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Calorimetric Properties of Dehydrating Pollen[1]

Analysis of a Desiccation-Tolerant and an Intolerant Species

Julia Buitink[2], Christina Walters-Vertucci*, Folkert A. Hoekstra, and Olivier Leprince[3]

United States Department of Agriculture, Agricultural Research Service, National Seed Storage Laboratory, Fort Collins, Colorado 80521 (J.B., C.W.-V., O.L.); and Department of Plant Physiology, Wageningen Agricultural University, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands (J.B., F.A.H.)

The physical state of water in the desiccation-tolerant pollen of *Typha latifolia* L. and the desiccation-sensitive pollen of *Zea mays* L. 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 (wc) values less than 0.21 (*T. latifolia*) and 0.26 (*Z. mays*) g H_2O/g dry weight. 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 wc values 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 wc. 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 behavior 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.

Living processes are dependent on water, which regulates biological reactions, serves as a fluid medium, and stabilizes macromolecular structure (reviewed by 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 by Crowe et al., 1992; Crowe et al., 1996). These organisms form suitable model systems to study properties of water in biological tissues, since they remain viable even at low wc.

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 wc. This led to the assignment of hydration levels distinguishable by the changes in these properties with wc (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 (Burke, 1986; 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 by Leopold et al., 1994).

The majority of pollens are desiccation tolerant, i.e. they can be dried to wc values of less than about 0.05 g H_2O g^{-1} dry weight without loss of viability (Hoekstra, 1986). However, some desiccation-intolerant species can be found among the Gramineae (Goss, 1968). For instance, maize (*Zea mays*) 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 (*Typha latifolia*, cattail) and a moderately desiccation-sensitive one (maize) to ascertain whether these two species exhibit different water characteristics.

MATERIALS AND METHODS

Plant Materials and wc Determinations

Mature male inflorescences of *Typha latifolia* L. 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 H_2O g^{-1} dry weight and subsequently stored at −20°C until used. *Zea mays* cv Gaspe Flint (maize) was grown in a

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[2] Present address: Department of Phytopathology, Wageningen Agricultural University, Binnenhaven 9, 6709 PD Wageningen, The Netherlands.

[3] Present address: Brassica and Oilseeds Research Department, John Innes Centre, Norwich NR4 7UH, UK.

* Corresponding author; email chrisv@lamar.colostate.edu; fax 1-970-221-1427.

Abbreviations: Cp, heat capacity; DSC, differential scanning calorimetry; ΔH, enthalpy; Tg, glass-to-liquid transition temperature; wc, water content.

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 g H_2O g^{-1} dry weight, was immediately used in the experiments.

wc of *T. latifolia* pollen was manipulated by equilibration over saturated salt solutions at room temperature for 48 h for both viability assays and DSC measurements (Rockland, 1960; Winston and Bates, 1960; summarized by Vertucci and Roos, 1993). 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 wc values for the viability assay. For DSC measurements, fresh maize pollen was equilibrated over the saturated salt solutions for 48 h. wc values 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. wc was expressed on a dry weight basis as g H_2O g^{-1} dry matter.

Viability Assays

Viability of *T. latifolia* pollen was determined by counting the percentage of empty, cytoplasmless grains in four replicates of 50 pollen tetrads 24 h after placing the pollen on a germination medium at 24°C (Hoekstra et al., 1992). 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 semisolid germination medium containing salts, according to Brewbaker and Kwack (1963), 15% Suc, and 0.125% agar and then placed in an open Petri dish on the laboratory bench. After 30 min the germinated grains were counted (four replicates of 50 grains each). To prevent imbibitional damage, pollen grains were prehydrated in water vapor at room temperature for 2 h prior to the germination assay.

DSC

Pollens, adjusted to different wc, were hermetically sealed into standard aluminum DSC pans. Grains with less than 0.1 g H_2O g^{-1} dry weight were packed into nonhermetically sealed pans to improve thermal conductivity.

Phase transitions in pollen at various wc 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 for energy with indium (28.54 J g^{-1}). 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°C/min. 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 ΔH of the transition was determined from the area encompassed by the peak and the baseline. The temperatures of Tg's, detected in pollen with low wc, 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 wc of the pollen. The slope of the linear relationship corresponds to ΔH g^{-1} H_2O, and the x intercept gives the wc 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 Cp at different wc values between 0.01 and 0.30 g H_2O g^{-1} dry weight and three different temperatures (30, −10, and −60°C) for maize and six different temperatures (30, 10, −10, −20, −40, and −60°C) for *T. latifolia*. Displacement from the DSC isothermal baseline was evaluated while samples were heated 4°C at 4°C/min, e.g. −12 to −8°C for the −10°C determination. Cp was measured at the highest temperature first, and then samples were cooled at 10°C/min to the next-lower assay temperature and allowed to equilibrate until a steady baseline was achieved (usually within about 5–10 min). The DSC was calibrated for Cp measurements using benzoic acid as a standard (Ginnings and Furukawa, 1953).

RESULTS AND DISCUSSION

Viability and wc

Pollen of *T. latifolia* 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 wc values (Fig. 1). Germination percentages of *T. latifolia* pollen were constant at about 90% for the entire wc range. In contrast, the viability of maize decreased sharply if grains were dried to wc values less than about 0.4 g H_2O g^{-1} dry weight, and no germination was observed when grains were dried to 0.1 g H_2O g^{-1} dry weight.

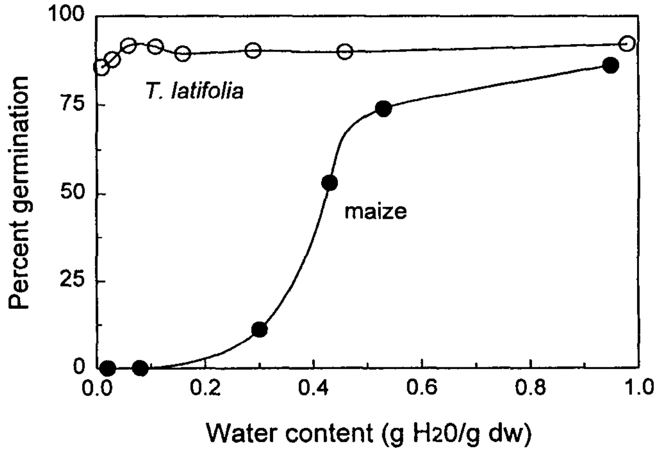

Figure 1. The germination percentage determined in vitro for *T. latifolia* (○) and maize (●) pollen dried to different wc values. dw, Dry weight.

Phase Behavior of Water at Different wc Values

The calorimetric properties of water in pollen of *T. latifolia* and maize were measured as a function of wc and temperature. In Figure 2, a selection of these thermograms is presented. Several transitions were observed within the temperature range of −80 to 70°C. At wc values greater than about 0.22 g H_2O g^{-1} dry weight, a broad endothermic peak at about −30 to −12°C was observed during warming runs for both pollens (Fig. 2, A and B, w). The area and temperature of the peak increased with increasing wc. 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^{-1} dry weight) 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 wc, a small endothermic peak was observed at about −20°C for *T. latifolia* and −25°C for maize (Fig. 2, C 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 wc and are similar to triglyceride transitions reported for seed tissues (Vertucci, 1989, 1990, 1992; Hoekstra et al., 1991).

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. 2, g). The temperatures at which these shifts occurred increased with decreasing wc. For example, in *T. latifolia* pollen with 0.27 g H_2O g^{-1} dry weight the baseline shift occurred at −75°C (Fig. 2A, g), whereas in samples with 0.042 g H_2O g^{-1} dry weight (Fig. 2C, g) this shift occurred at 50°C. An endothermic peak superimposing the baseline shift was observed in samples with wc values between 0.02 and 0.06 g H_2O g^{-1} dry weight 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 wc values between about 0.25 and 0.35 g H_2O g^{-1} dry weight, the baseline shifts were observed at subzero temperatures and were accompanied by exothermic reactions considered to be devitrification (ice-crystal formation) events (Franks, 1982; Vertucci, 1989) (Fig. 2, A and B, g and d). The baseline shifts occurring at elevated temperature with reduction in wc, the overshoot, and the devitrification events are typical of glass-to-liquid transitions (Franks, 1982; Perez, 1994). Glasses are a common feature of food polymers (Slade and Levine, 1991) and have also been detected in seed tissues by thermally stimulated depolarization current (Bruni and Leopold, 1992; Sun and Leopold, 1993; Sun et al., 1994) and DSC (Vertucci, 1989; Williams and Leopold, 1989; Leprince and Walters-Vertucci, 1995). As with seeds, the baseline shifts in pollen were not detected at wc values greater than about 0.35 g H_2O g^{-1} dry weight, probably because the 10°C/min cooling rate was not rapid enough to allow for glass formation at high wc values (Vertucci, 1989; Leprince and Walters-Vertucci, 1995). At wc values between 0.08 and 0.15 g H_2O g^{-1} dry weight, the shifts in the baseline were not measurable, probably because the melting transition of the triglycerides and the baseline shift occurred over the same

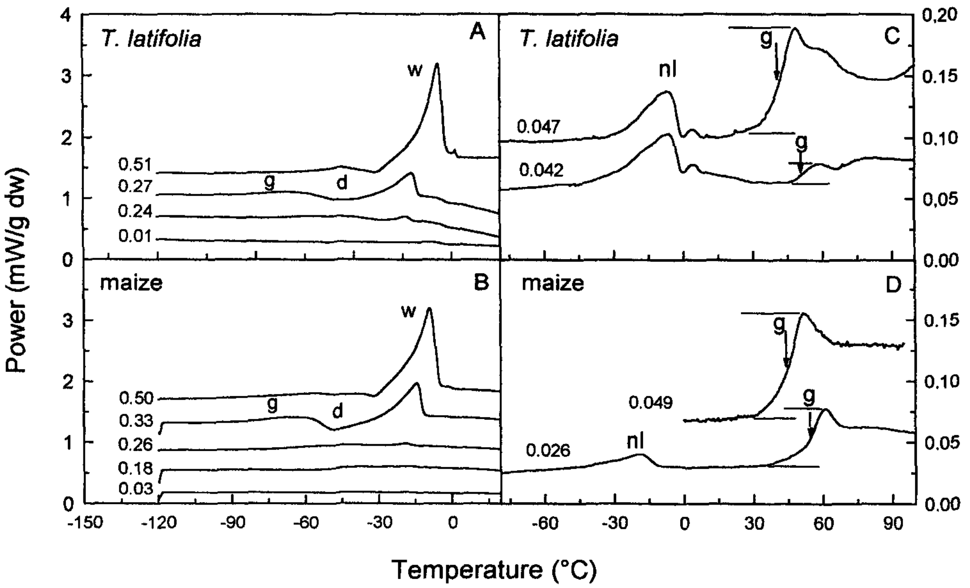

Figure 2. Heating thermograms of *T. latifolia* (A and C) and maize (B and D) pollen with varying wc values made using DSC. Samples were scanned at 10°C/min from −120 to 20°C (A and B) or from −80 to 100°C (C and D). w and nl, Water or neutral lipid melting transition, respectively. d, Devitrification event. g, Glass-to-liquid state transition. Numbers by the traces indicate wc (g H_2O g^{-1} dry weight). Arrows indicate the midpoint temperature of the glass transition. dw, Dry weight.

temperature range and the former event masked the latter one.

Plotting the ΔH of the freezing and melting transitions of water versus the wc of the dehydrating pollen may give insight into the different properties of water (Fig. 3). The ΔH g^{-1} H_2O of the melting and freezing transitions, calculated from the slopes of the linear relationship between ΔH g/dry weight versus wc ($r^2 > 0.99$ for all regressions), was approximately 300 and 330 J g^{-1} H_2O, respectively, for both pollens. A slight reduction in the value of ΔH g^{-1} H_2O compared to the value for pure water (333 J g^{-1} H_2O) is expected when the Tm is reduced due to the presence of solutes (Gekko and Satake, 1981). In contrast to these pollens, an unusually low ΔH of about 100 J g^{-1} H_2O has been observed in orthodox and minimally recalcitrant seeds at wc values between 0.15 and 0.45 g H_2O g^{-1} dry weight (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 wc relationships (Fig. 3), was 0.27 and 0.36 g H_2O g^{-1} dry weight for *T. latifolia* and maize, respectively, if determined from freezing transitions, or was 0.21 and 0.26 g g^{-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 wc at which freezing and melting transitions are not observable, water molecules may be sufficiently immobilized to limit the molecular reorganization necessary for crystallization events.

The viability of maize pollen declined if it was dried to wc values between 0.4 and 0.2 g H_2O g^{-1} dry weight (Fig. 1). This wc 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 some recalcitrant embryos (Pammenter et al., 1991, 1993). Pollination in maize is generally successful, with pollen having wc values in the range of 0.10 to 0.15 g H_2O g^{-1} dry weight (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 loss in the fertility of the sample. Further removal of the unfrozen water fraction to wc values less than 0.10 to 0.15 g H_2O g^{-1} dry weight leads to a loss in fertility. For the tolerant *T. latifolia* pollen, removal of unfrozen water is tolerated to much lower wc values.

Cp at Low wc Values

At wc values less than about 0.3 g H_2O g^{-1} dry weight, water freezing and melting transitions were not observed (Figs. 2 and 3). To investigate the properties of water at these low wc values, Cp was measured in both pollen species at temperatures between +30 and −60°C (Fig. 4). The Cp of pollen grains was a function of wc and temperature. The Cp changed sigmoidally as a function of wc, and three hydration regions (a, b, and c) could be distinguished (Fig. 4). Only two hydration regions were observed at −60°C. The wc values at which changes in the Cp-wc 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 wc values increased with decreasing temperatures (Fig. 4). Such changes in the properties of water with wc and temperature have been predicted on the basis of thermodynamic considerations (Vertucci, 1993; Vertucci and Roos, 1993).

The slopes of lines drawn for the Cp-wc relationship (Fig. 4) within each hydration region gave the Cp of the water in J $°C^{-1}$ g^{-1} H_2O for that region. The Cp of water in pollen containing less than 0.3 g H_2O g^{-1} dry weight and held at different temperatures differed from what has been reported for pure water in the liquid and hexagonal ice state (Fig. 5). In the first hydration region (Fig. 4, a), the Cp of water ranged between 0.1 and 2.2 J $°C^{-1}$ g^{-1} H_2O, depending on temperature, for both pollens. Except for the −60 and −40°C measurements in *T. latifolia*, the Cp of

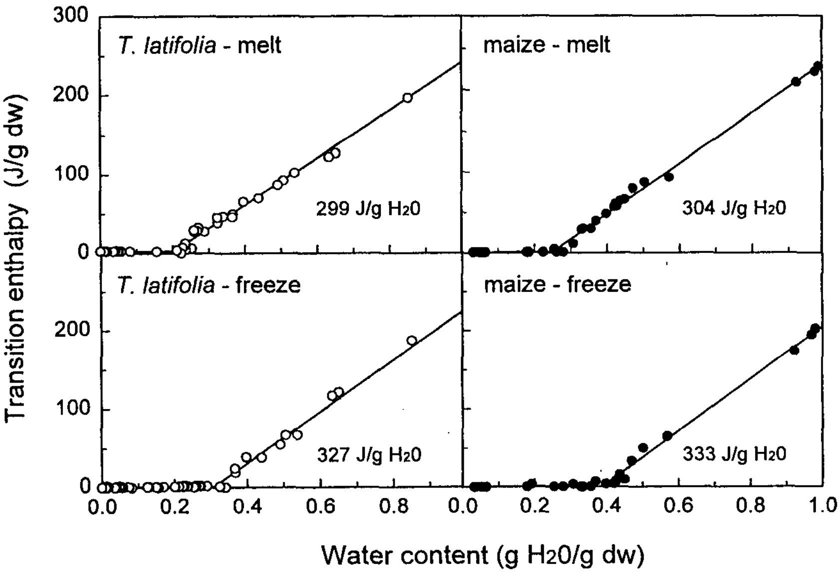

Figure 3. The relationship between the wc and the ΔH of melting and freezing transitions for pollen of *T. latifolia* (left panels) and maize (right panels). The lines drawn represent the least-squares best fit. The slopes of the lines represent the ΔH of the transition on a per g water basis. Areas of peaks were determined from thermograms similar to those in Figure 2. dw, Dry weight.

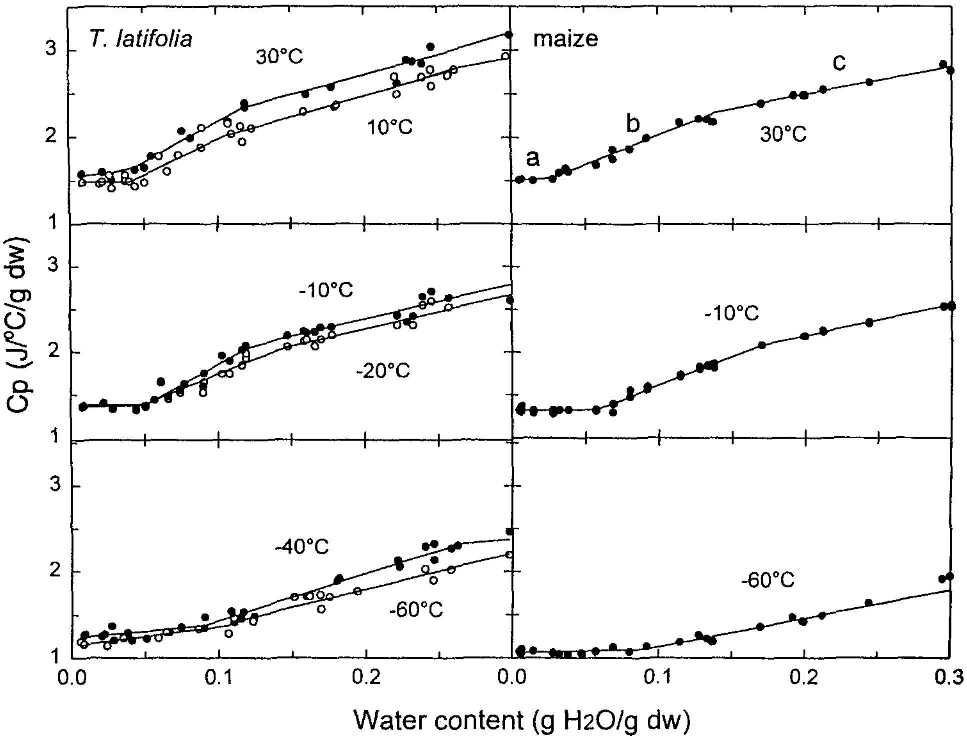

Figure 4. The relationship between the wc and the Cp of *T. latifolia* and maize pollen at temperatures between 30 and −60°C. Cp values were calculated from the ordinate displacement when samples were heated 4°C at 4°C/min. The lines are drawn according to the least-squares best fit of all the data. dw, Dry weight. a, b, and c, First, second, and third hydration regions, respectively. ●, Cp measurements at 30, −10, and −40°C (*T. latifolia*) or −60°C (maize only); ○, Cp measurements made at 10, −20, and −60°C (*T. latifolia* only).

water in region a is considerably lower than the Cp of pure water in any state (Fig. 5). Although the significance of water with such low Cp has not been established, it has been demonstrated that the longevity of seeds (Vertucci and Roos, 1990, 1993; Vertucci et al., 1994b) and *T. latifolia* pollen (J. Buitink, unpublished data) stored in this hydration range decreases with decreasing wc.

At intermediate wc values (region b), the Cp of water in the pollens ranged between 4 and 9 J $°C^{-1}$ g^{-1} H_2O, depending on temperature, and was considerably higher than the Cp of pure liquid water (ranging from 4–5 J $°C^{-1}$ g^{-1} H_2O at temperatures between −20 and +40°C) (Fig. 5). In this region glass-to-liquid transitions were observed as power shifts in the baseline (Fig. 2). Values of Cp that were higher than that of liquid water have also been reported for seed tissues (Vertucci, 1990).

As the wc of pollen grains was increased to between 0.12 and 0.20 g H_2O g^{-1} dry weight for temperatures between

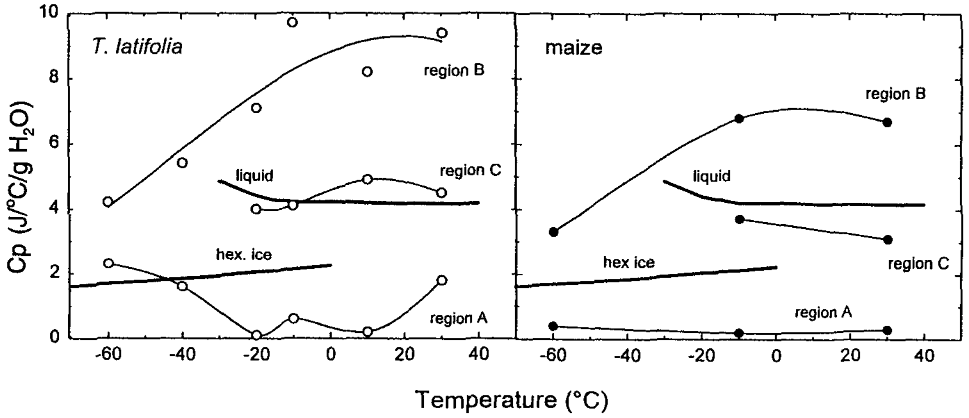

Figure 5. Cp of water measured in *T. latifolia* and maize pollen at different wc values and temperatures. Cp values were calculated from the slopes of regression lines given in Figure 4. Values of the Cp of pure water were taken from Angell (1982) (ice), Ginnings and Furukawa (1953), and Wakabayashi and Franks (1986) (liquid).

+30 and −20°C, respectively, the Cp of water approached that of pure liquid water. This third hydration region (region c) was not observed at −60°C, perhaps because the wc in this region (c) is close to where freezing and melting transitions become detectable. The viscosity of food polymers at comparable wc values is much lower than that of the glassy state, but it is considerably higher than that of liquid water (Slade and Levine, 1991; Kalichevsky et al., 1992; Angell et al., 1994). This condition is commonly referred to as a "rubbery state" (Slade and Levine, 1991; Peleg, 1993).

State Diagrams: Implication for Desiccation Tolerance

In previous studies, the impact of temperature and wc on the formation of glassy states and on the freezing behavior of water in seeds has been displayed in state/phase diagrams (Williams and Leopold, 1989; Bruni and Leopold, 1992; Vertucci et al., 1994a; Leprince and Walters-Vertucci, 1995). In Figure 6 such state/phase diagrams are depicted for *T. latifolia* and maize pollen. For both pollens, the onset temperature of ice melting was observed at higher temperatures and lower wc values than the onset temperature of water freezing, but no obvious differences could be observed between the pollens. The Tg, derived from an apparent shift in the thermogram baseline (Fig. 2), decreased with increasing wc values. The temperatures at which the glass transitions occur in these two pollen species is lower than has been reported for seeds (approximately 10–20°C difference; Leopold et al., 1994), with maize pollen having a slightly higher Tg than *T. latifolia* pollen.

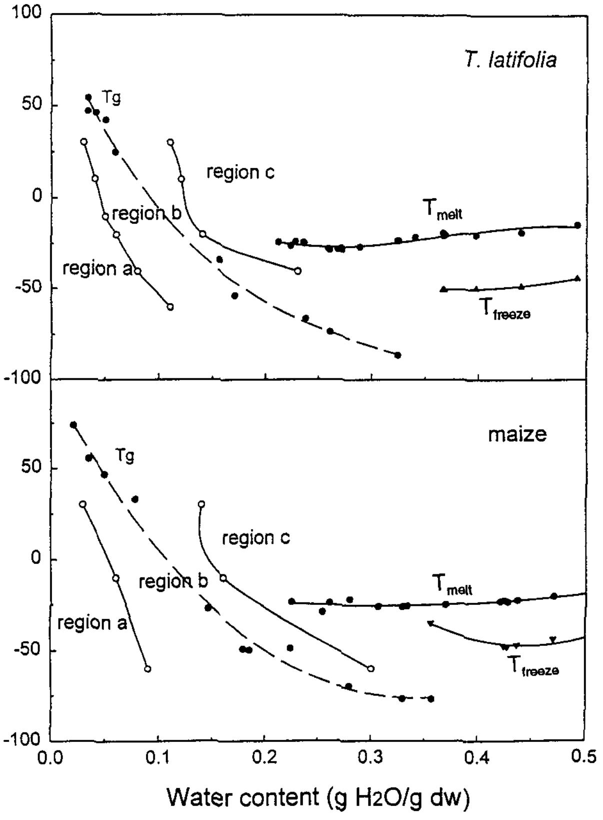

Figure 6. State/phase diagrams for pollen of *T. latifolia* and maize, describing the interaction between wc and temperature on glass-transition temperature, changes in apparent Cp, and freezing and melting events. The open circles represent the wc values at which changes in the slopes of the Cp versus wc relationships could be detected at various temperatures (Fig. 4). Onset temperature for water transitions were determined from thermograms similar to those in Figure 2. The curve used to illustrate the Tg-wc relationship is a third-order polynomial function fit to the data points. It is drawn as an aid to the eye. dw, Dry weight.

Also included in the state/phase diagrams are breaks from the Cp curves of Figure 4, which are indicative of changing water properties. The changes in Cp of water followed the wc-temperature relationship of the Tg. Region b, which was identified by an anomalously large Cp of water of the system, coincided with the glass-to-liquid (or perhaps rubbery) transition. This unusually high Cp might be due to alteration of the molecular structure of the water in the system when it is changing from the glassy state to the liquid (rubbery) state.

Considering sugars as major determinants of glass formation (Franks, 1982; Slade and Levine, 1991; Angell et al., 1994), it is interesting to note that the dry pollens contain Suc as the major (around 95%) intrinsic soluble sugar (12 and 23% of dry weight for maize pollen and *T. latifolia* pollen, respectively; Hoekstra et al., 1989, 1992), whereas maize and soybean embryos have, in addition to Suc, considerable amounts of oligosaccharides (Koster and Leopold, 1988; Kuo et al., 1988). Oligosaccharides generally result in higher Tg values for a given wc (Slade and Levine, 1991), which may explain the higher Tg values of the embryos (compare data from Williams and Leopold [1989] and Sun and Leopold [1993] with Tg values given in Fig. 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. The glassy state cannot be reached in maize pollen at room temperature without exceeding the critical minimum wc for viability (compare Fig. 6 with Fig. 1 and 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 hydrated seed tissues (Vertucci, 1989, 1993; Wesley-Smith et al., 1992) and frozen foodstuffs (Slade and Levine, 1991), 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 wc values 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 Figure 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 wc and temperature. Viability of maize pollen is progressively lost when unfreezable water is progressively removed from cells. In contrast, viability of *T. latifolia* pollen is unaffected by moisture contents as low as 0.01 g H_2O g^{-1} dry weight. The physical state of water changes as a function of both wc and temperature in both pollen species. Both pollens had comparable state diagrams, and no major differences were found in Cp, Tg, or ΔH of water melting and freezing transitions between desiccation-tolerant and -intolerant pollen. We conclude that even though the state of water is similar for desiccation-sensitive and -tolerant pollens, the response to loss of different states of water varies for desiccation-tolerant and -intolerant types.

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