The Role of Water in the Cryopreservation of Seeds

N.E. Zaritzky

Abbreviations

ΔH_L	Lipid melting enthalpy
ΔH_T	Total enthalpy
db	Dry basis
DSC	Differential scanning calorimetry
ERH%	Equilibrium relative humidity
GAB	Guggenheim-Anderson-de Boer *equation*
l	Latent heat of ice melting
LN	Liquid nitrogen
RH	Relative humidity
wb	Wet mass basis
WC	Water content of the seeds
WC_{50}	Seed desiccation sensitivity
WC_u	Unfrozen water content

1 Introduction

Plant genetic resources are vitally important for human beings and the sustainability of the planet. Biodiversity conservation is the practice of protecting and preserving the abundance and variety of all species (Lambardi et al. 2004; Walters 2006).

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Neotropical ecosystems are submitted to constant pressure by human activity; in these ecosystems the number of plants at risk of genetic depletion or extinction and the loss of important genetic resources is continuously increasing. Preservation of plant biodiversity avoids the risk that species and plant varieties may become extinct, producing a definitive loss of their genetic variability. Preservation of plants only in field collections is risky, as valuable germplasm can be lost (genetic erosion) because of pests, diseases, and adverse weather conditions.

Seed storage is the most effective and efficient method for the ex situ preservation of plant genetic resources; cryopreservation can provide an important contribution to long-term conservation of valuable germplasm. Cryobiology (the study of life at low temperature) and anhydrobiology (the study of life at low water content) have some features in common, because, in environmental freezing, one of the major causes of damage is freezing-induced dehydration.

A seed is a small embryonic plant enclosed in a covering called the seed coat, usually with some stored food. The seed is the site of partial development of the embryo and the linkage between successive generations, and a critical intermediate stage in the life cycle of angiosperms and gymnosperms, which guarantees the propagation and survival of the species. The formation of the seed completes the process of reproduction in seed plants; the biological function of seeds ensures propagation of the species.

Seeds are fundamentally a means of reproduction; most seeds are the product of sexual reproduction, which produces a remixing of genetic material and phenotype variability. Other important functions of the seeds include nourishment of the embryo, dispersal to a new location, and dormancy during unfavorable conditions. Seed storage longevity depends on intrinsic properties of the species and on external factors during storage, such as temperature, relative humidity (RH) and, to a lesser extent, composition of the gaseous atmosphere.

Five levels of hydration in seed tissues were identified by Vertucci (1990). At these levels, water exhibits different physical properties, and seeds show different metabolic status. Level V corresponds to a high water content (>0.75 $gH_2O\ g^{-1}$ wb, wet basis) and the water properties in the tissue are similar to those of a dilute solution; the metabolism is normal and seeds germinate. As the water content decreases (Level IV, 0.75–0.45 $gH_2O\ g^{-1}$ wb), properties of water are similar to a concentrated solution where the interaction between water and solutes becomes stronger, and the system deviates from "ideal" behavior; water content is inadequate for cell growth and germination, but respiration occurs and synthesis of protein and nucleic acid is produced. On removal of more water (Level III, 0.45–0.25 $gH_2O\ g^{-1}$ wb), synthesis of protein and nucleic acid is not significant, but some respiration occurs. In level II (0.25–0.08 $gH_2O\ g^{-1}$ wb) only low-level catabolic events are slowly produced. In levels III and II the solution becomes concentrated and viscous, having the properties of a glass. At very low water content (Level I, <0.08 $gH_2O\ g^{-1}$ wb), there is no metabolic activity; water is tightly associated with macromolecular surfaces and its mobility is reduced ("bound water") (Vertucci 1989b; Vertucci and Farrant 1995; Pammenter and Berjak 2000).

2 Relationships Between Seed Structure and Storage Behavior

Sensitivity to desiccation and low temperatures limits the storage potential of seeds and their genetic conservation. Research on over 9,000 plant species has demonstrated that seeds can be grouped according to their storage behavior in both orthodox seeds and nonorthodox seeds. A great number of tropical species are nonorthodox seeds, including recalcitrant (Roberts 1973) and intermediate seeds (Ellis et al. 1990, 1991). The main characteristics of these seeds, according to Bonner (2008) include the following:

- True orthodox seeds can be stored for long periods at seed moisture contents of 5–10 % and subfreezing temperatures.
- Sub-orthodox seeds can be stored under the same conditions, but for shorter periods due to high lipid content or thin seed coats.
- Temperate recalcitrant seeds cannot be dried at all, but can be stored for 3–5 years at near-freezing temperatures.
- Tropical recalcitrant seeds cannot be dried, and they do not survive at temperatures below 10–15 °C.

Orthodox seeds acquire desiccation tolerance during development, can be dried to low water contents and retain viability in the dry state for predictable periods (Vertucci and Roos 1990). Examples of orthodox seeds include bean (*Phaseolus vulgaris* L.), pea (*Pisum sativum*), mungbean (*Vigna radiata*), sunflower (*Helianthus annuus*), and soybean (*Glycine max*). Most agricultural crops produce orthodox seeds, since they undergo a drying phase as they mature on the plant and usually contain around 20–30 % water when mature and are ready to harvest. After collection, orthodox seeds survive further drying to at least 5 % water content. Mature orthodox seeds can be dehydrated without damage to very low levels of moisture. The stability of orthodox seeds in the dry state has been a crucial factor in the development of agriculture and human civilization. Low water content is known to slow deteriorative chemical reactions in dry seeds. Studies of seed storage over two centuries have confirmed this observation and thus the preservation of seeds at low or ultra-low water content has been advocated.

Intracellular glasses have been detected in a number of seeds. High levels of sugars and other biopolymers in seeds result in a rapid increase in cytoplasmic viscosity during drying, which prevents the cellular biological system from reaching physical and chemical equilibrium in a measurable time frame. As a result, cytoplasmic components become vitrified, and intracellular glasses form during drying. It has been suggested that the formation of intracellular glasses may be a strategy for seeds to survive desiccation (Burke 1986; Williams and Leopold 1989; Sun 1997). Cytoplasmic vitrification minimizes major changes in molecular organization and cellular structures during dehydration, thus leading to the preservation of biological structures. The high viscosity of the glassy state immobilizes cellular constituents, thus inhibiting diffusion and slowing deleterious reactions or

changes in structures and chemical composition. In orthodox seeds, all water is bounded unfrozen water (structural water), which seems to be a crucial factor to tolerate desiccation; in storage, the longevity of seeds increases with a reduction of the water content (Vertucci and Leopold 1987; Vertucci 1989a, c, 1990; Sun and Leopold 1993). Low water content enables orthodox seeds to be stored at freezing temperatures without harm, as there is insufficient water for lethal ice-crystals to form. Most orthodox seeds remain viable for many years, even under less than ideal storage conditions. The lower limit of moisture content varies substantially between crop species, i.e., about 6 % moisture content for pea (*Pisum sativum*) and mung bean (*Vigna radiata*), and about 2 % for sunflower (*Helianthus annuus*). These moisture contents coincide with 10–12 % equilibrium relative humidity at 20 °C (aw = 0.1–0.12). Orthodox seeds are able to withstand dehydration to water contents below 5 % wet basis (equivalent to 0.053 $gH_2O\ g^{-1}$ dry basis). Successful storage of orthodox seeds was achieved under 3–7 % moisture content and −18 °C (Walters 2006). Long-term storage of orthodox seeds has also been achieved by cryopreservation (or cryostorage) at ultra-low temperatures from −80 to −196 °C with liquid nitrogen (LN). An essential first step in seed cryopreservation is the determination of optimum (safe) moisture contents for each orthodox species, particularly those with oily seeds. Long-term storage stability of orthodox seeds was correlated with the presence of a glassy state.

Figure 1 shows a schematic diagram of glass transition temperatures as a function of water content in seeds (dry basis). The graph (adapted from Sun 1997), is based on information from three seed species, *Glycine max*, *Phaseolus vulgaris,* and *Pisum sativum*, as reported by Bruni and Leopold (1991, 1992);

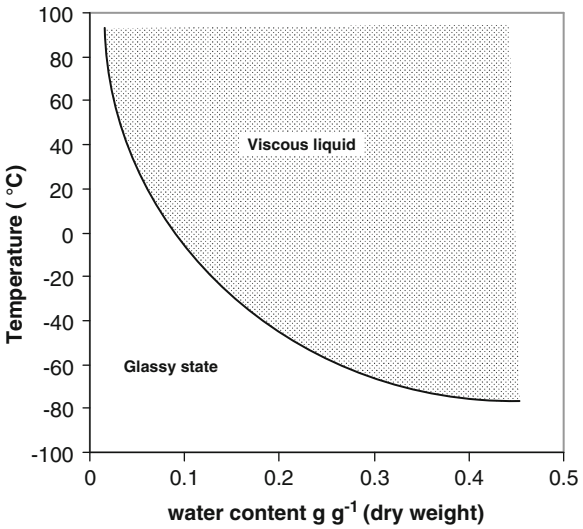

Fig. 1 Schematic representation of glass transition curve for different citrus seeds, based on data of three seed species: *Glycine max*, *Phaseolus vulgaris,* and *Pisum sativum*, as reported by Bruni and Leopold (1991, 1992), Sun and Leopold (1994), Sun et al. (1994), Leprince and Walters-Vertucci (1995), Williams and Leopold (1995), and Sun (1997)

Sun and Leopold (1994); Sun et al. (1994); Leprince and Walters-Vertucci (1995); and Williams and Leopold (1995). The effect of water content on storage stability appeared to be largely related to the plasticization effect of water on intracellular glasses in orthodox seeds.

In contrast, recalcitrant seeds do not undergo maturation drying maintaining a high water content; that is, they do not experience a reduced cellular metabolism and are sensitive to desiccation and to low temperatures (Pammenter et al. 1991; Pammenter and Berjak 2000). Recalcitrant seeds cannot be dried and cannot be stored at subzero temperatures because they are damaged by freezing injury resulting from ice formation. Examples of recalcitrant seeds included avocado (*Persea Americana*), mango (*Mangifera indica* L.), cacao (*Theobroma cacao*), and tea (*Camellia sinensis*). Due to the active metabolism in the hydrated state, viable recalcitrant seeds cannot be stored for long-term periods. The longevity of recalcitrant seeds is short, from a few weeks to a few months for species adapted to tropical environments, and up to about 3 years for several species adapted to temperate environments. Recalcitrant seeds neither tolerate nor survive desiccation. The dehydration of recalcitrant tissues produces membrane deterioration (plasmalema and mitochondria), protein denaturation, reduction of respiratory rate, and reduction in ATP level. When freshly harvested recalcitrant seeds are dried, viability is reduced considerably at certain moisture content ("critical moisture content" or "lowest safe moisture content"). If drying continues further, viability is eventually reduced to zero. The sensitivity of recalcitrant seeds to low temperatures is due to their high water content. Critical moisture contents for loss of viability on desiccation vary greatly among recalcitrant species, as well as among cultivars and seed lots, depending on the stage of seed maturity at time of collection. Recalcitrant seeds are present in at least 70% of tropical trees. Oxidative processes and free radical reactions seem to be involved in cellular and molecular deterioration; seeds show a strong resistance to rehydration, and loss of cellular integrity leads to a loss of viability. The water content at which recalcitrant seeds start to lose viability is relatively high; it is far above that at which a glassy state can exist at room temperature and nonfreezable water is lost.

Recalcitrant seeds are characterized by high water content at maturity 0.43–4.0 $gH_2O\ g^{-1}$ (dry basis, db), which is 30–80 % on a wet mass basis (wb); they cannot survive drying below around 20–30 % water content (wb). For example, *Avicennia marina* seeds are unable to survive water content lower than 0.5 $gH_2O\ g^{-1}$ (db) (33 % wb); cacao (*Theobroma cacao*) seeds rapidly lose germination when they are dried to 0.26 $gH_2O\ g^{-1}$ db. Highly recalcitrant seeds are unable to survive water content lower than 0.5 $gH_2O\ g^{-1}$ db (33 % wb). Recalcitrant seeds are unable to survive below 0.42–0.25 $gH_2O\ g^{-1}$ (30–20 % wb).

Recalcitrant and orthodox seeds differ greatly in their ecology and morphology. Recalcitrant seeds primarily come from perennial trees in the moist tropics. In some cases, they also come from temperate tree or aquatic species. Most orthodox seeds come from annual species grown in open fields. With respect to morphology, recalcitrant seeds differ from orthodox seeds not only in size, but also complexity and viability. Generally, recalcitrant seeds are covered with fleshy or juicy layers and impermeable testa. These structures maintain the seeds in a high-moisture

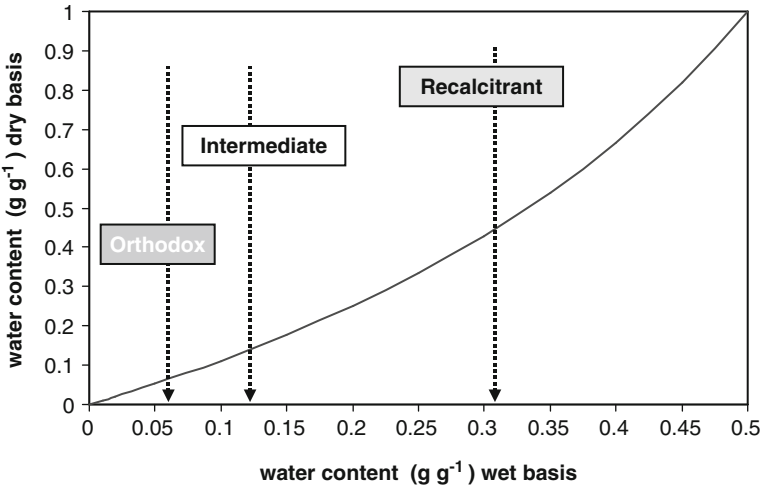

Fig. 2 Schematic diagram showing approximate values of safe moisture levels (minimum water content) for recalcitrant, intermediate, and orthodox seeds, expressed on a dry or a wet basis

environment. High moisture content of recalcitrant seeds makes them sensitive to desiccation and chilling injury. The large seed and impermeable seed coat benefit recalcitrant seeds, since they are less likely to be affected by minor fluctuations in relative humidity that might occur prior to germination.

Intermediate seeds have properties somewhat in between those of orthodox and recalcitrant seeds. Some intermediate seeds may be stored at subzero temperatures, but many are injured by freezing temperatures, while others (usually those of tropical origin) do not store well below 10 °C. Intermediate seeds are exemplified by many tropical and subtropical species, such as neem (*Azadirachta indica*), a tropical evergreen tree with medicinal properties, palm oil (*Elaeis guinensis*), coffee (*Coffea arabica*), and *Citrus* species (Dussert et al. 2001; Hamilton et al. 2008, 2009; Hor et al. 2005; Makeen et al. 2007; Sacandé et al. 2000). Intermediate seeds survive dehydration to minimum water content in the range of 0.114–0.176 g g^{-1} (10–15 % wb), but suffer desiccation injury if dried further.

Figure 2 schematically shows the approximate values of safe moisture levels (minimum water content) for recalcitrant, intermediate, and orthodox seeds, expressed on a dry and a wet basis.

3 Mechanisms Implicated in Desiccation Tolerance of Seeds

Pammenter and Berjak (2000) compared subcellular organization and metabolic activity during development of seeds of three species that attain different levels of desiccation tolerance: *Avicennia marina*, which is very sensitive to desiccation; *Aesculus hippocastanum*, which shows an increase in tolerance with development,

but at shedding was still sensitive; and *Phaseolus vulgaris*, an orthodox seed that increased in tolerance with development, and during maturation drying became even more tolerant.

Moisture level below 0.5 g g^{-1} db (33 % wb) was lethal for the highly recalcitrant tropical species *Avicennia marina*, a tropical wetland species commonly known as gray mangrove or white mangrove, a species of mangrove tree that develops in the zones of estuarine areas. The moderately recalcitrant *Aesculus hippocastanum* is a temperate terrestrial species that can tolerate dehydration between 0.42 and 0.25 g g^{-1} db (30–20 % wb). The orthodox seed *Phaseolus vulgaris* tolerates water contents as low as 0.08 g g^{-1} db (9 % wb) without viability loss.

Study of these seeds was conducted at three developmental stages: stage 1, immediately post histo-differentiation; stage 2, in the middle of the reserve accumulation phase; and stage 3, at the end of reserve accumulation. Different processes or mechanisms are proposed to confer protection against desiccation in seeds; their deficiency or absence could contribute to relative degrees of desiccation sensitivity.

The most important intracellular physical characteristics of dehydration resistance in seeds were determined according to Pammenter and Berjak (2000):

- Reduction of the degree of vacuolation that increases the mechanical resilience of cells to dehydration. *Avicennia marina* is a highly recalcitrant seed and one of the species most sensitive to desiccation. It is highly vacuolated, and this condition does not change with development, supporting the concept that drying of highly vacuolated material leads to mechanical damage. In contrast, the more tolerant species showed a decline in vacuolation with development.
- Integrity of the cytoskeleton, which is formed by microtubules and microfilaments; it is an integrated intracellular support system, and contributes to the organization of the cytoplasm and the nucleus.
- Conformation of DNA, chromatin, and nuclear architecture: maintenance of the integrity of genetic DNA material in the desiccated condition in orthodox seeds, and/or its rapid repair when seeds are dehydrated, is considered to be a fundamental requirement for desiccation tolerance.
- Intracellular de-differentiation is a characteristic of maturing desiccation-tolerant seeds. Intracellular structures are simplified and minimized (minimization of surface areas of membranes and mitochondria) in maturing desiccation-tolerant seeds. Mitochondria of the root meristem cells of recalcitrant seeds were highly differentiated and had the appearance of active mitochondria, while those in orthodox seeds were de-differentiated and appeared inactive. In orthodox seeds, there was a decline in the contribution of mitochondria to cell volume with development, and by the end of reserve accumulation, the degree of differentiation of mitochondria was very low.
- "Switching off" of metabolism: Decrease in respiratory rate is an essential event enabling an orthodox seed to withstand rapid loss of water. Recalcitrant seeds are, in contrast, metabolically active. Measured respiration rates were high in the

recalcitrant seeds *Avicennia marina* and *Aesculus hippocastanum* throughout development, while the respiration rates of the orthodox seeds of *Phaseolus vulgaris* were low, even though the seeds had not gone through maturation drying and were still hydrated.
- Presence and efficient operation of antioxidant systems (free-radical scavenging systems) should be maximally effective during maturation drying of orthodox seeds and when seeds take up water upon imbibition. In contrast, uncontrolled free-radical generation occurs during dehydration of recalcitrant seeds, thus producing damage.
- Accumulation of protective molecules, including late embryogenic accumulating/abundant proteins (LEA), (or dehydrin-like proteins).
- Accumulation of nonreducing sugars, sucrose and certain oligosaccharides, or galactosyl cyclitols; the glasses protect macromolecules against denaturation and also minimize liquid crystalline gel phase transformations of the lipid bilayer of membranes. Membrane phase behavior during dehydration and rehydration is important to the survival of seeds and other anhydrobiotic tissues.
- Deployment of endogenous amphipathic molecules into membranes upon water loss may be a prerequisite for desiccation tolerance; these molecules serve to maintain the integrity of membranes in the dry state in desiccation-tolerant organisms by lowering the water content at which the phase change of membrane lipids occurs. The essential property for desiccation tolerance is that they must be reversible, reestablishing the membranes in a functional condition upon rehydration.

Other mechanisms implicated in desiccation tolerance of seeds are (Pammenter and Berjak 2000):

- The presence of an effective peripheral oleosin layer around lipid bodies: All plant seeds that store triglycerides, such as sunflower, canola, and cottonseed, sequester these oils in specialized organelles called oleosomes, which are spherical in shape, 1–3 μm in diameter. Oleosomas are surrounded by a phospholipid layer, which is encapsulated by proteins called oleosins. Oleosins have a central, hydrophobic domain that interacts with the periphery of the lipid, and an amphipathic N-terminal domain that, with the C-terminal domain, facilitates interaction with the aqueous cytomatrix. The oleosin boundary of lipid bodies allows these hydrophobic masses to be accommodated as discrete entities in the aqueous cytomatrix under hydrated conditions, and their role during dehydration is to prevent the bodies from coalescing in desiccation-tolerant seeds.
- The presence and operation of repair mechanisms during rehydration (replacement of damaged rRNA, repair of DNA lesions, and protein-synthesizing systems).
- Amount and nature of insoluble reserves accumulated: Recalcitrant seeds stored reserves as soluble sugars, while the more tolerant species accumulated insoluble reserves during development.

4 Subzero Storage Temperatures of Seeds

Ex situ conservation practices are becoming a priority procedure in safeguarding genetic resources, especially through germplasm repositories, e.g., in field gene banks or in conventional germplasm gene banks at −20 °C. However, such methods may not represent ideal conditions for maintaining germplasm securely in the long term. In field gene banks, germplasm integrity is subjected to unexpected changes of environmental biotic and abiotic components.

Recommendations for conventional seed bank storage are 3–7 % seed moisture content (wb) and −18 °C in hermetically sealed containers. Seeds having intermediate or recalcitrant storage behavior are cold-sensitive; consequently, they cannot be stored in standard seed gene banks at −20 °C (Roberts 1973; Ellis et al. 1990). Although living organisms may suffer severe stress due to the freezing process, it has been postulated that biological activities are greatly minimized under cryogenic conditions.

Cryopreservation is proposed as the most favorable and safest technique for preserving germplasm and preventing its deterioration. Further studies are required to determine the feasibility of using cryogenic storage conditions, especially for nonorthodox seeds. Cryostorage techniques have an important application for preserving species, allowing seeds to be stored at ultra-low temperatures that reduce metabolic rates and deterioration. Long-term storage of orthodox seeds has been achieved by cryopreservation at ultra-low temperatures from −80 to −196 °C with liquid nitrogen (LN). In contrast, recalcitrant seeds do not survive drying and freezing during ex-situ conservation, i.e., they cannot resist the effects of drying or temperatures less than 10 ° C and cannot be stored for long periods like orthodox seeds because they can lose their viability.

Intermediate seeds can withstand partial dehydration, but they cannot be stored under conventional gene bank conditions because they are cold-sensitive and desiccation does not increase their longevity (Ellis et al. 1990). Intermediate seeds cannot be stored in LN without a previous partial dehydration process. The water content of seeds at the moment of immersion in LN must be regarded as the most critical factor in cryopreservation. One important factor is the cooling rate. If a liquid is cooled sufficiently quickly, freezing and vitrification can be avoided, thus forming an amorphous glass phase.

5 Cryopreservation of Citrus Seeds: A Case Study

Species that are freezing or desiccation tolerant have been observed to accumulate solutes, especially sucrose and trehalose. For nonorthodox seed species, cryopreservation is the only technique available for long-term germplasm conservation. In the case of intermediate seed-propagated species, seeds are partially desiccation tolerant and, therefore, the whole seed cryopreservation is the first option to be tested.

Seed moisture content and germination conditions need to be optimized to maximize freezing tolerance and recovery following the cryopreservation process (Dussert et al. 2001; Hor et al. 2005).

Graiver et al. (2011), analyzed the optimal moisture content hydration status for cryopreservation of different *Citrus* seeds: *Citrus sinensis* (sweet orange), *Citrus paradise* (grapefruit), *Citrus reticulata* var. Criolla and *Citrus reticulata* var. Dancy (mandarin).

The tolerance (viability) of these seeds to desiccation at different relative humidities was compared to the viability of the seeds that were desiccated prior to the LN treatment.

Seeds were extracted manually from freshly harvested mature fruits of the different *Citrus* species. After extraction, seed were surface-sterilized by immersion in ethanol aqueous solution, followed by treatment in sodium hypochlorite aqueous solution. Seeds were then rinsed twice in tap water and in distilled water and immediately surface dried. To analyze tolerance to desiccation, seeds without testa (endocarp) were placed under desiccation conditions by equilibration at 20 °C over seven saturated salt solutions (LiCl, $MgCl_2$, K_2CO_3, $NaNO_2$, NaCl, KCl, KNO_3) with constant equilibrium relative humidity (ERH%) of 11, 32, 43, 64, 75, 85, and 95 %, respectively. Water content of the seeds (WC) was determined gravimetrically after oven-drying the seeds at 103 °C until constant weight and expressed on dry basis ($gH_2O\ g^{-1}$ db). A desiccation period of 35 days was applied for the different assayed ERH, in order to achieve equilibrium conditions (constant weight). Water sorption isotherms were obtained from the data of WC and the corresponding ERH of the saturated salt solutions; the curves were modeled using Guggenheim-Anderson-de Boer (GAB) equation and the simplified D´arcy and Watt model proposed by Dussert et al. (2001).

Seed viability was analyzed using germination experiments. Seeds were sown in hermetic controlled germination conditions (humid sand in covered plastic box and kept in a growth chamber at 25 °C in the dark). The percentage of normal seedlings was evaluated 4–6 weeks after sowing.

Figure 3 shows the effect of the desiccation ERH on the viability of one of the tested citrus seeds (grapefruit, *Citrus paradise*). Similar results were reported by Graiver et al. (2011) for the other tested seeds. As can be observed, as the ERH% of the desiccation stage increased, the number of germinated seeds was higher. A decline in the germination percentage was observed for all of the tested *Citrus* seeds when desiccation was conducted at ERH < 75 %. Seed desiccation sensitivity (WC_{50}) was quantified by the quantal response model of Dussert et al. (1999).

In order to analyze the feasibility of cryopreservation of citrus seeds, the viability of seeds (germinability) submitted to different levels of desiccation, followed by LN treatment was measured. To study the tolerance to LN exposure, seeds were previously desiccated by equilibration at 20 °C over the seven saturated salt solutions, wrapped in aluminum foil and then immersed in LN 1 h. After the cooling period, seeds were immersed for 5 min in a water-bath at 37 °C and directly placed under germination conditions. Figure 3 shows the germinability percentages of seeds previously desiccated at different ERH, and then submitted to LN; as can be observed, viability was significantly lower than in the case of seeds that were only desiccated. A similar pattern of sensitivity to LN exposure after desiccation was observed for all of the tested citrus seeds. No survival was achieved at the

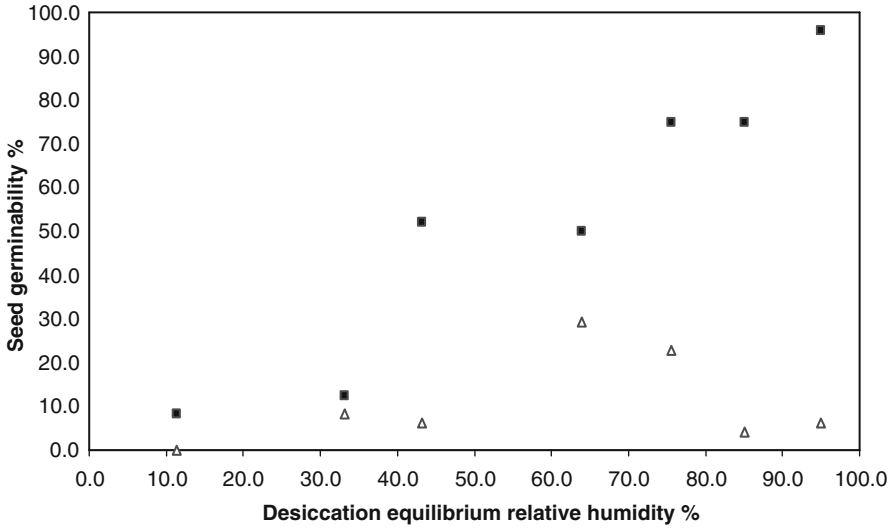

Fig. 3 Viability of *Citrus paradisi* seeds determined by % of germinability (normal seedling percentage) after desiccation at different ERH% (*filled square*) or desiccation at different ERH%, followed by liquid nitrogen treatment (*open triangle*). (Adapted from Graiver et al. 2011)

lowest assayed ERH; a maximum survival was observed between 64 and 85 % desiccation ERH, depending on the species, and a decline in seed viability percentage was found at higher ERH values. The optimal ERH ranges to achieve maximum germinability in cryopreserved seeds were 75–85 % for *C. sinensis* and 64–75 % for *C. paradisi* (Graiver et al. 2011).

In order to relate the obtained results of viability with the amount of frozen water in the seeds, differential scanning calorimetry (DSC) was used to determine thermal transitions of water and lipid melting phenomena during warming of cotyledon tissue. *Citrus* seeds, previously equilibrated at different ERH ranging between 11 and 95 % (Graiver et al. 2011) were cooled in the DSC to −120 °C at a rate of 20 °C min^{-1}; after 2 min at this temperature, the samples were heated from −120 to 100 °C at a warming rate of 10 °C min^{-1}. Enthalpies of water melting transition were determined for each previous desiccation condition, and the corresponding frozen water contents were calculated. After DSC analysis, pans were punctured and the sample dry weight was determined. Final water content of dried seeds ranged between 35 and 52 g H_2O kg^{-1} db for the tested species. Seed oil was extracted from the samples after 10 days drying over silica gel; dry seeds were ground and oil was extracted using the Soxhlet method (petroleum ether). Extracted oils were measured gravimetrically; the lipid content of the tested *Citrus* seeds ranged between 336 and 435 g kg^{-1} db. Seeds that were desiccated on silica gel were also analyzed by DSC to identify the melting transition of the seed lipids.

Figure 4 (inset) shows DSC thermal transitions of three samples: (a) untreated seed (seed that was not submitted to dehydration), with a large peak of water melting; (b) seed dehydrated in silica gel; and (c) extracted seed lipid phase. It

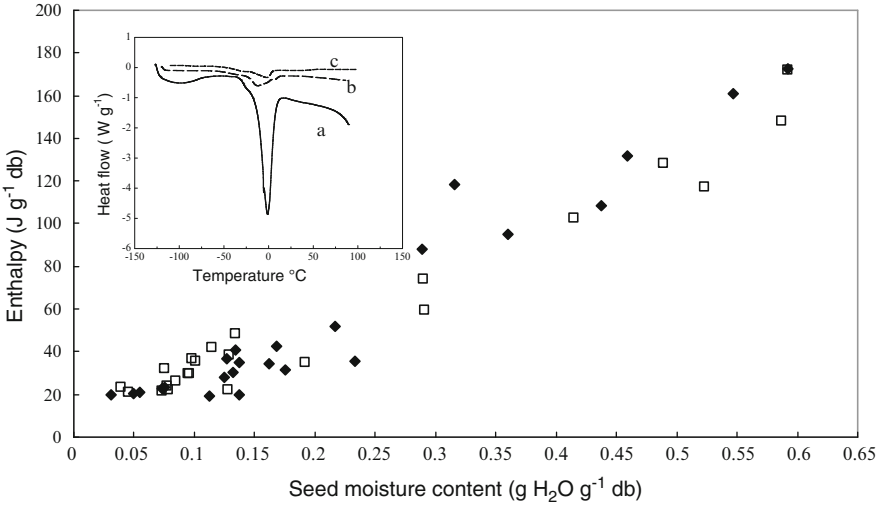

Fig. 4 Total enthalpy measured by DSC as a function of moisture content of seed samples for *C. paradisi* (*filled diamond*) and C. *sinensis* (*open square*). *Inset*: DSC heating thermograms of *Citrus* seed samples: (*a*) untreated seed, (*b*) seed desiccated on silica gel, (*c*) seed lipid extract. (Adapted from Graiver et al. 2011)

can be noted that the endotherm of the silica gel dehydrated seed was similar to that of the lipid extract. In addition, lipid and ice melting events are overlapping; therefore, to calculate the unfrozen water fraction, the lipid enthalpy must be subtracted from the total enthalpy values. Additionally, in Fig. 4 the peak areas of the DSC thermograms (total enthalpy) for *C. paradisi* and *C. sinensis* seeds that were dehydrated at different ERH previous to DSC cooling and warming programs were plotted as a function of the moisture content of the seed samples. It can be observed that at low moisture contents in the tissue, asymptotic values of enthalpy (20 J g^{-1} dry basis) can be attributed to the presence of lipids in the seeds were reached. Unfrozen water content (WC_u) was calculated as follows:

$$WC_u = WC - \left(\frac{\Delta H_T - \Delta H_L}{\lambda}\right)(1 + WC)$$

where: WC = total water content (dry basis); ΔH_T = total enthalpy measured by DSC (J g^{-1} dry basis); λ = latent heat of ice melting; ΔH_L = lipid melting enthalpy (J g^{-1} dry basis). The obtained average values of unfrozen water content (WC_u) expressed as g H_2O g^{-1} db were 0.14 and 0.13 for *C. sinensis* and *C. paradise*, respectively. The values of WC_u in *Citrus* sp. were found to be negatively correlated to seed lipid content (Hor et al. 2005; Graiver et al. 2011). Finally, it is important to relate the unfrozen water content in the seeds with LN tolerance.

Using the water sorption isotherms of each citrus seed, the equilibrium relative humidity (ERH%) of the desiccation atmosphere leading to the WC_u determined by DSC was obtained. For the measured values of WC_u (0.14 g H_2O g^{-1} db for

C. sinensis and 0.13 g H_2O g^{-1} db for *C. paradisi*) the obtained ERH% were 81 % for *C. sinensis* and 67 % for *C. paradisi* . These ERH% are included in the optimum ranges for seed cryopreservation determined by the germinability tests, resulting in 75–85 % for *C. sinensis* and 64–75 % for *C. paradisi*. Therefore, seed survival was maximized when dehydration previous to LN treatment was performed at the highest ERH%, leading to the absence of frozen water in the tissue.

6 Conclusions

The controlled dehydration process before liquid nitrogen exposure constitutes a satisfactory method by which nonorthodox oily seeds withstand cryopreservation processes. In cryopreservation, the usual goal is to achieve intracellular vitrification while avoiding intracellular ice formation and membrane damage. The limit of dehydration previous to LN treatment in intermediate *Citrus* species corresponds to the unfrozen water content in the seed. The current results offer additional evidence that lipid-rich seeds do not withstand the presence of frozen water in their tissues during the cooling/thawing process. These results agree with the findings of Vertucci (1990) and Hor et al. (2005). The *Citrus* species studied by Graiver et al. (2011) shared an important common feature for the response of the seeds to LN exposure, which is that the optimal desiccation ERH ranged between 64 and 85 %, corresponding to the higher values of *C. sinensis* and *C. reticulata* var. Criolla and var. Dancy and the lowest to *C. paradisi*.

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