

PREDICTING THE OPTIMUM STORAGE CONDITIONS FOR SEEDS USING THERMODYNAMIC PRINCIPLES

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ABSTRACT

Determination of the optimum moisture content for seed storage is difficult because deterioration is very slow at conditions close to the optimum. We have used thermodynamic principles to relate water phase behavior with different mechanisms of seed deterioration. We conclude that the optimum moisture level for storage represents a compromise between slowing aging reactions and preventing lethal ice formation by drying, and retaining the structural integrity of cellular constituents by supplying sufficient structural water. For desiccation sensitive tissues, there may not be a moisture content and temperature combination at which aging reactions are sufficiently slowed, and lethal freezing injury and desiccation damage are prevented. In this case, storage protocols require vitrification, a process by which the thermodynamically stable state is avoided by cooling very rapidly. The thermodynamic principles invoked in this paper may indicate the optimum conditions for seed storage, but they are not sufficient to describe the rate of aging under these or other conditions.

Additional index words: Seed storage, seed aging, seed longevity, bound water, calorimetry, glass, vitrification, recalcitrant, freezing injury, desiccation damage, water content, ultradry

Abbreviations: dw, dry weight; NMR, nuclear magnetic resonance, IR, infrared; ESR, electron spin resonance; TSDC, thermally stimulated direct current; DSC, differential scanning calorimetry; DTA, differential thermal analysis; DMA, dynamic mechanical analysis

INTRODUCTION

One of the primary problems associated with germplasm preservation is determining the optimum methods to store seeds. Traditionally, optimum protocols have been predicted using equations such as Harrington's Thumb Rules (Justice and Bass, 1978) or the Viability Equations (Roberts, 1973; Ellis and Roberts, 1980), which extrapolate trends of longevity observed during storage at high temperatures and supra-optimal moisture levels to more optimal conditions typically used in germplasm banks. Extrapolation of data is not grounds for criticism; unless deterioration rates at the given temperature and moisture content combination are measured directly (which may take hundreds of years), there is no other

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way to predict longevity. We have used a biophysical approach to the problem of predicting seed longevity under conditions where collection of actual aging data is impractical (Vertucci and Roos, 1990, 1993). We believe that the nature of water binding in seeds and how bound water affects the physiological status of cells (Clegg, 1978; Leopold and Vertucci, 1989; Vertucci, 1989a, 1991; Vertucci and Farrant, 1994) influences the nature and kinetics of deterioration (Rockland, 1969; Karel, 1975; Vertucci and Roos, 1990, 1993; Vertucci, 1992b). This knowledge ultimately may lead to predictive tools based on thermodynamic principles. The purpose of this paper is not to provide the answers, but to describe the approach.

The optimum condition for seed storage is one which thermodynamically or kinetically limits deleterious reactions. Thermodynamics is the study of what is possible—or more importantly, what is impossible. From thermodynamics, we know that all matter eventually arrives at an equilibrium condition (Atkins, 1982). Seed deterioration may be described as reactions leading to equilibrium. Basic principles in thermodynamics (i.e. Le Chatelier's principle) state that if temperature, pressure or composition change, the resultant equilibrium conditions also change. This means that, in a complex system where several reactions are possible, the amount of or the nature of products, which may contribute to the loss of seed viability, will vary depending on the environmental parameters. At this point, we can not specify what the "products" are, mostly, because we do not know by what mechanism(s) seeds deteriorate (Priestley, 1986). Thermodynamic principles can be used to define the nature of aging reactions and they suggest that the reactions could vary according to the storage conditions.

Equilibrium thermodynamics do not tell us the kinetics at which deteriorative reactions occur. Reaction rates are determined by physical barriers, such as the fluidity of the system and the degree to which reactants are mixed, concentration of reactants, catalysts, and inhibitors, and the activation energy of the reaction (Atkins, 1982). The collective contribution of many of these factors can be estimated by van't Hoff analyses of aging rates at various temperatures, assuming that the composition and conformation of the reactants do not change (Atkins, 1982). Before models that predict seed aging rates using thermodynamic principles can be developed, the assumptions need to be evaluated.

THE PHYSICAL PROPERTIES OF WATER

Water content and temperature are the most important factors determining seed viability and longevity (Priestley, 1986). It has long been established that drying tissues or freezing them preserves their chemical integrity. In effect, these preservation procedures alter the thermodynamic properties of water. Thus, to understand seed aging from a thermodynamic standpoint, it is important to understand the properties of water and how these are affected by moisture content and temperature.

There are a number of ways to measure water properties. The mobility of molecules can be evaluated using spectroscopic (i.e. NMR, IR, ESR) or dielectric techniques (i.e. permittivity, TSDC). Phase properties can be determined directly by calorimetry (DSC or DTA) or inferred from mechan-

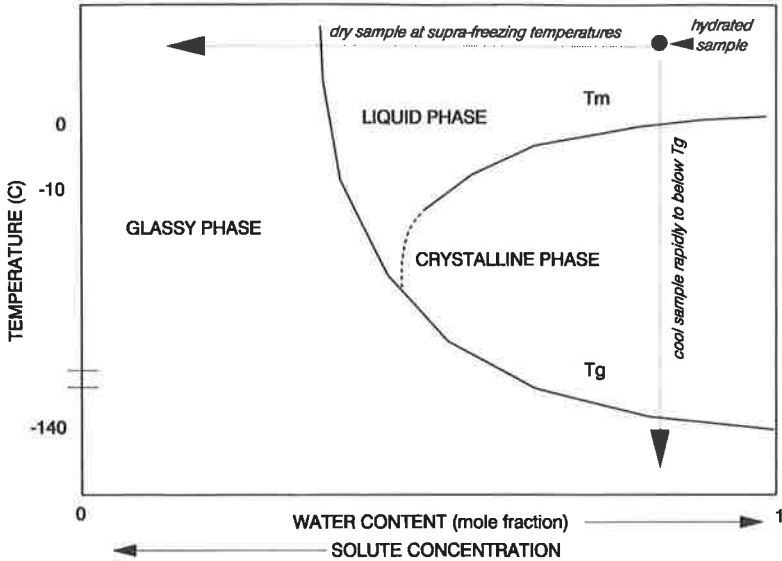


Figure 1. Schematic of a phase diagram of a water and sugar solution. T_m represents the equilibrium freezing temperature of water and T_g represents the glass transition temperature of water. The two ways of achieving a glass in a hydrated sample are demonstrated by the dotted lines.

ical properties (DMA). Discrete changes in properties are called phase changes, and these occur at a specific chemical potential (Atkins, 1982). The work presented here describes measurements made using differential scanning calorimetry (DSC).

The interaction of water content and temperature on the phase properties of water is described by a phase diagram. For a simple system of sugar and water, three main phases can be distinguished: liquid water, ice and a "glass" (Fig 1). A glass appears as a solid, but it is actually a super-viscous liquid (Franks, 1982) (e.g. a lollipop). As solute concentration increases, the glass transition temperature also increases so that, with a sufficiently concentrated solution, glasses can be observed above 0°C . In contrast, the temperature at which water freezes declines as solute content increases; this is known as the freezing point depression. Whereas ice and liquid water represent equilibrium phases, glasses do not. This means that with time, aqueous glasses revert to the equilibrium state (i.e. liquid water or ice depending on the temperature). Because they are super-viscous, the reversion to the equilibrium state is believed to be slow; thus, glasses are considered to be kinetically but not thermodynamically stable (Franks, 1982).

Glasses can be formed in two ways: 1) concentrating a solution so that the glass transition temperature is greater than the freezing temperature and then cooling the sample to ensure a more stable glass or 2) cooling

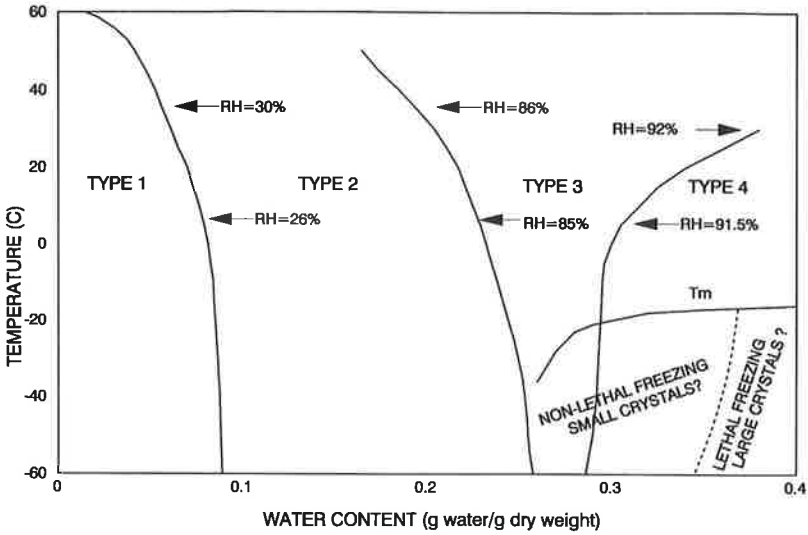


Figure 2. Phase diagram of the different states of water in soybean seeds. Solid lines were determined from moisture sorption isotherm data or calorimetry (Data are taken from Vertucci 1992b). Dashed lines are suggested from freezing injury studies (Vertucci, 1989b,c).

a dilute solution to temperatures below the glass transition temperature so rapidly (several thousands of degrees/sec) that ice crystals do not have sufficient time to grow (Franks, 1982; Fahy et al., 1984; Burke, 1986). Because glasses are believed to be important for preventing freezing injury (Fahy et al., 1984) and desiccation damage (Burke, 1986; Williams and Leopold, 1989; Koster, 1991; Bruni and Leopold, 1992;) and for promoting longevity in the desiccated state (Vertucci and Roos, 1990; Bruni and Leopold, 1992; Vertucci and Farrant, 1995), glass formation and glass stability (how rapidly a glass reverts to the equilibrium state) is critical in preservation research.

The properties of water in a seed system are far more complex than what is observed in a simple binary solution (Williams and Leopold, 1989; Vertucci, 1990; Bruni and Leopold, 1992). Different "types" of water have been identified based on their thermodynamic properties (Vertucci, 1990). These types of water can be described as different phases, and whether they represent equilibrium or non-equilibrium (i.e. glasses) phases is unknown (Williams and Leopold, 1989; Vertucci, 1990; Bruni and Leopold, 1992). A phase diagram relating temperature and the moisture content at which different types of water are present can be calculated by assuming that phase transitions occur at a given chemical potential of water (Vertucci, 1992b; Vertucci and Roos, 1993) (Fig 2).

The phase diagram in Figure 2 shows at least 4 types of water that have been identified (Vertucci, 1990). While we are not sure of the physical sig-

nificance of these different types of water, we use a globular protein as a conceptual model to explain how water interactions with different molecular surfaces can result in the different properties of water observed (Rupley et al., 1983). In a dilute protein solution, type 5 water (not shown in Fig 2) has properties similar to dilute solution water and so it would be interpreted as free water. If the solution is concentrated so that the water potential is between about -2 and -4 MPa, type 4 water can be identified by its extremely low melting temperature. Type 4 water is believed to be water that remains in pores or capillaries and does not interact directly with the protein surfaces. Further concentration of the protein to water potentials within -4 to -11 MPa (water content of about 0.25 g H_2O/g dw) reveals water that has some glassy characteristics at subfreezing temperatures. We believe this water (type 3) forms bridges over hydrophobic moieties of amino acids, necessary to fully 'wet' the molecule. When type 3 water is removed by further drying, water freezing is no longer observed. Unfreezable water (traditionally called "bound" water) can be classified as type 2 (water interacts with hydrophilic moieties of the protein) when water contents are between 0.08 and 0.25 g H_2O/g dw or type 1 (water interacts very strongly with charged groups) when water content is less than 0.08 g H_2O/g dw. Type 2 water has some solution properties, but it is extremely viscous (Vertucci and Roos, 1990) and has been described as a glass (Williams and Leopold, 1989; Bruni and Leopold, 1992). Type 1 is believed to be important for the structural integrity of the macromolecule (Vertucci, 1990; Vertucci and Roos, 1990; Bruni and Leopold, 1992).

BIOLOGICAL RESPONSES TO HYDRATION LEVELS

Physiological activity corresponds to the different types of water present (Clegg, 1978; Rupley et al., 1983; Leopold and Vertucci, 1989; Priestley, 1986; Vertucci, 1989a, 1991; Vertucci and Farrant, 1995) (Fig 3). Seed germination can only be completed if type 5 water is present, although some metabolism necessary for germination (i.e. protein synthesis) occurs in the fourth hydration level (type 4 water) (Vertucci and Farrant, 1995). Respiratory activity is first observed in the third hydration level (type 3 water) (Leopold and Vertucci, 1989; Vertucci 1989a). Some catabolic reactions catalyzed by enzymes occur in the second hydration level (type 2 water) (Leopold and Vertucci, 1989). Very few enzyme-mediated reactions occur when seeds are dried to within the first hydration level (type 1 water); however, light reactions and reactions occurring in the lipid phase can be measured (Rockland, 1969; Leopold and Vertucci, 1989).

The sensitivity of seeds to various stresses can be described by the hydration level. For example, cooling seeds hydrated to within the fourth or fifth level usually results in lethal freezing injury (Vertucci, 1989b,c; Vertucci et al., 1991; Wesley-Smith et al., 1992). Mature recalcitrant seeds do not survive complete removal of type 3 water (Pammenter et al., 1991, 1992; Berjak et al., 1993), while seeds with intermediate storage behavior do not survive complete removal of type 2 water (Vertucci and Roos, 1993; Vertucci, unpublished data). Seed aging is observed at all hydration levels except type 5 (Pammenter et al., 1991; Berjak et al., 1993; Vertucci and Farrant, 1995).

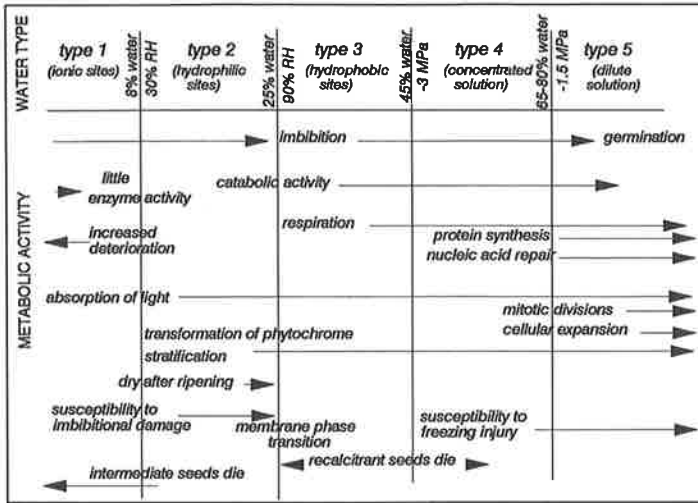


Figure 3. The effect of different hydration levels on physiological activity in seeds. Data are taken from Leopold and Vertucci (1989), Vertucci (1989a), and Vertucci and Farrant (1995).

We suggest that the mechanisms of seed aging vary with hydration level because the nature of physiological reactions differ at different moisture levels. Seeds deteriorated under "accelerating aging" (Delouche and Baskin, 1973) or "controlled deterioration" (Matthews, 1980) conditions are usually within hydration level 3, where mitochondria are functional (Fig 3). Enzyme activity is observed at hydration level 2, and so deterioration at these moisture contents may be a function of how easily substrates are transported to reaction centers, i.e. the fluidity of the system (Burke, 1986; Vertucci and Roos, 1990; Bruni and Leopold, 1992). We believe that deterioration within hydration level 1 is a result of intermolecular interactions made possible because water has been removed from reactive sites. This type of damage could be manifested by ionic bonding of charged sites on proteins, membrane phase transitions, or free radical attack on molecules (Rockland, 1969; Karel, 1975; Vertucci and Roos, 1990; Bruni and Leopold, 1992; Webb et al., 1993; Vertucci and Farrant, 1995). Since water protects macromolecules from these interactions, one would expect increased deterioration with the progressive removal of water within the first hydration level (Vertucci and Roos, 1990). Measurements of various effects of aging supports the idea that the mechanisms of aging differ at different hydration levels (Vertucci, 1992b) (Table 1). Mitochondrial activity is severely impaired when seeds are stored at high water contents, but does not appear to be affected by storage at low water contents (Table 1, oxygen uptake at 0.35 g/g). In contrast, lipid properties are affected by storage at low, but not high, humidities (Table 1, lipid transition enthalpy).

Table 1. The effect of different storage regimes on mitochondrial activity and lipid properties in soybean seeds.

Temperature, water content ^a , and time in storage	Storage Environment					stderr ^b
	5°C 0.06 6 mths	5°C 0.06 30 yrs	5°C 0.06 6 yrs	35°C 0.01 16 wks	35°C 0.35 5 d	
% germination	100	8	50	12	15	
oxygen uptake at 0.35 g H ₂ O/g dw ^c	0.65	0.78	nd ^d	0.9	0.2	±0.06
oxygen uptake at 0.12 g H ₂ O/g dw ^e	0.07	0.12	nd ^d	0.15	0.07	±0.02
lipid transition enthalpy ^f	13.1	nd ^d	9.5	10.5	14.2	±0.04

^a Water content expressed on a g H₂O/g dw basis.

^b Standard error of the mean of 5 replicates.

^c In units of μ moles/hr/g dw. Mitochondrial respiration is represented at this moisture content.

^d Not determined

^e In units of μ moles/hr/g dw. Mitochondrial respiration does not occur at this moisture content.

^f In units of J/g dw. The method is described in Vertucci (1992a). Data are taken from Vertucci (1992b).

The hypothesis that the mechanisms of seed deterioration vary with hydration level is also supported by the observation that the kinetics of seed deterioration change according to the moisture content of storage (Fig 4). In the third hydration level (water contents between 0.25 and 0.5 g H₂O/g dw), deterioration occurs within days. Deterioration is slower (within months or years) when seeds are stored at moisture contents corresponding to the second hydration level (between 0.25 and 0.08 g H₂O/g dw), and the rate is directly related to the water content. Deterioration occurs at a comparable rate when seeds are stored at water contents within the first hydration level (less than 0.8 g H₂O/g dw), but the rate is inversely related to the water content. The Viability Equations (Roberts, 1973; Ellis and Roberts, 1980) accurately predict longevity for seeds stored at hydration level 2 but fail at higher and lower moisture levels (Ellis and Roberts, 1980; Ellis et al., 1989). This suggests that the fluidity of the seed matrix is responsible for deterioration rates at this moisture level (Burke, 1986; Vertucci and Roos, 1990; Bruni and Leopold, 1992), since both longevity (Ellis and Roberts, 1980) and viscosity (Atkins, 1982; Vertucci and Roos, 1990) are exponential functions of water content in hydration level 2.

OPTIMIZING STORAGE CONDITIONS TO REDUCE SEED DETERIORATION

The idea that different deteriorative reactions occur at different hydration levels has two ramifications: 1) progressively drying tissues does not necessarily enhance seed longevity (as is implied by the longevity equations),

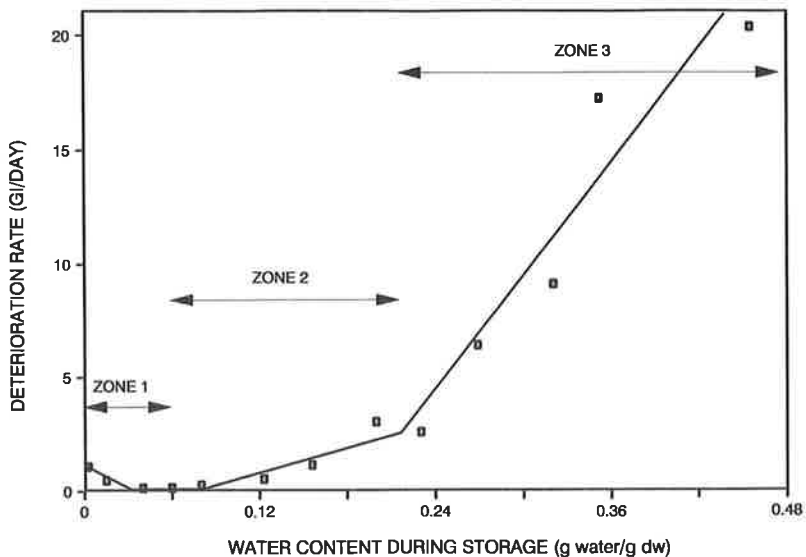


Figure 4. The effect of water content on the deterioration rate of soybeans stored at 35°C. Deterioration rate is expressed as the rate of decline in the germination index (radicle length x % germination) over the storage period. This was calculated from the regression of GI vs time stored.

and 2) optimizing storage conditions requires determining the water content at which the collective contribution of all deteriorative reactions is minimized. This principle of minimizing damage by optimizing water content can be applied to storage of both recalcitrant and orthodox seeds.

Studies of deterioration in soybean suggest that the optimum moisture content for storage is between 0.08 and 0.05 g H₂O/g dw (Fig 4). We have also demonstrated that optimum moisture levels for storage exist for other orthodox seeds, but that the moisture content varies according to the chemical composition of the seed (Vertucci and Roos, 1990) (Table 2). When expressed in terms of the equilibrium relative humidity required to obtain the optimum moisture contents, the hydration level for the various seed species is constant and corresponds to the boundary between hydration levels 1 and 2 (Vertucci and Roos, 1990) (Table 2) when the phase, as well as the functional, properties of water change. This finding suggests that the optimum moisture level for storage of any orthodox seed can be easily achieved by equilibrating to the proper relative humidity and that this practice can be justified by thermodynamic principles.

What is the optimum relative humidity for storage? This is a matter of some debate (Ellis et al., 1989, 1991; Vertucci and Roos, 1990, 1991, 1993a,b; Smith, 1992) which will only be resolved by further experimentation. Previously, we proposed that optimum moisture level for storage at 25°C could be obtained by equilibrating seeds at 20 to 25% RH (Vertucci

Table 2. The optimum moisture contents and relative humidities for longevity during storage at 35°C for five seed species with varying chemical compositions.

Crops	Lipid Content (g lipid/g dw)	Moisture level that gives maximum after-storage:	
		Water Content (g H ₂ O/g dw) ^a	Relative Humidity (%) ^b
Sunflower	0.58	0.031	36
Peanut	0.45	0.043	38
Lettuce	0.37	0.047	34
Soybean	0.18	0.059	33
Pea	0.02	0.071	30

^a Data are taken from Vertucci and Roos (1990).

^b Data are derived from 35°C isotherms given in Vertucci and Roos (1993) and unpublished data.

and Roos, 1990); others have proposed an optimum RH less than this value (Ellis et al., 1989, 1991; Smith, 1992). More recently, we have suggested that the optimum RH or water content cannot be considered independently of the storage temperature (Vertucci and Roos, 1993). Since the chemical potential of water is an important factor in the phase behavior of water as well as the physiological status of seeds, we must expect the optimum water content and corresponding relative humidity to change with temperature (Atkins, 1982). For soybeans, this relationship is described by the phase diagram in Figure 2. Theoretically, the optimum moisture level for storage of orthodox seeds corresponds to the boundary between hydration levels 1 and 2, and so optimum water content for soybean would range from 0.035 g H₂O/g dw at 65°C to 0.11 g/g at -150°C (Vertucci and Roos, 1993). To achieve these moisture contents, seeds dried at 25°C would need to be equilibrated to relative humidities of 6 and 61%, respectively (Vertucci and Roos, 1993).

The previous arguments can also be applied to storage of recalcitrant seeds. Since these seeds remain metabolically active at non-lethal moisture contents, the moisture level must be adjusted to limit both desiccation damage and freezing injury. For several recalcitrant seeds, this corresponds to hydration level 3. Successful storage of recalcitrant embryonic axes of *Landolphia kirkii* at -70°C has been achieved using these principles (Vertucci et al., 1991). However, the phase diagram in Figure 2 suggests the moisture content required for type 3 water increases and the moisture content at which lethal ice crystals form decreases with decreasing temperature. This implies that the window of allowable moisture contents gets progressively smaller as storage temperature is reduced, and eventually freezing injury cannot be prevented without inducing a lethal desiccation damage.

The protocols for seed storage described in the preceding paragraphs assume relatively stable or equilibrium conditions. Clearly, these protocols are not possible for very desiccation sensitive embryos. An alternative approach is to obtain a kinetically, rather than a thermodynamically, stable

condition — i.e. a glass. The procedure, known as “vitrification,” produces a glass using three principles: 1) concentrate the solution so that the freezing point is depressed and the glass transition temperature is increased, 2) cool the sample as fast as possible to minimize the time spent at temperatures below the freezing temperature and above the glass transition temperature, and 3) maintain the sample at a temperature well below the glass transition temperature to prevent the solution from “devitrifying” (Franks, 1982; Fahy et al., 1984). Since rapid treatment is essential for success, only small samples can be used. We have successfully applied this procedure to embryonic axes of *Camellia sinensis* (Wesley-Smith et al., 1992) and other species (Wesley-Smith, Berjak, and Pammenter, unpublished; Vertucci and Crane, unpublished) by cooling partially dried axes at about 2000°C per second. The rapid cooling rates were achieved by injecting axes into sub-cooled liquid nitrogen, a technique that has recently been adopted to cryopreserve *Drosophila* larvae (Mazur et al., 1992). Freeze fracture electron microscopy of cells from *C. sinensis* axes treated in this way show no signs of lethal ice formation (Wesley-Smith et al., 1992).

CONCLUSIONS

Thermodynamic principles relating to the phase behavior of water can be used to predict optimum moisture levels for storage of both orthodox and recalcitrant seeds. The optimum moisture level corresponds to the point at which deterioration resulting from aging reactions, desiccation damage, and freezing injury are minimized. This moisture level is considerably lower for orthodox compared to recalcitrant seeds; but for both seed types, the optimum moisture content is predicted to increase with decreasing temperature.

While the models presented in this paper suggest that optimum moisture levels for storage exist, they do not indicate the actual kinetics of deterioration. The longevity equations provide guidelines for orthodox seeds stored within the second hydration level. When orthodox seeds are stored within the first hydration level, the difference between the actual and optimum moisture contents may indicate seed longevity. There are few studies to suggest the longevity of recalcitrant seeds, but it is likely to be related to the stability of the glasses formed during vitrification. Studies of the kinetics of deterioration and the stability of aqueous glasses are presently a focus of our research.

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APPENDIX

In this paper, water content is expressed in g H₂O/g dw. This convention simplifies the interpretation of thermodynamic data. In addition, expression of water content on a dry weight basis gives an absolute scale which is expanded at high moisture levels (when compared with the scale calculated on a fresh weight basis). Water content on a dry weight basis can easily be converted to water content on a fresh weight basis (the more common convention) by the equation

$$fwb = dwb/(dwb+1)$$

where fwb is g H₂O/g fresh weight and dwb is g H₂O/g dry weight.