Dielectric Relaxation of Water and Water-Plasticized Biomolecules in Relation to Cellular Water Organization, Cytoplasmic Viscosity, and Desiccation Tolerance in Recalcitrant Seed Tissues[1]

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To understand the relationship between the organization of cellular water, molecular interactions, and desiccation tolerance, dielectric behaviors of water and water-plasticized biomolecules in red oak (*Quercus rubra*) seeds were studied during dehydration. The thermally stimulated current study showed three dielectric dispersions: (a) the relaxation of loosely-bound water and small polar groups, (b) the relaxation of tightly-bound water, carbohydrate chains, large polar groups of macromolecules, and (c) the "freezing in" of molecular mobility (glassy state). Seven discrete hydration levels (water contents of 1.40, 0.55, 0.41, 0.31, 0.21, 0.13, and 0.08 g/g dry weight, corresponding to −1.5, −8, −11, −14, −24, −74, and −195 MPa, respectively) were identified according to the changes in thermodynamic and dielectric properties of water and water-plasticized biomolecules during dehydration. The implications of intracellular water organization for desiccation tolerance were discussed. Cytoplasmic viscosity increased exponentially at water content < 0.40 g/g dry weight, which was correlated with the great relaxation slowdown of water-plasticized biomolecules, supporting a role for viscosity in metabolic shutdown during dehydration.

Properties of water in biological systems were studied extensively using isothermal sorption measurement (Schneider and Schneider, 1972; Clegg, 1978; Lusher-Mattli and Ruegg, 1982; Rupley et al., 1983), calorimetric method (Ruegg et al., 1975; Bakradze and Balla, 1983; Vertucci, 1990), infrared and Raman spectroscopy (Careri et al., 1979; Luck, 1985; Cameron et al., 1988), nuclear magnetic resonance (NMR) spectroscopy (Mathur-de Vre, 1979; Seewaldt et al., 1981; Rorschach and Hazlewood, 1986; Ratkovic, 1987), quasi-elastic neutron-scattering spectroscopy (Lehmann, 1984; Trantham et al., 1984), and dielectric relaxation techniques (Harvey and Hoekstra, 1972; Kamiyoshi and Kudo, 1978; Clegg et al., 1982; Pissis et al., 1987; Bruni and Leopold, 1992; Pissis et al., 1996). These studies revealed the great structural complexity of water in biological systems. Interfacial water, which is close to macromolecules and membranes, plays an important role in determining the properties and structures of macromolecules and membranes. These water molecules, being part of a network of biological interfaces, are dynamically oriented and exhibit restricted motion (i.e. "bound"). In consequence, the mobility and the ordering of water molecules are very different from those of pure bulk or "free" water. Changes in thermodynamic and motional properties of water at different hydration levels indicate the existence of different fractions of water, which may vary in structures and properties and presumably play different biological roles.

Anhydrobiotes, such as *Artemia* cysts, seeds, and pollen, were frequently used to study the role of water in biological functions because of their ability to survive desiccation and to resume active life upon addition of water. Isothermal sorption measurements showed the presence of three hydration regions: a strong water-binding region at low water content (WC), a weak binding region at intermediate WC, and a very loose binding region at high WC (Clegg, 1978; Vertucci and Leopold, 1987). More sensitive differential scanning calorimeter (DSC), NMR, and dielectric techniques identified the existence of at least four or five fractions of water, presumably relating to different interactions between water and cellular constituents (Clegg, 1986; Ratkovic, 1987; Vertucci, 1990; Pissis et al., 1996). These hydration levels are correlated to the onset of various metabolic activities in organisms. Physical properties of cells are often directly related to the behavior of cellular water (Clegg et al., 1982; Clegg, 1986; Bruni et al., 1989).

The relationship between cellular water property and desiccation tolerance was examined in a number of studies. Seeds that can tolerate essentially complete desiccation are called "orthodox" seeds, whereas seeds that lose their viability after drying to a critical WC are called "recalcitrant" (desiccation-intolerant)

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seeds. "Bound" water was reported to play an important role in desiccation tolerance of organisms (Vertucci and Leopold, 1987; Farrant et al., 1988; Pritchard, 1991; Sakurai et al., 1995). Some workers proposed that desiccation intolerance of some seeds and vegetative tissues is associated with the lack of strong water-binding sites (Vertucci and Leopold, 1987), whereas others suggested that desiccation intolerance is related to the requirement of structure-associated water to maintain ongoing metabolism and cellular membrane integrity (Farrant et al., 1988). However, the loss of viability in many recalcitrant seeds occurs at WC that is much higher than that of bound-water or unfreezable water (Pammenter et al., 1991; Berjak et al., 1992). The extent to which desiccation can be tolerated varies among recalcitrant species. No consistent difference in water-binding characteristics or the amount of freezable water is found between recalcitrant and orthodox seeds (Sun, 1999b). The relationship between cellular water organization and desiccation tolerance is not resolved yet.

The thermally stimulated current (TSC) technique is a powerful tool for investigating the mode of hydration in biological systems. This technique is capable of providing information concerning the mobility and rotational freedom of hydration water, hydration sites, and mechanisms (Mascarenhas, 1980; Pissis et al., 1987; Bruni and Leopold, 1992; Pissis et al., 1996). TSC is based upon the dependence of the microdynamics of water dielectric relaxation on their surroundings resulting in different dielectric relaxation times for water in different fractions, and on the influence of water on the dielectric relaxation mechanisms of other biomolecules. This method is very sensitive for detecting small amounts of water in different phases, and dipole concentration as low as 0.1 ppm can be measured accurately (Pissis et al., 1991). The objective of the present study was to investigate the dielectric relaxation properties of water and water-plasticized biomolecules in red oak (*Quercus rubra*) seeds. The study offers valuable insight into the organization of cellular water, molecular interactions between water and other biomolecules, and relationships between cytoplasmic viscosity, molecular mobility, and desiccation tolerance.

RESULTS

Critical WC and Water Potential (Ψ) of Desiccation Tolerance

Red oak seeds were unable to tolerate full desiccation after maturation (Fig. 1A). Seed germination decreased after embryos (axes and cotyledons) were dried to WC $< 0.35 \pm 0.02$ g/g dry weight (80% relative water loss). Cellular leakage increased abruptly after seed tissues were dried to WC below the critical level. The critical WC was similar to reported values (Pritchard, 1991; Finch-Savage, 1992). The effect of drying rate on desiccation tolerance was reported elsewhere, using the same seeds. Drying rate did not affect desiccation tolerance of red oak seeds (Sun, 1999c).

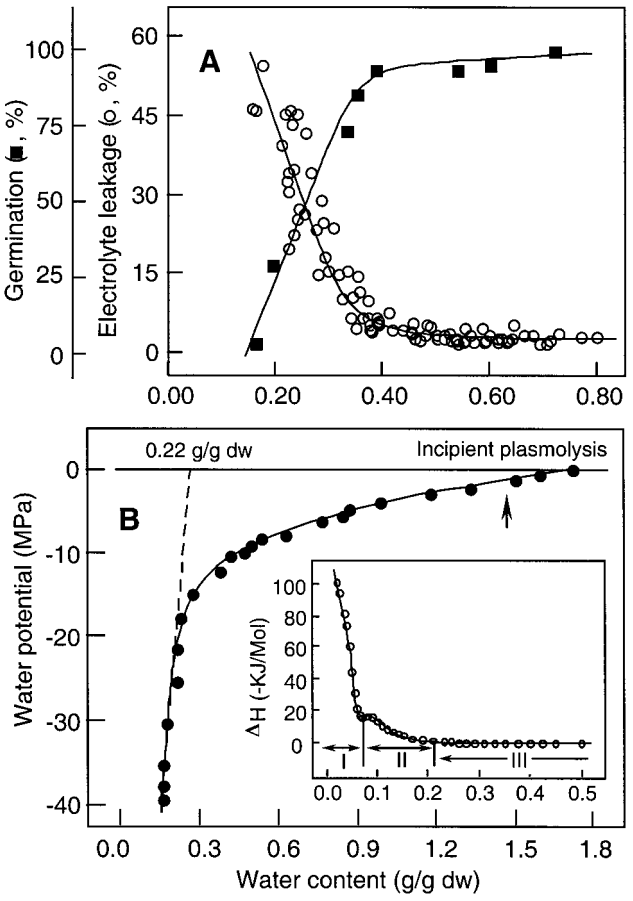

Figure 1. A, Desiccation tolerance of recalcitrant red oak seeds, as determined by seed germination and electrolyte leakage method. B, Ψ of seed tissues at different WC. Ψ at incipient plasmolysis was estimated from pressure-volume curves (not shown). The content of "matric" water was estimated to be 0.21 ± 0.01 g/g dry weight. Data at $\Psi < -40$ MPa were not shown. The inset shows three types of water according to the differential hydration enthalpy at different seed WC.

Figure 1B shows the Ψ of seed tissues with different WC. The pressure-volume curve indicated that cells started to plasmolyze at WC = approximately 1.4 to 1.5 g/g dry weight (−1.3 to −1.5 MPa). The critical Ψ of desiccation tolerance for red oak seeds was about −12.5 MPa (0.35 g/g dry weight). Using extrapolation, the amount of matrically bound water in tissues was calculated to be 0.22 ± 0.01 g/g dry weight (−24.5 MPa). This result was consistent with the data of differential hydration enthalpy (ΔH) calculated from desorption isotherms (Fig. 1B, inset). Primary hydration completed at WC = 0.21 g/g dry weight when ΔH reaches zero, according to Lusher-Mattli and Ruegg (1982) and Rupley et al. (1983). At WC < 0.21 g/g dry weight, water molecules were adsorbed at the strong and weak binding sites. The

amount of strongly bound water was estimated to be 0.08 g/g dry weight.

Interpretation of TSC Thermograms

TSC plots of red oak seeds showed three dielectric dispersions (Fig. 2). These relaxation peaks were denoted as peaks A, B, and C, according to the sequence of their occurrence during warming. Peak A occurred at temperature between −150°C and −100°C. Peak B and peak C were normally observed at temperature from −100°C to 0°C and from −50°C to 40°C, respectively. Peaks A, B, and C were due to the dipolar disorientation. The possibility that their occurrence was due to space charge relaxations of the ionic origin was excluded. Experimental data were in an excellent agreement with Equation 4 that describes dipole depolarization processes (Fig. 3A).

The information obtained from TSC studies on cellulose (Pissis, 1985), proteins (Pissis, 1989), saccharides (Daoukaki-Diamanti et al., 1984; Pissis and Daoukaki-Diamanti, 1986), and plant tissues (Pissis et al., 1987; Bruni and Leopold, 1992, 1996) enables a detailed evaluation on the biological and physical nature of three relaxation peaks. A main contribution to peak A comes from bulk water and loosely bound water in seeds, i.e. the reorientation of water in frozen water clusters or water layers around primary hydration sites. Bulk water in a dilute biological solution exhibits a TSC peak similar to that of macroscopic polycrystalline pure ice (Pissis et al., 1991). Water molecules that bind at strong hydration sites are generally "irrotational," and do not contribute to peak A. At very low WC, peak A is dominated by the relaxation of small polar groups, such as short sidechains of saccharides and proteins (Pissis and Daoukaki-Diamanti, 1986; Pissis, 1989). Figure 3B shows a typical TSC spectrum for a sample at very low WC. The sample displayed three low-temperature dispersions (A_1, A_2, and A_3) instead of a single peak. The data suggested a multiplicity of relaxation times representing several relaxation processes for peak A. The appearance of peak A as a single peak at high WC was therefore assumed to be due to the superposition of several processes with a distribution of relaxation time and activation energy. The dependence of the depolarization peak maximum temperature (T_m) of peak A

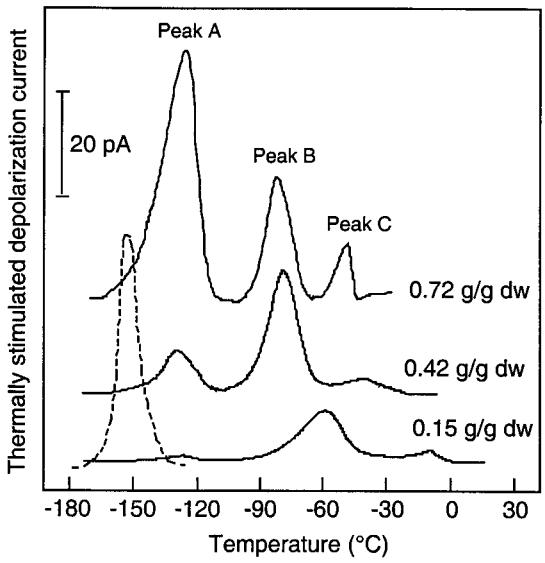

Figure 2. Representative plots of TSC versus temperature for red oak seeds. Conditions of TSC experiments: polarization dc field, 3 kV/cm; polarization temperature, approximately 22°C; polarization time, 3 min; and heating rate, 3°C/min. TSC plot of macroscopic polycrystalline pure ice (dashed line) was superimposed as a reference (data from Apekis and Pissis, 1987).

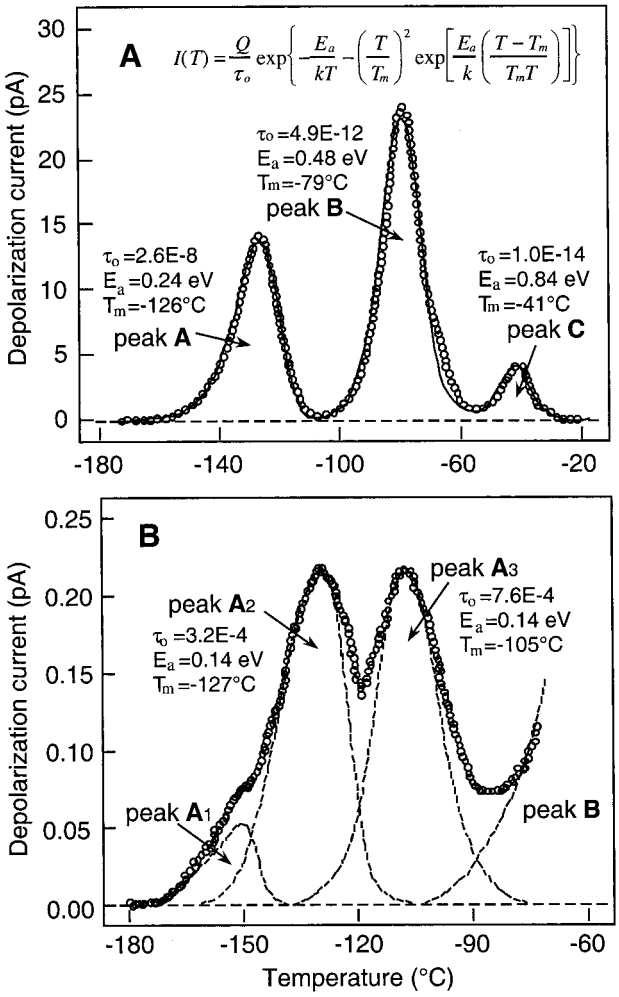

Figure 3. A, The iterative curve fitting of a TSC spectrum with Equation 6. Symbols are data points and the solid line represents the curve fitting. WC of the sample was 0.37 g/g dry weight. B, The TSC spectrum at the low-temperature region for a sample with WC = 0.10 g/g dry weight. The sample displayed three low temperature dispersions (A_1, A_2, and A_3, indicated by the dashed lines). The magnitude was normalized to a dry weight of 0.50 g. Estimated values of peak parameters are shown near individual peaks. The uncertainties of curve fitting: ±1°C in T_m, ±0.02 eV in E_a, and a factor of approximately 3 in τ_o.

on WC is given in Figure 4. The T_m of peak A remained almost constant during drying down to WC as low as 0.13 g/g dry weight. Below this hydration level, peak A began to split into three peaks, A_1, A_2, and A_3 (Fig. 3). T_m of peak A_2 remained constant, whereas T_m of peak A_3 increased rapidly upon further dehydration, indicating that the relaxation mechanism of peak A_3 was hydration-dependent (Fig. 4). This hydration threshold corresponded to the appearance of water molecules capable of undergoing fast reorientation (Seewaldt et al., 1981), or the boundary between very tight and intermediate water-binding sites (Vertucci and Leopold, 1987).

Active water adsorption centers were previously detected in seeds (Kamiyoshi and Kudo, 1978). Since the possibility that peak B arises from space charge relaxation is excluded, the contribution to peak B comes mainly from the relaxation of water bound tightly to hydroxyl groups, the main carbohydrate chains, and the large polar parts of macromolecules. The relaxation of large polar parts of macromolecules depends heavily on the plasticizing action of water. In view of the presence of high concentrations of soluble carbohydrates in seeds, it is suggested that the dipolar relaxation responsible for peak B is probably governed by the hydrated $-CH_2OH$ groups (Bruni and Leopold, 1992). WC greatly affected the relaxation process of peak B. Figure 4 shows that peak B shifted to higher temperatures when seeds were dried to WC $\leq$ 0.20 g/g dry weight, which correlates to the fraction of "matrically bound" water (Fig. 1B). The decline in T_m indicates that relaxation mechanisms get faster with increasing WC. The dielectric relaxation behaviors of water and water-plasticized biomolecules in red oak seeds will be analyzed in greater detail under separate subheadings.

TSC technique is very sensitive to transitions and is used to study glass transition of polymers (van Turnhout, 1980). The high-temperature dielectric dispersion is related to the "freezing-in" of molecular mobility (i.e. glass transition) in some systems (Smith and Schmitz, 1988; Pissis et al., 1991, 1992a). WC had a large influence on the T_m of dipole relaxation for peak C in red oak seeds (Fig. 4). As WC decreased, peak C shifted to higher temperatures. Our DSC study confirmed the association between glass transition and dielectric relaxation for peak C. Figure 5 shows a comparison between T_m of peak C and glass transition temperature (T_g). At WC $<$ 0.25 g/g dry weight, the T_m for peak C was almost identical to the T_g, as measured by DSC. At WC $>$ 0.25 g/g dry weight, T_m was lower than the T_g. The cooling rate in DSC study was from 2.5°C to 10°C/min, whereas the cooling rate in TSC study was much higher ($>$ 20°C/min). Because freeze-induced dehydration could be more severe at a slower cooling rate, the difference in the cooling rate may account for the observed discrepancy between T_g and T_m at WC $>$ 0.25 g/g dry weight. The relationship between glass transition and desiccation tolerance of red oak seeds was discussed elsewhere (Sun et al., 1994), and therefore the significance of peak C will not be evaluated further in this study.

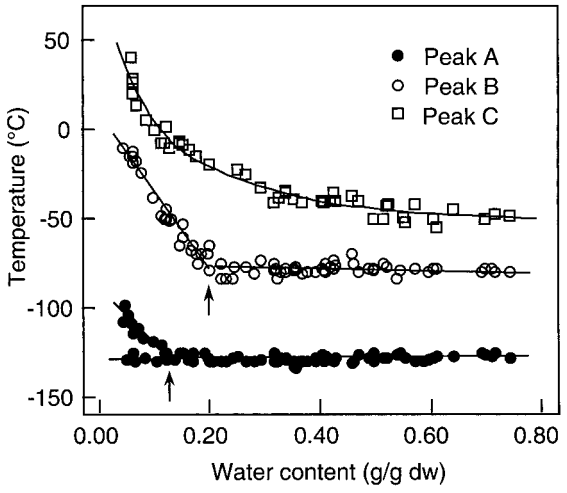

Figure 4. The dependence of TSC T_m on WC in red oak seeds. Solid lines represent the best fit.

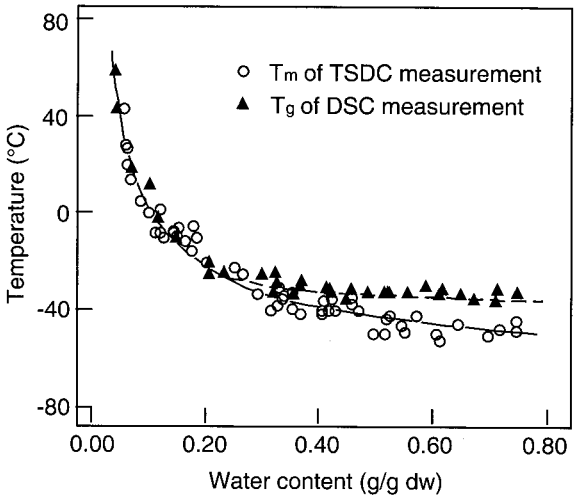

Figure 5. A comparison between TSC peak C T_m and T_g obtained with the DSC method. Samples were cooled and warmed at a rate of 2.5°C to 10°C/min during DSC measurements.

Seed WC and Magnitude of TSC Signals

Depolarization discharge dielectric relaxation (depolarization) discharge (Q), corresponding to the area under a peak, is a measure of the number of relaxation units that contribute to the peak. The magnitude of Q of TSC peaks was a function of WC (Fig. 6). The Q of peak A, which was mainly attributed to the presence of bulk water and loosely bound water molecules, showed an interesting behavior as seed WC decreased. The WC/Q relationship appeared to exhibit a discontinuity at WC = approximately 0.55 g/g dry weight. The Q of peak A decreased slowly

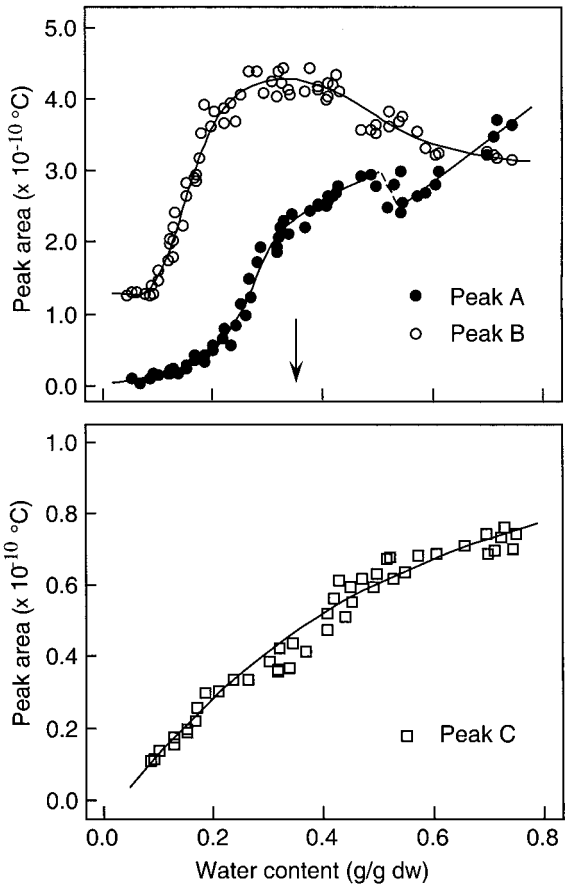

Figure 6. *Q* of individual TSC peaks in red oak seed samples with different WC. The depolarization charge corresponded to the peak area that was normalized to a dry weight of 0.50 g. Arrow indicates the critical WC of desiccation tolerance. Solid lines were drawn by eye.

during dehydration down to WC = 0.40 g/g dry weight. The rapid decline in the *Q* of peak A at WC = 0.40 to 0.20 g/g dry weight corresponded to the loss of seed viability (Fig. 1A). At WC < 0.20 g/g dry weight, the *Q* of peak A was very small, showing that matrically bound water did not contribute much to peak A.

The WC/*Q* relationship varied among three TSC peaks. The *Q* of peak B, which was due to the presence of water on active water adsorption centers and its plasticizing action on carbohydrate chains and the polar groups of macromolecules, registered an interesting increase when WC decreased from 0.80 to 0.40 g/g dry weight, but a sharp decline from 0.30 to 0.08 g/g dry weight. Thus the removal of water, even at a WC far above the critical desiccation tolerance level, has profound impact on the relaxation property and mobility of biomolecules at biological interfaces. The decrease in the *Q* of peak B at WC < 0.30 g/g dry weight represented a significant loss of dipole relaxation units. At WC < 0.08 g/g dry weight, the *Q* of peak B remained essentially unchanged, indicating a minimum hydration level of relaxation mechanisms. The *Q* of peak C, related to glass transition, showed a steady decline during dehydration, an observation similar to that of synthetic polymers (Pissis et al., 1992a).

Figure 7 shows the relative magnitude of *Q* for individual TSC peaks. The relative magnitude is expressed as the percentage of total depolarization charge of all three peaks. It indicates the relative pool size of dipole relaxation in samples at different WC. At WC below the critical desiccation tolerance level, the relative magnitude of peak A decreased rapidly, whereas that of peak B increased quickly. The change in the relative magnitude of peak A and peak B reflects not only the decrease in the number of relaxation units in peak A, but also a shifting role of water molecules at different hydration levels. The relative magnitude of peak C remained unchanged, accounting for about 8% of the relaxation activity.

Dielectric Relaxation of Water in Red Oak Seeds

Information on the structure of water can be derived by evaluating the peak parameters at different WC. Figure 8 shows the values of pre-exponential factor τ_o, activation energy E_a, and the contribution of peak A to static permittivity $\Delta\epsilon$ as a function of WC. These values should be considered as the "apparent" values or "weighted" averages with a distribution of relaxation times and activation energies, because there exists a multiplicity of relaxation processes for peak A. As WC was reduced from 0.84 to 0.30 g/g dry weight, E_a declined slowly from approximately 0.26 to 0.22 eV, whereas τ_o increased slightly in the order of 10^{-8} s. At WC < 0.30 g/g dry weight, E_a decreased rapidly, whereas τ_o increased exponen-

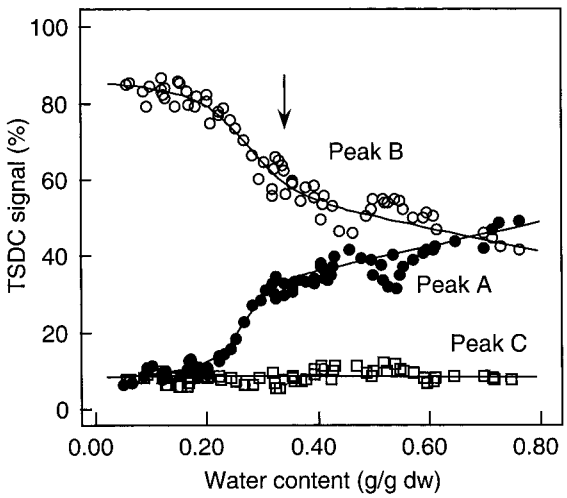

Figure 7. The change in the relative magnitude of the *Q* of individual TSC peaks in red oak seed samples as a function of WC. The relative magnitude of individual peaks are expressed as the percentage of total depolarization charge of all three peaks (A, B, and C). Arrow indicates the critical WC of desiccation tolerance. Solid lines were drawn by eye.

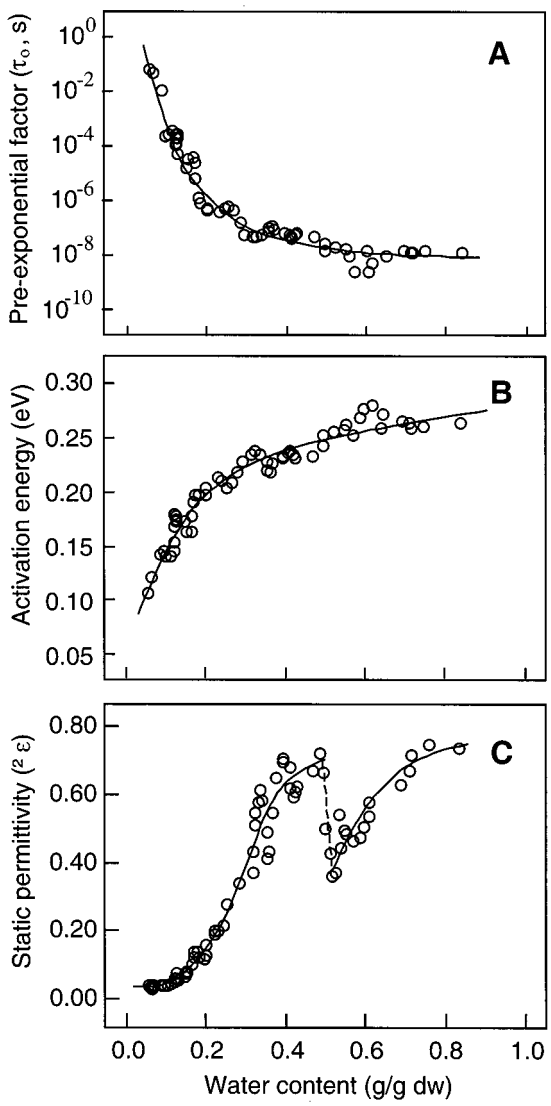

Figure 8. The values of τ_o (A), E_a (B), and the contribution to $\Delta\epsilon$ (C) of peak A in red oak seed samples as a function of seed WC. Solid lines were drawn by eye.

tially. E_a and τ_o changed in opposite directions. The τ_o for water in well-hydrated tissues was much longer ($>10^{-9}$ s) than that of water in macroscopic polycrystalline pure ice (1×10^{-10} s). It appears that in seed tissues no fraction of water behaves dielectrically like "free" water. Interfacial water has a longer relaxation time compared with pure water due to the increased hydrogen bond connectivity in the vicinity of hydrophilic surfaces. Figure 8C shows the contribution of peak A to the static permittivity. The $\Delta\epsilon$ exhibited a discontinuity at WC = approximately 0.52 g/g dry weight (Fig. 8C). When WC decreased from 0.84 to 0.52 g/g dry weight, $\Delta\epsilon$ decreased from 0.75 to 0.40, but then increases to 0.70. The $\Delta\epsilon$ decreased again upon further dehydration and reached a minimum at WC = 0.12 g/g dry weight.

Figure 9 shows the linear relationship between ln τ_o and E_a for peak A, known as the compensation effect (Peacock-Lopez and Suhl, 1982):

$$\ln \tau_0 = \ln\tau_0' - (E_a/kT_c) \quad (1)$$

where τ_o is a constant and T_c is the compensation temperature. The compensation effect exists in many processes that require activation energy to proceed. The derived equation is given in Table I. The T_c for peak A is calculated to be −135°C from the slope. This value is much lower than those reported for ice microcrystals in oil (−118°C), plant leaves (from −101 to −91°C), and frozen carbohydrate solutions (−117°C; Daoukaki-Diamanti et al., 1984; Pissis et al., 1987). The physical nature of T_c is still unclear (Crine, 1987). The validity of the compensation effect law indicated that relaxation mechanisms of different water structures for peak A are related to each other (Daoukaki-Diamanti et al., 1984; Pissis et al., 1987).

Dielectric Relaxation of Water-Plasticized Polar Groups

Figure 10 shows the values of τ_o and E_a of peak B as well as the contribution of peak B to $\Delta\epsilon$ as a function of WC. WC/τ_o, WC/E_a, and WC/$\Delta\epsilon$ relationships changed somewhat erratically as WC decreased to 0.55, 0.41, 0.31, 0.21, 0.13, and 0.08 g/g dry weight. The relaxation property and mobility of polar groups at biological interfaces were significantly altered when WC decreased to these hydration levels. The complex pattern of dielectric relaxation behaviors for water-plasticized polar groups was not unexpected, as water plays a variety of structural roles in seeds. However, the nature of such molecular interactions remains to be resolved in future studies.

Figure 11 shows the relationship between WC and dielectric relaxation time τ of water-plasticized bi-

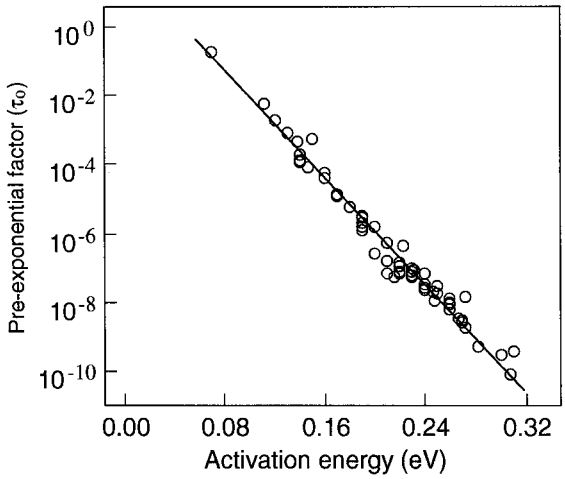

Figure 9. The relationship between lnτ_o and E_a of peak A (the compensation effect). The linear regression is: lnτ_o = 2.76–84.2 E_a (r^2 = 0.97). The compensation temperature T_c is −135°C.

Table I. *The compensation effect of dielectric relaxation of water and water-plasticized polar groups in red oak seed tissues*

Peak	WC	Compensation Effect	T_c	R^2
	g/g dry wt		*°C*	
A	$0.04 < WC < 0.84$	$\ln \tau_o = 2.76 - 84.2\ E_a$	−135	0.97
B	$WC \leq 0.08$	$\ln \tau_o = 3.65 - 47.4\ E_a$	−39	0.99
B	$0.08 < WC \leq 0.20$	$\ln \tau_o = -1.64 - 44.7\ E_a$	−25	0.99
B	$WC > 0.20$	$\ln \tau_o = 0.89 - 54.0\ E_a$	−68	0.95

omolecules. As WC decreased to <0.40 g/g dry weight, the apparent τ increased exponentially, showing that the relaxation mechanisms contributed to peak B are greatly restricted and the molecular mobility becomes increasingly slower. This increase of τ might be related to the rapid decrease in the relaxation discharge Q of peak A (Figs. 6 and 7).

Figure 12 shows the relationships between τ_o and E_a of peak B. This compensation effect plot revealed the existence of three different, WC-dependent compensation laws for water-plasticized molecular relaxation in red oak seeds. Three compensation laws operated at different hydration levels, i.e. WC ≤ 0.08 g/g dry weight, 0.08 g/g dry weight < WC ≤0.20 g/g dry weight, and WC > 0.20 g/g dry weight. These hydration levels corresponded to monolayer hydration water (i.e. strongly bound water), weakly bound water, and multilayer molecular sorption water, respectively (Fig. 1B). This result offers insight into how different fractions of water in seed tissues may contribute to the relaxation properties of other biomolecules. T_c for three hydration levels was calculated to be −39, −25, and −68°C, respectively (Table I). These T_c values are similar to those reported for other biological systems (Pissis et al., 1992a).

DISCUSSION

Cellular Water Organization in Recalcitrant Red Oak Seeds

The organization of water in the cell is a critical component in desiccation tolerance. Because irreversible changes occur in desiccation-intolerant organisms during dehydration, our knowledge on the properties of intracellular water is mainly derived from studies using desiccation-tolerant anhydrobiotes. The cell has a variety of fractions of interfacial water, and their physical properties change with hydration levels (Clegg, 1978, 1986; Bruni et al., 1989; Vertucci, 1990). However, little is known about the differences in the organization of cellular water between desiccation-tolerant and desiccation-intolerant organisms.

Two hydration models have been proposed to explain the organization of water in the cell according to the hydration behaviors of macromolecules and the physical properties of intracellular water. The first model depicts the formation of multilayers of water molecules around primary hydration sites of macromolecular structures. With increasing distance from surfaces, the binding energy decreases and approaches to the value of bulk water at a remote distance from macromolecular structures (Rupley et al., 1983). Within the cell, however, no fraction of water behaves like bulk or free water, as shown by

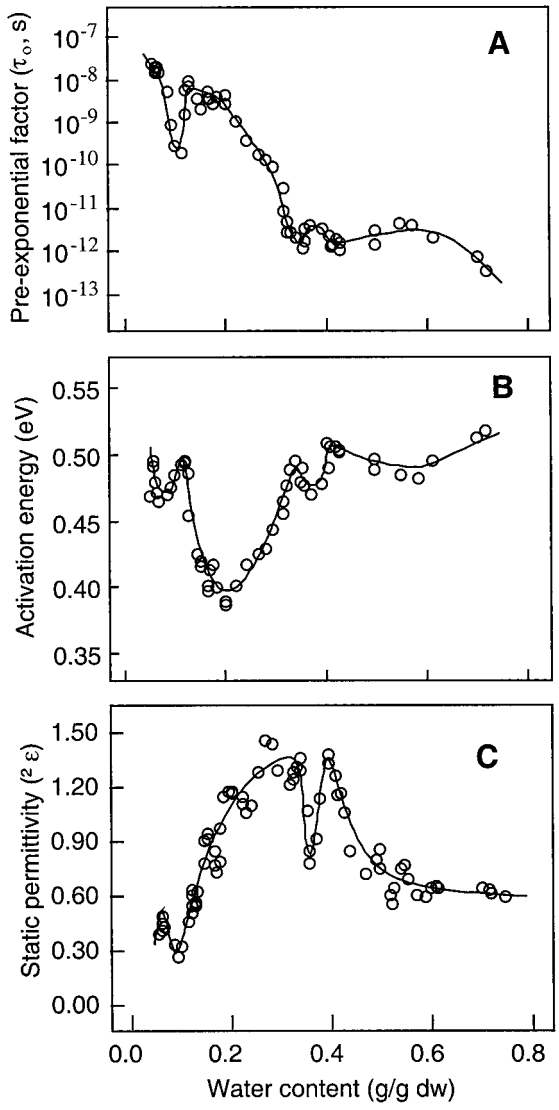

Figure 10. The values of τ_o (A), E_a (B), and the contribution to $\Delta\epsilon$ (C) of peak B in red oak seed samples as a function of seed WC. Solid lines were drawn by eye.

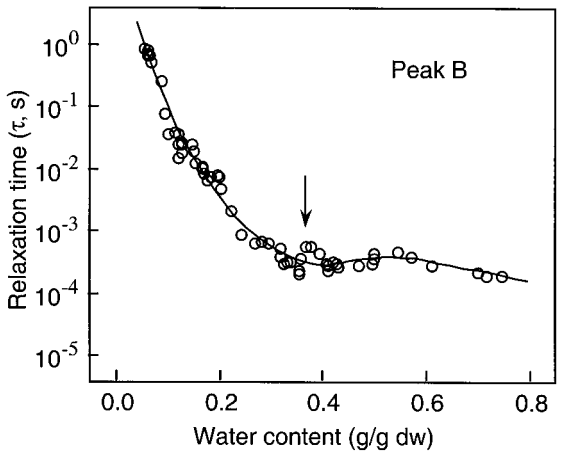

Figure 11. The τ of water-plasticized polar groups in red oak seeds at 25°C as a function of WC. Data are calculated according to Equation 2 from results shown in Figure 12. Arrow indicates the critical WC of desiccation tolerance. Solid lines were drawn by eye.

the present TSC study on red oak seed tissues. NMR study showed that even in maximally hydrated *Artemia* cysts the properties of water were markedly different from those of bulk or free water (Clegg, 1978; Seitz et al., 1981). The second model depicts that water molecules are organized into discrete domains and clusters within the cell. The "vicinal" water network model suggests that the presence of various biological interfaces in the cell leads to the structural differences of water among discrete water domains or clusters. Different domains or clusters are bridged by additional water to form "soluble pathways" or "channels of continuity" for the transfer of metabolites and energy (Clegg, 1978; Drost-Hansen, 1982).

The results from the present study support the second model for the organization of water in red oak seeds, i.e. water molecules existing in discrete domains and clusters within the cell rather than in loosely bound multilayers around the primary hydration sites. First, according to the first model the τ and T_m of loosely bound water molecules are expected to decrease with increasing WC, whereas based on the second model the τ and T_m are likely to be independent of WC (Pissis et al., 1987). Our study showed that the T_m of peak A in red oak seed tissues remained constant at WC from 0.13 to 0.80 g/g dry weight (Fig. 4). The multiplicity of peak A as shown in Figure 3 also suggests the existence of a variety of water structures in the tissues. Second, according to the first model, the Q is expected to decrease with decreasing WC. The results in the present study, however, showed discontinuity in the WC/Q relationship for peak A at certain hydration levels, and even an increase in the Q of peak B during early dehydration (Fig. 6). Third, the complex patterns of dielectric relaxation behaviors for water molecules and water-plasticized biomolecules (Figs. 8, 10, and 12) provide convincing evidence for the existence of an array of specific interactions between water and cellular constituents. In consequence, water molecules in different domains and clusters may exhibit distinctive physical properties.

Relevance of Cellular Water Organization to Desiccation Tolerance

The extent to which desiccation can be tolerated varies among recalcitrant species (Vertucci and Farrant, 1995). A literature survey has revealed that their critical water potentials appears at certain discrete levels (around −1.5, −4, −12, −23, and −75 MPa; Probert and Longley, 1989; Ellis et al., 1990, 1991; Pritchard, 1991; Berjak et al., 1992; Poulsen and Eriksen, 1992, 1993; Pritchard et al., 1995; Farrant and Walters, 1998; Pritchard and Manger, 1998; Tompsett and Pritchard, 1998). We have recently reported the critical WC of desiccation tolerance for 64 recalcitrant seeds that exhibited in a continuous scale ranging from 0.10 to 1.40 g/g dry weight (Sun, 1999a, 1999b). When the critical hydration level of many recalcitrant seeds was expressed in terms of water potential, the values of critical water potential were observed to form clusters around several discrete levels (approximately −1.5, −4, −8, −12, −23, and −73 MPa). Specific mechanisms associated with the degree of desiccation tolerance in recalcitrant seeds remain unknown. It is conceivable that dehydration might initiate deleterious events at specific hydration levels and that the seed may not survive to a lower hydration level without relevant adaptive and protective mechanisms. Using the sorption isotherm measurement and TSC technique, the present study revealed seven discrete hydration levels in red oak seed tissues, corresponding to −1.5, −8, −11, −14, −24, −74, and −195 MPa, respectively. Relaxation properties and mobility of intracellular biomolecules change significantly when WC decreased to these hydration

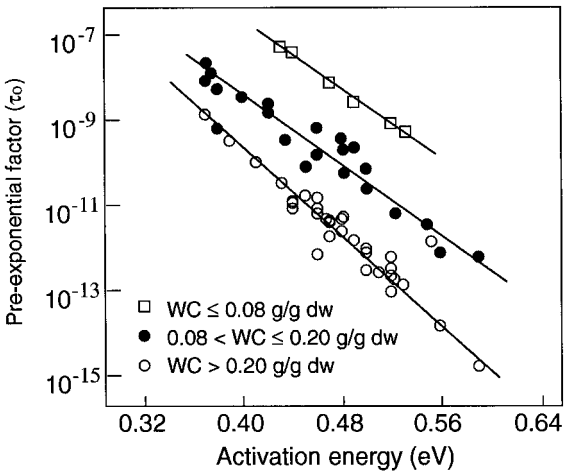

Figure 12. The relationship between ln τ_o and E_a of peak B (the compensation effect). Note that the compensation effect of peak B is hydration dependent.

levels (Figs. 4, 6–8, and 10–12). These changes may be associated with the loss of certain domains or clusters of intracellular water during dehydration. These discrete hydration levels were closely related to the varying degrees of desiccation tolerance expressed by various desiccation-intolerant seeds when compared in terms of water potential. This observation is suggestive of possible physiological significance for these discrete hydration levels in desiccation tolerance. The present study represents an important step elucidating molecular interactions between water and cellular constituents and the relationship between the organization of cellular water and desiccation tolerance.

Molecular Relaxation and Changes in Cytoplasmic Viscosity

τ of water molecules is related to the viscosity near biological interfaces (Nimtz and Weiss, 1987). The τ of water molecules was used to calculate cytoplasmic viscosity in red oak seed tissues. The cytoplasmic viscosity increases exponentially as WC decreased to $<$0.40 g/g dry weight (Fig. 13). At WC $<$ 0.40 g/g dry weight, the relaxation of water-plasticized biomolecules (peak B) is greatly retarded (Fig. 11). The change in relaxation behaviors of biomolecules is apparently associated with this rapid increase in viscosity during dehydration. The slowdown of molecular mobility, as a result of the rapid increase in viscosity, may be related to the depression of metabolism during dehydration. The levels of hydration in anhydrobiotes are well correlated to the onset of various biological functions (Clegg et al., 1982, 1986; Bruni et al., 1989; Vertucci, 1990). In general, there is essentially no metabolic activity occurring at WC $<$ 0.10 g/g dry weight. Upon the completion of the primary hydration process, WC may increase to 0.20 to 0.30 g/g dry weight, corresponding to a hydration region with "restricted" metabolism in which concerted enzymic activities in enzyme complexes can be detected (Clegg, 1978, 1986; Bruni et al., 1989). After the completion of the primary hydration process, respiratory activity becomes measurable (Vertucci, 1990). Higher levels of metabolic coordinations or conventional metabolism occur only at much higher hydration levels (WC $>$ 0.40 g/g dry weight for seeds and $>$0.60 g/g dry weight for *Artemia*), which permit the long-range transfer of metabolites and energy sources between various organelles and other compartments within the cells. During dehydration, a progressive arrest of metabolic activities is observed in many anhydrobiotes. The precise mechanism that triggers the arrest of metabolism is not clear yet. It has been proposed that an increase of cytoplasmic viscosity during dehydration and an eventual cytoplasmic vitrification may bring the intracellular processes to a halt (Bruni and Leopold, 1992; Leopold et al., 1994; Sun and Leopold, 1997). However, there is still lack of convincing experimental evidence to support this hypothesis. Previous studies showed that cytoplasmic vitrification alone was not sufficient to protect biological system during desiccation and that the glass transition behaviors of desiccation-tolerant and desiccation-intolerant seeds and pollen appeared to be identical (Sun et al., 1994; Buitink et al., 1996; Sun and Leopold, 1997).

In the present study the rapid increase in cytoplasmic viscosity during dehydration is correlated with the great decrease in molecular relaxation and mobility at a hydration range that is associated with the arrest of metabolism in cysts, seeds, and pollen (Figs. 11 and 13). The significant decrease in molecular mobility could impede the diffusion of reactants and conformational changes of macromolecules that are required for metabolic activities. The data support a role for cytoplasmic viscosity in the metabolic shutdown during dehydration. A rapid increase in viscosity may serve as a mechanism regulating the metabolic depression in dehydrating tissues before the cytoplasm enters into a glassy state.

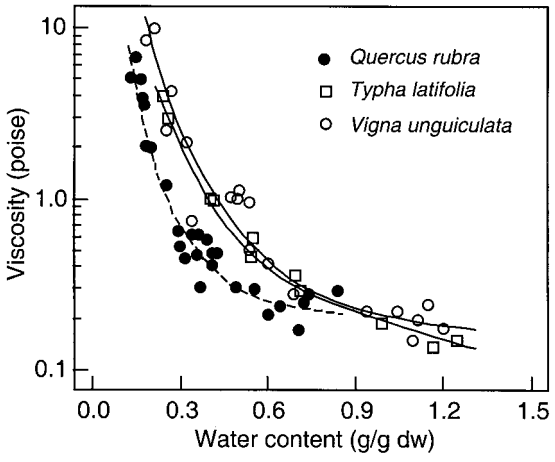

Figure 13. The change of cytoplasmic viscosity in red oak cotyledons during dehydration. Cytoplasmic viscosity was determined from the dielectric relaxation time of water. Data of cattail pollen and cowpea cotyledons were taken from Leprince and Hoesktra (1998).

Relationship between Cytoplasmic Viscosity and Desiccation Tolerance

The cytoplasmic viscosity of desiccation-intolerant red oak seeds was compared with that of desiccation-tolerant cattail pollen and cowpea cotyledons. At WC $>$ 0.6 g/g dry weight, the viscosity in all three tissues were measured to be similar (Fig. 13). At WC $<$ 0.6 g/g dry weight, the viscosity of desiccation-intolerant red oak seeds was much lower than that reported for desiccation-tolerant seeds and pollen. The viscosity in cattail pollen and cowpea cotyledons increased exponentially at WC $<$ 0.8 g/g dry weight (Leprince and Hoekstra, 1998), whereas a significant increase in the viscosity occurred in red oak seeds

only when WC decreased to <0.40 g/g dry weight (Fig. 13). The observed difference cannot be discounted by differences in techniques used and cellular compositions. Red oak and cowpea produce starchy seeds, with low protein content and little lipid, and therefore, the difference in their hydration behaviors would not change the observed pattern significantly. The comparison on viscosity is quite interesting in view of the possible role of viscosity in desiccation tolerance. The regulated shutdown of metabolism is essential to achieve desiccation tolerance (Leprince and Hoekstra, 1998). The lower viscosity in red oak seed tissues (increased significantly only after desiccation to WC < 0.4 g/g dry weight) may not be able to effectively control the formation of reactive oxygen species during desiccation and to minimize the oxidative damages. The loss of viability of desiccation-intolerant seeds during drying is commonly associated with various oxidative damages (Leprince et al., 1990; Finch-Savage et al., 1994; Li and Sun, 1999).

MATERIALS AND METHODS

Plant Material

Red oak (*Quercus rubra*) seeds were collected at the natural seed-shedding period when WC declined to approximately 45% (i.e. approximately 0.8 g/g dry weight). To prepare samples for TSC measurements, acorns were transversely cut into discs with a thickness of approximately 1.5 mm and a diameter of approximately 1.2 cm. Transverse sections were fully hydrated in distilled water, and then dehydrated to various WC by equilibrating over saturated solutions of NaCl (76% RH) and KCl (85% RH) at 5°C. WC of seed tissues decreased to approximately 0.16 g/g dry weight within 6 to 8 d in 76% RH.

Determination of Desiccation Tolerance

Desiccation tolerance of acorns was determined by methods of germination and electrolyte leakage according to Sun and Leopold (1993). In the germination test, acorns that were air dried to different WC (slow drying) were germinated in moist sand. Electrolyte leakage was measured using one cotyledon. The cotyledon was sliced into small pieces (5 × 5 × 0.5 mm) that were immediately rinsed for 2 to 3 min with distilled water to remove electrolyte solutes on the surface. Sliced tissues were then imbibed in distilled water. The conductivity of imbibition water was measured after 1 h, and the leakage was expressed as a percentage of total electrolyte leakage after boiling for 10 min. The other cotyledon was used for WC measurement. The WC, below which seed viability decreased rapidly or electrolyte leakage increased sharply, was considered as the critical WC of desiccation tolerance.

Measurement of Seed Water Potential

An isothermal equilibration method was used to determine water potential (Ψ) in tissues because osmometric, psychrometric, and hydrometric methods were limited to the nominal range of Ψ to −8 or −10 MPa. Sliced tissues were equilibrated in closed containers at 5°C, 15°C, and 25°C for 7 to 12 d over a series of glycerol solutions and saturated salt solutions. The relationship between WC and Ψ at equilibrium was derived. Water potentials of seed samples are given by:

$$\Psi = RT\ln(RH/100)/\overline{V} \quad (2)$$

where R is gas constant, T is temperature, $\overline{V}$ is the partial molal volume of water, and RH is relative humidity. ΔH was calculated according to the Clausius-Claperon equation, using data at 5°C and 25°C.

TSC Measurement

The principles and procedures of the TSC technique have been described extensively elsewhere (van Turnhout, 1980; Pissis et al., 1987). TSC measures the current generated by the thermally activated release of stored dielectric polarization during controlled heating, and basically consists of three steps: (a) The polarization of a sample by a strong dc electric field at a temperature T_P, (b) "freeze in" the polarization by cooling down to a sufficiently low temperature T_o while the field is still on, and (c) the measurement of the TSC spectrum during heating after the dc field is disconnected. Our TSC measurements were carried out with a sample holder configuration and electrode arrangement that were described previously (Bruni and Leopold, 1992). The arrangement using insulating electrodes excluded the possibility of space charge relaxation of ionic origin (i.e. dc conductivity). To perform a measurement, the sample was polarized by a direct electrical field of 3 kV/cm at approximately 22°C for 3 min, and rapidly cooled (>20°C/min) with liquid nitrogen to −180°C while the field was on. TSC was measured during warming at a constant rate of 3°C/min. After measurement, the sample was dried at approximately 95°C under vacuum for at least 24 h to determine its WC.

Analysis of TSC Peaks

Temperature dependence of dipole relaxation follows the Arrhenius equation:

$$\tau(T) = \tau_0 exp(E_a/kT) \quad (3)$$

where τ_o is the pre-exponential factor, E_a is activation energy for dipole reorientation, and k is the Boltzmann constant. In the case of dipole disorientation with a single relaxation time, the depolarization current, $I(T)$, is given by the following equation:

$$I(T) = \frac{Q}{\tau_o}\exp\left(\frac{-E_a}{kT}\right)\exp\left[\frac{-1}{\beta\tau_o}\int_{T_o}^{T}\exp\left(\frac{-E_a}{kT'}\right)dT'\right] \quad (4)$$

where Q is the initial polarization (relaxation discharge, area under the peak), β is heating rate, and T_o is temperature at which depolarization current starts to appear. The

contribution of a peak to static permittivity, $\Delta\epsilon$ is obtained from the equation:

$$\Delta\varepsilon = Q/(A\varepsilon_0 E_p) \quad (5)$$

where A is the cross-sectional area of the sample, ϵ_o is the permittivity of free space, and E_p is the polarization field (3 kV/cm). Peak parameters E_a and τ_o were determined by resolving Equation 4, using the following mathematical approximation according to Christodoulides et al. (1988) and Bruni and Leopold (1992):

$$I(T) = \frac{O}{\tau_o}\exp\left\{-\frac{E_a}{kT} - \left(\frac{T}{T_m}\right)^2 \exp\left[\frac{E_a}{k}\left(\frac{T-T_m}{T_m T}\right)\right]\right\} \quad (6)$$

where T_m is temperature at which maximum depolarization current occurs in a peak (Bruni and Leopold, 1992). The iterative curve-fitting analysis of TSC peaks was performed using a procedure that was developed by Dr. F. Bruni and inserted as a macro into the commercially available software "Igor" (WaveMetrics, Lake Oswego, OR). The uncertainties of curve fitting were ±1°C in the peak temperature T_m, ±0.02 eV in the activation energy E_a, and a factor of approximately 3 in the pre-exponential factor τ_o. A total of 87 TSC experiments using tissues at different WC were examined. Data of τ_o, E_a, $\Delta\epsilon$, and τ were smoothed by two points (Figs. 8, 10, and 11), and the data of the integrated peak signal were smoothed by three points (Fig. 6).

Determination of T_g

T_g of samples was determined using a DSC (DSC-2, Perkin-Elmer, Foster City, CA and DSC-131, Setaram, Caluire, France). Samples were hermetically sealed in aluminum pans and scanned at cooling and heating rates from 2.5°C to 10°C/min using an intracooling device. T_g was the mid-point temperature in the change of specific heat capacity associated with glass transition.

Calculation of Cytoplasmic Viscosity

The viscosity η of the intracellular liquid is calculated with the Debye equation:

$$\eta = \tau Tk/(4\pi\alpha^3) \quad (7)$$

where τ is dielectric relaxation time of water molecules, α is the radius of a water molecule, k is the Boltzmann constant, and T is temperature (298 K; Grant et al., 1978). The τ of water molecules in seed tissues was calculated according to Equation 3 with τ_o and E_a of peak A (see "Results"). Note that the ratio of dielectric relaxation time between ice and liquid water is 1.1×10^6 at 0°C (Grant et al., 1978) and that the τ derived from Equation 2 has to be corrected by a factor of 1.1×10^6. At WC < 0.15 g/g dry weight, cytoplasmic viscosity cannot be calculated accurately because the relaxation of small polar groups such as short side chains of saccharides and proteins would have a significant contribution to peak A.

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