanken, bleek de optimale levensduur bereikt te worden rond 0.12 g H₂O g dw⁻¹. Daarnaast voorspelde de extrapolatie een langere levensduur bij hogere watergehaltes dan voorspeld door de viability equation. De voorspellingen aan de hand van de beweeglijkheid gaan in tegen de theorie dat de levensduur verbetert door een verdere

verlaging van het watergehalte en waarschuwen tegen te ver doordrogen van zaden en stuifmeel wanneer opslag bij lage temperaturen wordt uitgevoerd.

De geformuleerde hypothese dat suiker een belangrijke component is van het intracellulaire glas was gebaseerd op de observatie dat uitdroogtolerante organismen een grote hoeveelheid suikers bevatten en de wetenschap dat suikers bekende glasvormers zijn. Daarnaast maakt de literatuur melding van een mogelijke correlatie tussen de hoeveelheid oligosacchariden en de levensduur van zaden. De wetenschap dat oligosacchariden een hogere T_g hebben dan saccharose heeft tot het idee geleid dat oligosacchariden de stabiliteit van intracellulaire glazen verhogen en dus de verouderingssnelheid verlagen. In Sectie IV werd de rol van oligosacchariden in de stabiliteit van intracellulaire glazen nader bekeken.

Een voorbehandeling van zaden in een oplossing met een lage osmotische potentiaal ("priming") resulteerde in een verlaging van de oligosaccharideconcentratie en levensduur (hoofdstuk 9 en 10). Bepaling van de T_g en de moleculaire beweeglijkheid van CP in het cytoplasma van erwten-, impatiens- en paprikazaden voor en na de voorbehandeling liet echter zien dat geen van deze twee parameters veranderden. Hieruit werd geconcludeerd dat oligosacchariden in zaden blijkbaar niet een belangrijke rol spelen in de stabilisatie van de intracellulaire glasstructuur, en dat de verlaagde levensduur na de voorbehandeling niet het resultaat is van een toegenomen cytoplasmatische beweeglijkheid.

Om een beter inzicht te krijgen in de samenstelling van intracellulaire glazen werd de moleculaire beweeglijkheid van suikerglazen en intracellulaire glazen onderzocht met behulp van ST-EPR, $^1\text{H-NMR}$ en FTIR spectroscopie. Verhoging van de temperatuur van suiker- en eiwitglazen resulteerde in twee abrupte veranderingen in de moleculaire beweeglijkheid. De eerste toename in beweeglijkheid kwam overeen met de T_g . De tweede toename, waar de beweeglijkheid een versnelling liet zien van een aantal orden van grootte, bleek overeen te komen met de temperatuur T_c waar het systeem veranderde van een vaste structuur in een vloeibare toestand. Het interval tussen T_g en T_c nam toe met de grootte van het moleculaire gewicht van de suikers, en varieerde van 17°C voor saccharose tot 35°C voor stachyose. Verhoging van de temperatuur van biologische systemen resulteerde alleen in een verandering van de beweeglijkheid rond T_g . Zelfs bij temperaturen $> 50^\circ\text{C}$ boven T_g had de tweede overgang nog steeds niet plaatsgevonden. Aangezien de cytoplasmatische matrix beneden T_c zich nog in een vaste toestand bevindt, resulteert de hoge T_c in biologische systemen in een grote stabiliteit van het cytoplasma, zelfs bij temperaturen ver boven T_g . Een vergelijkbaar groot interval (50°C) werd gevonden voor een glas bestaande uit het polypeptide poly-L-lysine, wat erop wijst dat eiwitten mogelijk een belangrijker rol spelen in de eigenschappen van het intracellulaire glas dan suikers. Het is te verwachten dat de buitengewoon hoge T_c van intracellulaire glazen zorgt voor een buitengewone stabiliteit van droge organismen, door een lage beweeglijkheid te handhaven in het cytoplasma bij temperaturen ver boven T_g .

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Curriculum Vitae

Julia Buitink werd geboren op 24 november 1970 te Amsterdam. Na het behalen van het VWO diploma aan het Comenius College te Hilversum werd in 1989 begonnen aan de studie Biologie aan de Landbouwniversiteit te Wageningen. Het doctoraal examen werd in juni 1995 behaald met als hoofdvakken plantenfysiologie en moleculaire fytopathologie, en een onderzoeksstage aan het "National Seed Storage Laboratory, USDA-ARS", Colorado, USA. Na een aantal maanden onderzoek uitgevoerd te hebben op de vakgroep Moleculaire Fytopathologie is zij in april 1996 begonnen als onderzoeker in opleiding voor de Stichting Technische Wetenschappen gefinancierd door de Nederlandse Organisatie van Wetenschappelijk Onderzoek bij de vakgroep Plantenfysiologie, in samenwerking met de vakgroep Moleculaire Fysica van de Landbouwniversiteit te Wageningen. Het uitgevoerde onderzoek heeft geleid tot dit proefschrift. Tijdens het promotieonderzoek heeft zij een maand doorgebracht in het "Institute of Chemical Kinetics and Combustion" in Novosibirsk, Rusland, bij Dr. Sergei A. Dzuba.