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Is There a Role for Oligosaccharides in Seed Longevity? An Assessment of Intracellular Glass Stability[1]

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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 glass transition temperature of the cytoplasm of impatiens (*Impatiens walleriana*) and bell pepper (*Capsicum annuum*) seeds that were osmo-primed to change oligosaccharide content 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 content, while the sucrose content increased. No difference in the glass transition temperature was found between control and primed impatiens seeds at the same temperature and water content. Similarly, there was no difference in the rotational motion of the spin probe in the cytoplasm between control and primed impatiens and bell pepper seeds. We therefore conclude that oligosaccharides in seeds do not 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.

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 a low tissue water content and low temperatures. The presence of glasses has been associated with improved storage stability (Sun and Leopold, 1993; Sun, 1997; 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 aging (Sun and Leopold, 1993; Sun, 1997). 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).

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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 amount 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 stabilization of intracellular glasses by increasing viscosity and the glass-to-liquid transition temperature (T_g) (Leopold et al., 1994; Bernal-Lugo and Leopold, 1995; Sun, 1997). The addition of oligosaccharides to Suc glasses in a model system will increase the T_g considerably (Levine and Slade, 1988; Koster, 1991; Wolkers et al., 1998a). In this study, we examined the suggested role of oligosaccharides in seed storage via increased glass stability.

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., 1998). Saturation transfer electron paramagnetic resonance (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 refs. therein), in organic liquids at low temperatures (Ito, 1983), and in 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 was demonstrated previously 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 aging rates. In particular, emphasis was 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 annuum* 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 d 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% relative humidity [RH]) for 2 d at room temperature. Subsequently, the seeds were kept over saturated salt solutions of various RHs at 25°C or 30°C for storage experiments, or used for EPR experiments and determination of the sugar content. At intervals during storage, approximately 100 seeds were imbibed at 20°C to determine the final percentage of germination. The half-viability time (P_{50}) was determined as the time over which the percentage of germination decreased to 50%. Water contents were analyzed 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 2 d 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 of 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 (Speed-Vac, Savant Instruments, Holbrook, NY). The residue was dissolved in distilled water, and after appropriate dilution, sugars were analyzed by HPLC on a Carbopac PA-1 column (Dionex, Sunnyvale, CA) 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, and then the seed coats were removed. Seeds were then incubated for 60 min in a 10-mL solution of 1 mM 3-carboxyproxyl (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. Labeling with the spin probe was done as described above for impatiens seeds. After labeling, the seed tissues were dried in dry air (3% RH) for 24 h and then 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 (model 300E, Bruker Analytik, Rheinstetten, Germany). Instrument settings were according to Buitink et al. (1999). For each EPR measurement, 20 mg of 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 content was analyzed 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 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 0.5 mT, 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 obtained by interpolation of the calculated L″ to L ratio (see Fig. 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 in impatiens 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 longevity and the molecular motion of CP in the cytoplasm. The τ_R was determined from spectra as

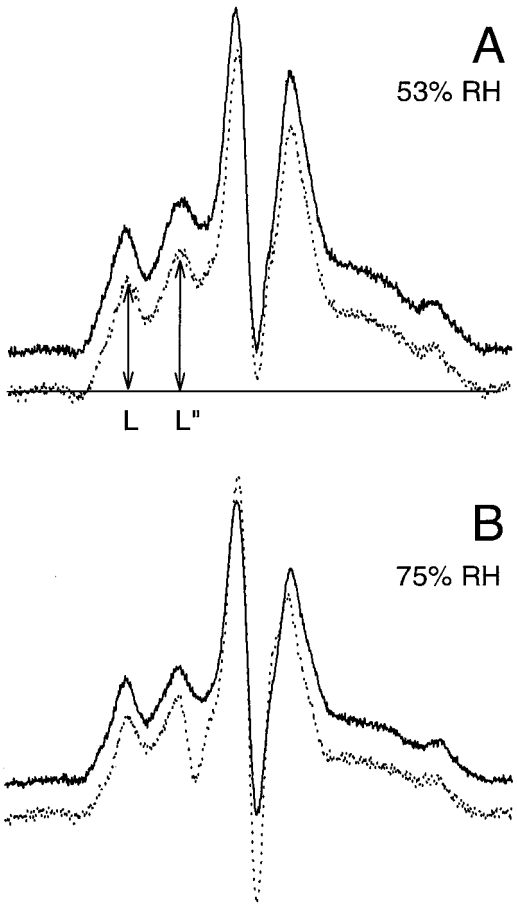

Figure 1. ST-EPR spectra of CP in untreated (solid line) or 7-d-primed impatiens seeds at −1.0 MPa and 20°C (dashed lines), equilibrated at 53% RH (A) or 75% RH (B). Spectra were recorded at 30°C. The parameters L″ and L are indicated.

shown in Figure 1. The ratio of L″ to 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., 1998). The spectra in Figure 1, A and B, show that with increasing RH, the L″ to 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 was only seen at high water content and temperature. Similar partitioning of CP into the lipid phase was observed previously in pea 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″ to L ratio from the ST-EPR spectra, allowing the rotational motion to be calculated.

The relationship between the τ_R of the polar spin probe CP in the cytoplasm and the half-viability times of impatiens seeds with different water contents at both 25°C and 30°C is shown in Figure 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 longevity, indicating that longevity is possibly related to the molecular mobility in the cytoplasm, as suggested previously (Leopold et al., 1994; Sun, 1997; Buitink et al., 1998a, 1998b, 1999).

Osmopriming-Induced Changes in Sugar Composition and Longevity

To assess how longevity of impatiens 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. 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. 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 with primed seeds were similar under the same conditions of storage. Differences in longevity were therefore not due to differences in water content during storage.

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, the Suc content increased from 1.1 μg/mg for untreated seeds to 21 μg/mg after 7 d of priming, whereas the concentration of an unknown trisaccharide decreased with priming from about 40.9 to 6.3 μg/mg after 7 d of priming (Fig. 4). During priming, the

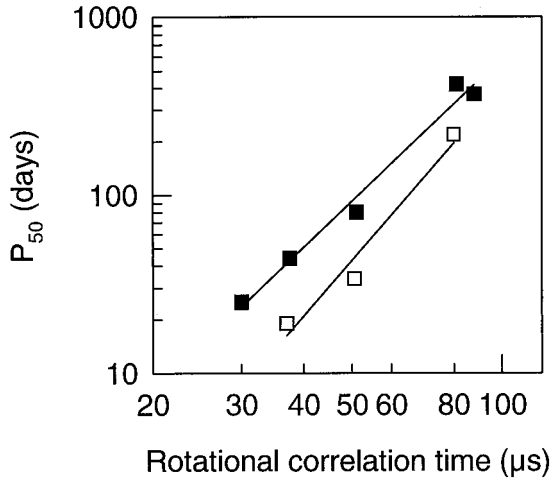

Figure 2. Relationship between rotational correlation time and half-viability times (P_{50}) for impatiens seeds. P_{50} represents days of storage until germination decreased to 50%. Symbols represent samples of different water contents at 25°C (■) or 30°C (□).

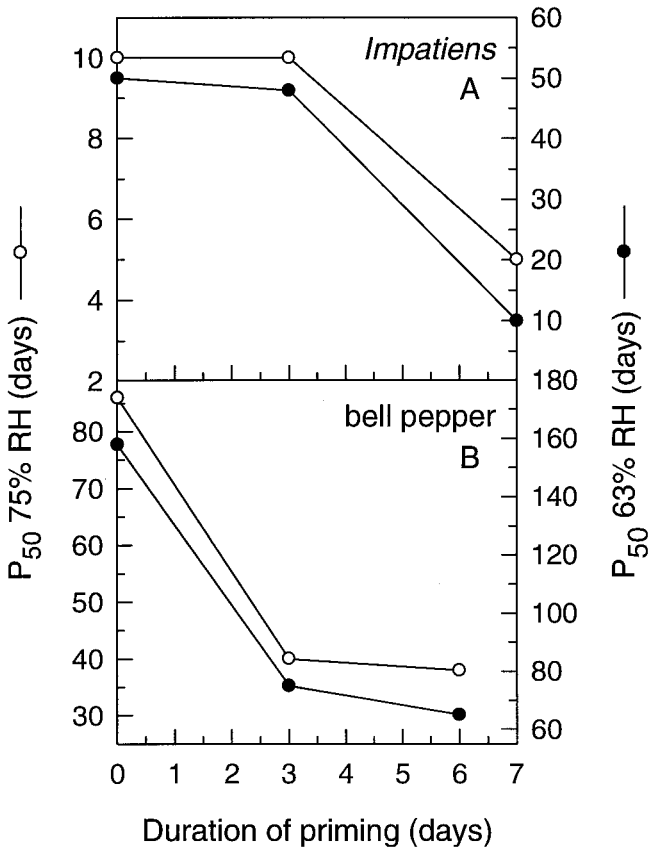

Figure 3. Relationship between half-viability times (P_{50}) at 75% RH (○) and 63% (●) at 30°C as a function of duration of priming at −1.0 MPa and 20°C. After priming, seeds were dried back and equilibrated over saturated salt solutions. A, Impatiens seeds; B, bell pepper seeds.

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. 5). In untreated seeds, the embryo contained a higher amount of Suc (41.2 μg/mg) than the endosperm (33.8 μg/mg). The Suc concentration increased in the embryo up to 70.9 μg/mg after 6 d of priming, but remained constant in the endosperm. Untreated embryos contained more of an unknown trisaccharide (31.1 μg/mg) than the endosperm (7.6 μg/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 μg/mg), whereas in the same time, the oligosaccharide content in the endosperm only slightly decreased to 5.5 μg/mg. After 6 d of priming, the oligosaccharide content in the endosperm decreased to 1.7 μg/mg.

Changes in Sugar Composition Do Not Change Cytoplasmic Glass Properties

After establishing that there is a relationship between cytoplasmic mobility and longevity (Fig. 2), it was possible to determine how the oligosaccharide content affects mobility and the resulting longevity. For that purpose, osmopriming 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 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 relationship between $2A_{zz}$ and temperature revealed a break (Fig. 6). Previously, this break was found to coincide with the T_g as measured by differential scanning calorimetry (Buitink et al., 1998a). Using this method, no difference could be found in the distance between the $2A_{zz}$ in relationship to temperature for untreated impatiens seeds or seeds primed for 7 d and equilibrated to the same RH (Fig. 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. 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 8 shows the τ_R of CP present in

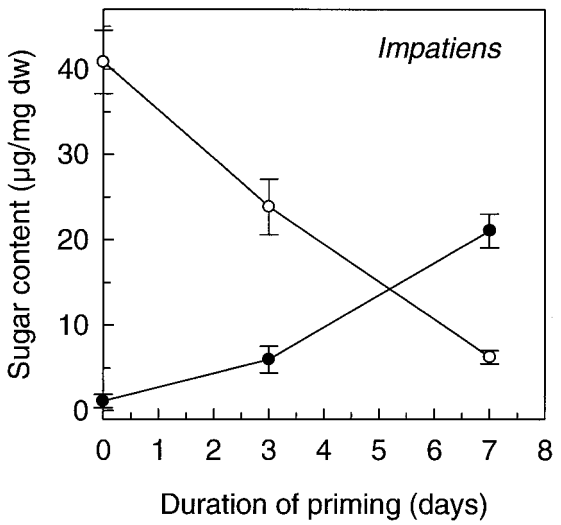

Figure 4. Changes in Suc (●) and oligosaccharide (○) content in whole seeds of impatiens in relation to the duration of priming. Error bars represent the SD of three replicates.

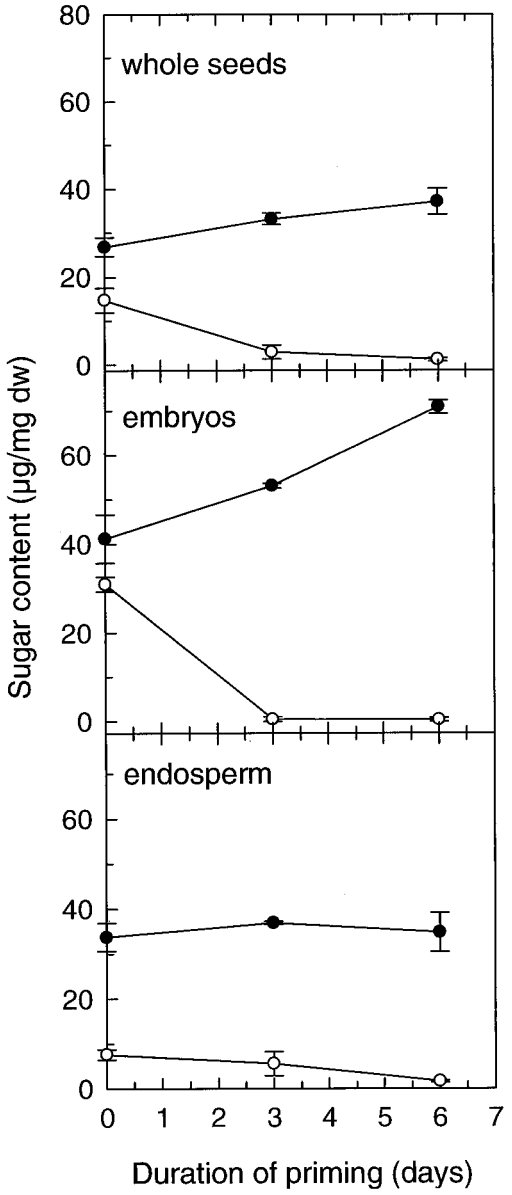

Figure 5. Changes in Suc (●) and oligosaccharide (○) content in various tissues of bell pepper seeds in relation to the duration of priming. Error bars represent the SD of three replicates.

the cytoplasm of impatiens and bell pepper seeds as a function of duration of priming. The τ_R changed in correlation with 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. 8A) or in bell pepper embryos (Fig. 8B) or endosperm (data not shown) after priming and re-drying. Using the same technique on model glasses, large differences could be found in the temperature dependence of τ_R of CP in a dry Suc glass compared with a dry raffinose glass. For example, at 70°C, the rotational motion of CP in Suc was found to be more than 4 orders of magnitude higher than that in raffinose at the same temperature (J. Buitink, unpublished results), a

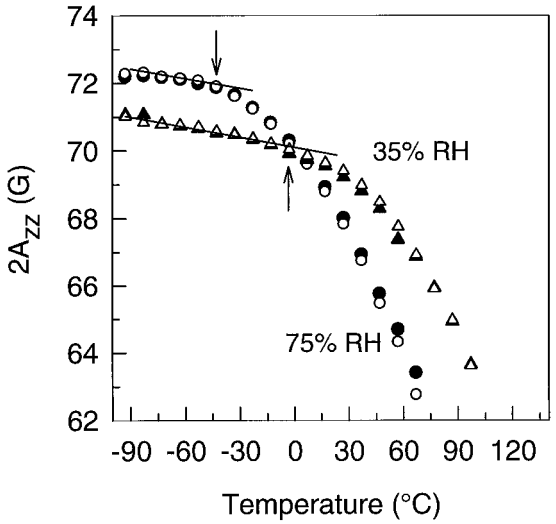

Figure 6. Distance between the outer extrema ($2A_{zz}$) derived from EPR spectra of CP in impatiens seeds as a function of temperature. The seeds containing CP were equilibrated to 75% RH (circles) or 35% RH (triangles) and then spectra were recorded. Seeds were untreated (black symbols) or primed for 7 d at −1.0 MPa, 20°C (white symbols). The arrows indicate the point of deviation from a straight line, representing the onset of the melting of the glassy state (T_g).

temperature that resulted in melting of the amorphous Suc but not of the amorphous raffinose (Levine and Slade, 1988; Wolkers et al., 1998a).

DISCUSSION

The proposed role of oligosaccharides in seed longevity was derived from experiments performed on model systems (Levine and Slade, 1988; Koster, 1991; Wolkers et al., 1998a). Oligosaccharides are known to increase T_g and viscosity in model Suc glasses, and this increased viscosity is likely to slow down detrimental aging reactions. So far,

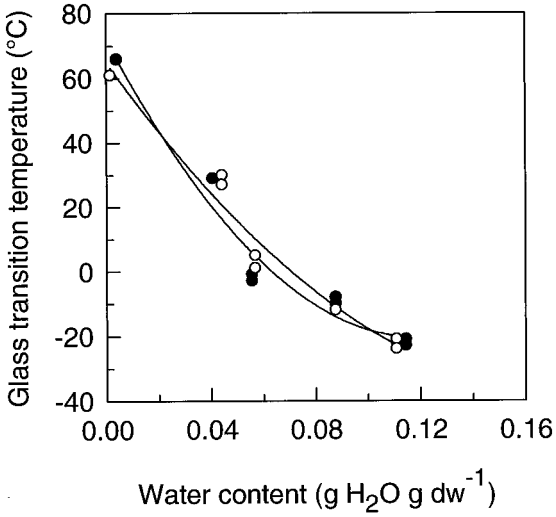

Figure 7. State diagram of untreated (●) or 7-d-primed (○) impatiens seeds. The T_g was determined as the point of deviation from a straight line (shown in Fig. 6).

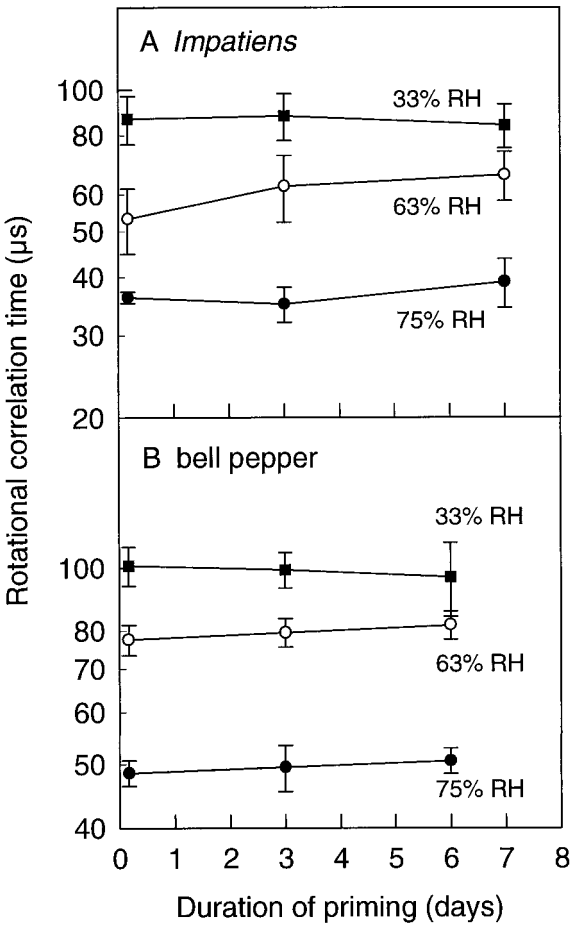

Figure 8. Rotational correlation times of CP in impatiens seeds (A) and bell pepper embryos (B) in relation to time of priming at −1.0 MPa and 20°C. Symbols represent the rotational motion of CP in the tissues equilibrated to various RHs. Data (±SD) are the average of four replicates.

most studies concerning the relationship between oligosaccharides and longevity in seeds have been based on correlative evidence (Horbowicz and Obendorf, 1994; Lin and Huang, 1994; Steadman et al., 1996; Sun and Leopold, 1997). The ratio of oligosaccharide to 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 Fig. 3 with Figs. 4 and 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 to total sugar ratio (0.01) than impatiens seeds (0.23).

Because a correlation cannot 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 found that increasing the temperature above 70°C resulted in a much higher rotational motion of a dry Suc glass compared with 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 in relation to water content (Fig. 2). Evidently, longevity of seeds is related to the molecular mobility in the cytoplasm, as has been suggested previously (Leopold et al., 1994; Sun, 1997; Buitink et al., 1998a, 1998b).

The osmo-priming treatment is a good model system with which 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 aging 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 in bell pepper endosperm or embryos with different sugar compositions (Figs. 7 and 8). Previously, we reported a similar result for pea axes in which the oligosaccharide content was reduced considerably after an osmotreatment, but no differences were found in the T_g measured by differential scanning calorimetry (Buitink et al., 2000). Apparently, there is no measurable change in the mobility of the cytoplasm that can be held responsible for the faster aging 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 to weak binding sites (Sun et al., 1997). The authors argued that such water redistribution might lead to enhancement of molecular mobility in the primed seeds. However, although we found a strong effect of water on the mobility of CP in the cytoplasm of seeds (see Fig. 8), a possible water redistribution after priming did not appear to influence the mobility of the spin probe in seeds.

The above observations do not support the hypothesis that oligosaccharides decrease molecular mobility in intracellular glasses. Apparently, other molecules in addition to soluble sugars play an important role in intracellular glass formation (Leopold et al., 1994; Leprince and Walters-Vertucci, 1995; Wolkers et al., 1998b; 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 intracellular glass, then do they have a role in increasing longevity? It has been suggested that oligosaccharides prevent crystallization of Suc during storage (Caffrey et al., 1988; Koster, 1991; Leopold et al., 1994). While model systems indicate that this crystallization phenomenon can

occur (Caffrey et al., 1988), to our knowledge, no studies exist in which crystallization was found in vivo in seeds (Sun and Leopold, 1993). It is likely that the mixture of all of the different components in the cytoplasm prevents crystallization of Suc, 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 stabilize the macromolecules during drying; however, Suc molecules have better hydrogen-bonding properties than do oligosaccharides (Wolkers et al., 1998a, 1998b). The observation that during priming oligosaccharides disappear and Suc content increases would suggest a better stabilization of macromolecules after priming, an observation in apparent contrast to 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 stabilization of the cytoplasmic matrix in seeds.

The discovery that reduced longevity of seeds after priming can be partially restored by a combined heat shock and dehydration treatment (Bruggink et al., 1999) could mean that protective chaperones that are lost during priming 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.

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