Research Article

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Partial dehydration and cryopreservation of *Citrus* seeds

Natalia Graiver,[a]* Alicia Califano[a] and Noemí Zaritzky[a,b]

Abstract

BACKGROUND: Three categories of seed storage behavior are generally recognized among plant species: orthodox, intermediate and recalcitrant. Intermediate seeds cannot be stored in liquid nitrogen (LN) without a previous partial dehydration process. The water content (WC) of the seeds at the moment of immersion in LN must be regarded as the most critical factor in cryopreservation. The purpose of this study was to investigate the basis of the optimal hydration status for cryopreservation of *Citrus* seeds: *C. sinensis* (sweet orange), *C. paradisi* (grapefruit), *C. reticulata* (mandarin) in LN.

RESULTS: To study the tolerance to dehydration and LN exposure, seeds were desiccated by equilibration at relative humidities between 11 and 95%. Sorption isotherms were determined and modeled; lipid content of the seeds was measured. Seed desiccation sensitivity was quantified by the quantal response model. Differential scanning calorimetry (DSC) thermograms were determined on cotyledon tissue at different moisture contents to measure ice melting enthalpies and unfrozen WC. Samples of total seed lipid extract were also analyzed by DSC to identify lipid transitions in the thermograms.

CONCLUSIONS: The limit of hydration for LN *Citrus* seeds treatment corresponded to the unfrozen WC in the tissue, confirming that seed survival strictly depended on avoidance of intracellular ice formation.

Keywords: *Citrus* seed; cryopreservation; desiccation; water activity; DSC; unfrozen water

INTRODUCTION

Seeds have been categorized into two main groups according to their response to desiccation and their storage physiology: orthodox (desiccation tolerant) and recalcitrant (desiccation sensitive) seeds.[1] A third category of intermediate seeds has also been recognized.[2,3] The intermediate seeds are relatively desiccation tolerant, but they will not withstand desiccation down to water contents as low as those tolerated by orthodox seeds and they are freezing sensitive.[4] The rate at which hydrated biological samples are cooled to subfreezing temperatures has a great effect on their subsequent viability.[5,6]

Citrus germplasm has traditionally been conserved in *ex situ* field collections of botanic gardens and research stations because of its non-orthodox (e.g. intermediate) seed storage behavior.[7] These collections are vulnerable to pests, disease and natural disasters.

Citrus is an ancient perennial crop and one of the most economically important genera among cultivated fruit trees, growing in tropical and subtropical regions and cultivated over a wide range of latitude. The long history of cultivation and dissemination, natural and human selection played an important role in the large diversity existing nowadays within the genus *Citrus*. The taxonomy of *Citrus* is complicated due to the sexual compatibility between *Citrus* and related genera, as well as between species within the genus *Citrus*, the high rate of bud mutations and the asexual reproduction through nucellar embryony, which is characteristic for several *Citrus* species. This led to a discrepancy between classification systems related to the number and kind of species. According to the two most accepted classification systems (those of Swingle[8] and Tanaka[9]) there are 16 genera and correspondingly 162 citrus species, while Scora[10] and Barret and Rhodes[11] determined only three true *Citrus* species, Pummelo (*C. grandis* (L) Osb.), Citron (*C. medica* L.) and Mandarin (*C. reticulata* Blanco) and suggested that all other *Citrus* species originated by crosses between these main species or between them and other related genera.[12]

Oranges, mandarins, lemons and grapefruits are used for production mainly of fresh fruit, but also by-products, such as juice and marmalade. A large part of fruit production is based on local cultivars, most of them considered as clones of the widespread economically important cultivars.

Citrus seeds have been considered recalcitrant for many years. However, following an initial report demonstrating that *Citrus limon* seeds were tolerant to desiccation, additional investigations were performed on the response of seeds from other *Citrus* species and related genera to desiccation and cryopreservation.[13]

Intermediate seeds cannot be stored in liquid nitrogen (LN) without a previous partial dehydration process. The water content (WC) of seeds at the moment of immersion in liquid nitrogen

* *Correspondence to: Natalia Graiver, Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA), CONICET, Facultad de Ciencias Exactas, UNLP, 47 y 116, La Plata (1900), Argentina. E-mail: natalia@quimica.unlp.edu.ar*

a *Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA), CONICET, Facultad de Ciencias Exactas, UNLP, 47 y 116, La Plata (1900), Argentina*

b *Depto. de Ingeniería Química, Facultad de Ingeniería, UNLP, 47 y 116, La Plata (1900), Argentina*

must be regarded as the most critical factor in cryopreservation. Seed moisture should be reduced to such an extent to avoid the formation of intra-cellular ice crystals during ultra-rapid freezing (produced by the immersion of seeds in liquid nitrogen), thus preserving the integrity of seed tissues.[14]

For non-orthodox 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.

The limits of hydration for intermediate seed cryopreservation were reported by Dussert *et al.*[15] using nine different coffee species, and by Hor *et al.*[16] in the case of citrus species (*C. aurantifolia, C. grandis, C. madurensis, C. reticulata*).

Argentina produces 2285 thousand tons per year of citrus and stands as the eighth largest international producer of citrus, according to statistics released by the Argentine Citrus Federation.[17] However, in Argentina, little has been reported on whole seed desiccation and cryopreservation tolerance of different *Citrus* species.

Seed moisture content and germination conditions need to be optimized to maximize freezing tolerance and recovery after cryopreservation process. Differential scanning calorimetry (DSC) provides a useful tool for the non-invasive determination of phase transitions (water and lipid-melting phenomena) in seed tissues.[15,18] For oil-rich seeds, such as *Citrus* spp. and *Coffea* spp., DSC has been used to analyze the lipid phase transitions and the unfrozen water content.[16]

The objectives of this study were: (1) to investigate 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*); (2) to analyze the tolerance (viability) to LN exposure of seeds of the genus *Citrus*; (3) to determine the relationship between the lipid content of the seeds and their unfrozen water content; and (4) to establish the relationships between equilibrium relative humidity (ERH), seed water content, presence of frozen water and germination percentage after immersion in LN.

MATERIALS AND METHODS

Plant materials

Seeds were extracted manually from freshly harvested mature fruits of three *Citrus* species: *Citrus sinensis* (sweet orange), *Citrus paradise* (grapefruit), *Citrus reticulata* var. Criolla and *Citrus reticulata* var. Dancy (*mandarin*). After extraction, seed were surface-sterilized by 5 min immersion in 700 mL L^{-1} ethanol aqueous solution, followed by 10 min treatment in 20 g L^{-1} sodium hypochlorite aqueous solution. Then seeds were rinsed three times (5 min each), twice in tap water and, lastly, in distilled water and immediately surface dried with paper cloth.

Taking account of the findings of Cho *et al.*,[13] who reported a higher survival percentage for citrus seeds without testa than with testa, in the present work, the experiments were carried out removing by hand the testa (endocarp) from the seeds and placing the seeds directly under desiccation conditions.

Desiccation tolerance

To study tolerance to desiccation, seeds of each cultivar were desiccated 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 humidities (ERH %) of 11, 32, 43, 64, 75, 85, and 95%, respectively.

For each salt solution, the seeds were hermetically closed inside glass jars with screw caps placed inside a chamber at 20 °C in the dark. To determine the time to reach equilibrium, samples were weighed periodically until constant weight. A desiccation period of 35 days was applied for the different assayed ERH, in order to achieve equilibrium conditions in all cases.

A minimum of 200 seeds of each of the four tested *Citrus* were equilibrated at each tested ERH in order to perform the different assays.

Seed viability by germination experiments

Seeds were sown in hermetically 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 seedling was evaluated 4–6 weeks after sowing.

The analyzed factors for seed viability were: seeds of four tested *Citrus* and seven ERH%. For each of the 28 combined conditions (treatments), 48 seeds, distributed in lots of six seeds in each germination box, were used to determine seed survival.

Seed water content

The water content (WC) of seeds at equilibrium expressed on dry basis (g H_2O g^{-1} db) was determined gravimetrically after oven-drying the seeds at 103 °C until constant weight using ten replicates. ISTA methods[19] recommend a drying time of 17 h; however, in preliminary studies it was found that constant weight was not achieved in that period, therefore in the present work experiments were performed until a constant weight was reached.

Sorption curves

Using experimental data of water content of the seeds and the corresponding ERH of the saturated salt solutions, the sorption isotherms were plotted.

Lipid content determination

Ten seeds from each of the tested *Citrus* were used to determine the lipid content. Seed oil was extracted from the samples after 10 days drying over silica gel. Experiments were run in duplicates. Dry seeds (final water content of 35–52 g H_2O kg^{-1} db) were ground and oil extracted using Soxhlet (petroleum ether). Extracted oils were measured gravimetrically and the lipid content (LC) was reported on a seed dry weight basis (g kg^{-1} db)

Desiccation sensitivity

Seed desiccation sensitivity (WC_{50}) was quantified by the quantal response model of Dussert *et al.*:[20]

$$Y = \frac{V_i}{1 + \exp[-\beta(x - WC_{50})]} \quad (1)$$

where Y is the survival (%), V_i the initial viability (%), β the parameter describing the seed to seed variation for desiccation tolerance (intra-specific variation), x the water content (g H_2O g^{-1} db) and WC_{50} (g H_2O g^{-1} db) is the water content at which 50% of initial viability was exhibited. Systat software version 10.0 was used to fit the model to the experimental data

Germinability of seeds submitted to desiccation followed by liquid nitrogen treatment

To study the tolerance to liquid nitrogen (LN) exposure, seeds were previously desiccated by equilibration at 20 °C over the seven saturated salt solutions already mentioned. Seeds were wrapped in aluminum foil envelopes and then immersed in LN during 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 to determine seed viability as described above.

Differential scanning calorimetry

Analysis of thermal events during warming of cotyledon tissue of the tested *Citrus* seeds in equilibrium with seven different ERH was performed using a DSC Q100 controlled by a TA 5000 module (TA Instruments, New Castle, Delaware, USA), with a quench-cooling accessory, under a N_2 atmosphere (nitrogen flow, 0.33 mL s^{-1}). Seed samples (about 10 mg) were analyzed by placing individual samples in aluminium pans, with an empty aluminium pan used as a reference. A cooling rate of 20 °C min^{-1} was used to reach −120° C; after 2 min at this temperature a warming rate of 10 °C min^{-1} was used to heat from −120 to 100 °C. After differential scanning calorimetry (DSC) analysis, pans were punctured and the sample dry weight was determined. Seeds that were desiccated on silica gel were also tested by DSC.

Heating thermograms were analyzed for the determination of peak and onset temperatures, and enthalpy of the water melting transition; from these results the unfrozen water content was calculated.

To identify the thermal transitions of the seed lipids, about 7 mg of the total lipid extracted from the seeds, were analyzed in the thermograms using triplicates.

Statistical analysis

Statistical analyses were done using SYSTAT version 10.0 (SYSTAT Inc., USA, 1996). Analysis of variances (ANOVA) was carried out to analyze significant effects. Least significant difference tests (LSD) were used to perform pair-wise comparisons between means. Differences in means and *F*-tests were considered statistically significant when $P < 0.05$.

As the results of germinability tests correspond to a binomial distribution, the arcsine transformation[21] was applied to the binary data in order to normalize them before ANOVA test was run. As in the performed germination tests the number of seeds per box (n) was six ($n < 50$) the following equation was used:

$$Y' = \arcsin\{[(y + 3/8)/(n + 3/4)]^{1/2}\} \quad (2)$$

where Y' is the transformed variable, and y is the number of germinated seeds in each germination box. The ANOVA test was carried out then on the transformed variable Y'.

RESULTS AND DISCUSSION

Desiccation sensitivity

Preliminary germination tests carried out with *Citrus sinensis* and *Citrus paradisi* showed that the extraction of the testa from the seeds improved in 10% the germination performance.

Table 1 shows the effect of ERH on the experimental data of normal seedling percentage (viability). As can be observed as the ERH increased the number of germinated seeds was higher.

Table 1. Normal seedling percentage (viability determined as the percentage of the seed germinated after desiccation) at different equilibrium relative humidity ERH (%) for *C. reticulata* var Dancy, *C. sinensis*, *C. paradise* and *C. reticulata* var. Criolla

ERH (%)	*C. reticulata* var. Criolla	*C. reticulata* var. Dancy	*C. sinensis*	*C. paradisi*
11	27 (3)	27 (5)	6 (2)	8 (2)
33	44 (5)	68 (4)	8 (3)	12.5 (4)
43	79 (4)	75 (3)	22 (4)	52 (6)
64	89 (4)	89 (4)	29 (5)	50 (6)
75	94 (3)	100 (0)	77 (5)	75 (4)
85	83 (5)	92 (3)	60 (4)	75 (4)
95	98 (2)	100 (0)	72 (6)	96 (3)

Standard deviations are indicated between parentheses.

By fitting Equation 1 to the experimental data of normal seedling percentage as a function of seed water content (db), two parameters (WC_{50} and β) were calculated for each tested *Citrus* (Fig. 1).

Table 2 shows differences among the estimated parameters of the four types of tested seeds representing the desiccation tolerance. WC_{50} (g g^{-1}) ranged from 0.05 for *Citrus reticulata* (v. Dancy) to 0.11 for *Citrus paradisi*. Statistical analysis showed significant differences ($P < 0.05$) among the calculated parameters.

Two-way analysis of variance of the transformed viability values showed that main factors (ERH and the four *Citrus* seeds) have significant effects ($P < 0.05$) on the germinability after desiccation. Thus according to the LSD test the mean viability values of the different seeds were: *Citrus sinensis* < *Citrus paradisi* < *Citrus reticulata (*v. *Criolla)* < *Citrus reticulata* (v. Dancy).

A decline in the germination percentage was observed in all the tested *Citrus* seeds when desiccation was conducted at ERH < 75% and the lowest values were observed at 11%. *Citrus* with lower β values (*Citrus paradisi* and *Citrus sinensis*) were more sensitive to desiccation than *Citrus reticulata* seeds.

Water sorption isotherms

The water sorption isotherm is the relation between the equilibrium moisture content of a material (expressed as mass of water per unit mass of dry matter) and water activity, at a given temperature. Water sorption in biological materials is one of the main factors that affect their physical state and stability.[22] Knowledge of water sorption properties is extremely important in predicting the physical state of biological materials at various conditions.

Sorption isotherms were determined using the simplified D'arcy and Watt model Equation 3, proposed by Dussert *et al.*:[20]

$$WC = K' + C\frac{p}{p_0} + \frac{kk'(p/p_0)}{1 - k(p/p_0)} \quad (3)$$

where WC is the seed water content (g H_2O g^{-1}, dry basis, db), p/p_0 is the relative vapor pressure, and corresponds to aw = ERH/100; K', C, k and k' are specific parameters. The GAB model (Equation 4) was also applied:

$$X_{aw} = \frac{X_m C_g K_{aw}}{(1 - K_{aw})\left[1 + (C_g - 1)K_{aw}\right]} \quad (4)$$

where X_{aw} is the seed water content (g H_2O g^{-1} db), X_m monolayer moisture content in db, C_g is the Guggenheim constant, aw is

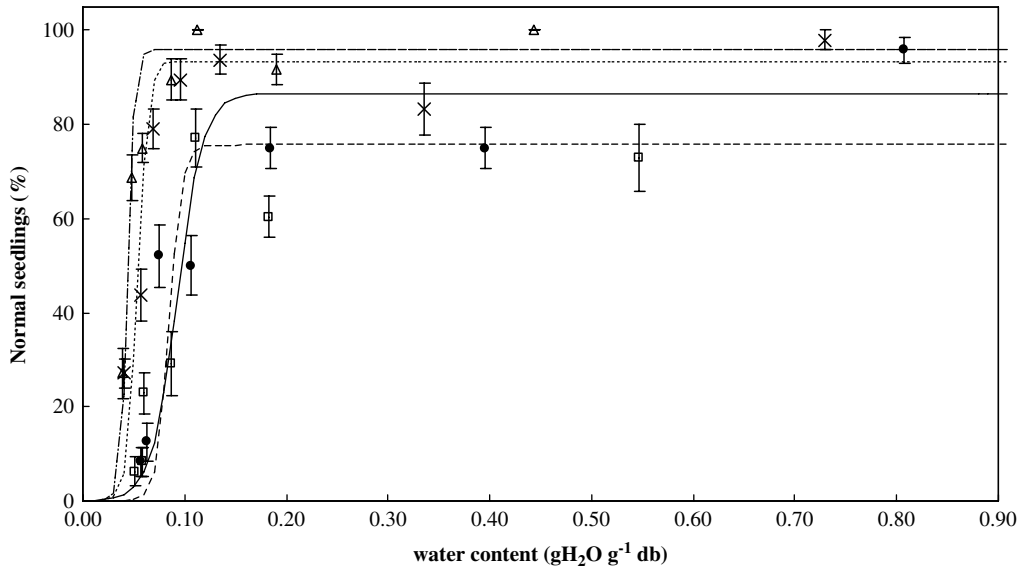

Figure 1. Normal seedling percentage (viability determined by germination percentage after desiccation) as a function of water content for different *Citrus* seeds: *C. reticulata* var Dancy, (△, –··–··–); *C. reticulata* var. Criolla (×, - - - -); *C sinensis* (□, – – – –); *C. paradise* (●, ———). Lines correspond to the model of Equation 1. Bars indicate standard deviations of the means.

Table 2. Seed desiccation sensitivity quantified by the quantal response model of Dussert *et al.*[20] (Equation 3)

Citrus species	WC_{50} (g g^{-1})	β
C. sinensis	0.09[a]	63[a]
C. paradisi	0.11[b]	23[b]
C. reticulata var. Criolla	0.06[c]	77[c]
C. reticulata var. Dancy	0.05[d]	109[d]

WC_{50} is the water content at which half of the initial germinability was lost, and β the seed-to-seed variation.
[a,b,c,d] Means that have no superscript in common are significantly different from each other ($P < 0.05$) calculated by least significant difference test (LSD).

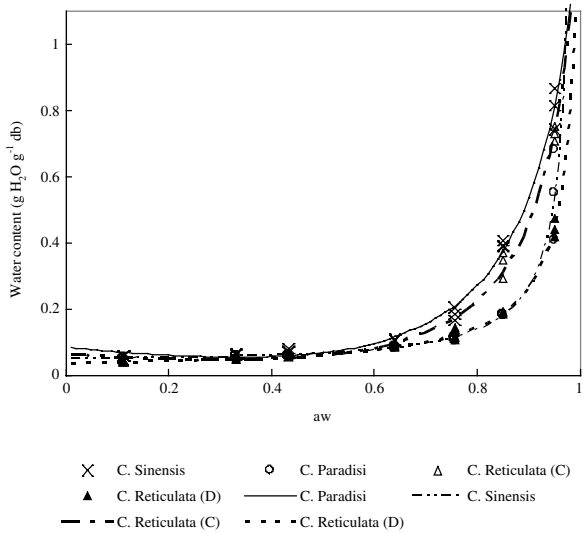

Figure 2. Experimental values and predicted sorption isotherms of the different Citrus seeds at 20 °C. *C. paradise* (×, ———), *C. reticulata* var. Criolla (△, – – – –), *C. sinensis* (○, –··–··–), *C. reticulata* var Dancy (▲, - - - -). Lines correspond to the simplified D'arcy and Watt model (Equation 3).

the water activity, and *K* is a factor correcting properties of the multilayer molecules relative to the bulk liquid.

Fitted water sorption isotherms are shown in Fig. 2 using Equation 3. The curves followed the expected shape in the range of relative humidities studied, with a sharp increase at aw > 0.7. Table 3 shows the obtained parameters K′, *C*, *k* and *k*′ of the D'arcy model and those of the GAB isotherm model[23] (Guggenheim–Anderson–de Boer). High significant correlation coefficients were obtained with both models; however, Equation 3 shows a better fitting of the experimental results, mainly at low values of aw.

Total lipid content

The total lipid content of the tested *Citrus* seeds ranged between 336 and 435 g kg^{-1} db in *C. sinensis* and *C. reticulata* var. Criolla, respectively (see first column of Table 4). ANOVA test showed that significant differences were observed in the lipid content among the three *Citrus* species ($P < 0.05$).

Seed germinability after LN exposure

Figure 3 shows that germinability percentages of the tested seeds, that were previously desiccated at different ERH, submitted to LN and rewarmed were significantly lower than in the case of seeds (Table 1) that were only desiccated ($P < 0.05$).

Regardless of the *Citrus* species, a similar sensitivity to LN exposure after desiccation was observed for seed germinability (Fig. 3). No survival was achieved at the lowest assayed ERH; a maximum was observed between 64 and 85% desiccation ERH and a decline in seed germinability percentage was found at ERH values higher than 85%.

Figure 3 allows the determination of the optimal desiccation ranges (ERH %) for seed tolerance to LN exposure. The values obtained were: 75–85% for *C. sinensis* and *C. reticulata* var. Criolla and var. Dancy; in the case of *C. paradisi* the optimum range of ERH was 64–75%.

Table 3. Parameters of the applied water sorption models: D'arcy and Watt model (Equation 3) and GAB (Guggenheim–Anderson–de Boer) model (Equation 4)

Model	Parameter	*C. sinensis*	*C. paradisi*	*C. reticulata* var. Criolla	*C. reticulata* var. Dancy
D'arcy and Watt	K'	0.051	0.085	0.064	0.036
	C	−0.023	−0.304	−0.197	−0.009
	k	1.00	0.908	0.937	0.978
	k'	0.026	0.162	0.106	0.032
	R^2 (%)	97	99	99	99
GAB	X_m	2.9	5.4	3.2	4.4
	C_g	1.3E10	6.2E10	1.8E10	−1.1E10
	K	0.996	0.982	0.978	0.989
	R^2 (%)	96	99	99	99

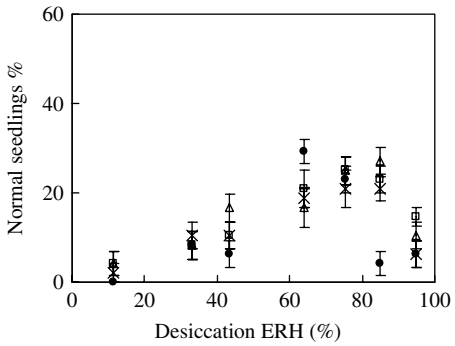

Figure 3. Effect of desiccation at different ERH% (before the liquid nitrogen treatment) on the viability of cryopreserved seeds (germinability percentages or normal seedling percentage) of different *Citrus*: (△) *C. reticulata* var Dancy, (□) *C. sinensis*, (●) *C. paradise*, (×) *C. reticulata* var. Criolla. Bars indicate standard deviations of the means.

Effect of the previous desiccation step on the frozen water fraction in the seed tissue by DSC

In order to determine the effect of the seed desiccation conditions on the frozen water fraction in the tissue, DSC thermograms of all the tested treatments (4 *Citrus* seeds × 7 ERH) were obtained. All the samples were submitted to the previously described *in situ* cooling and warming procedure. As an example Fig. 4 shows the heating thermograms of C. *sinensis* seed cotyledons that were desiccated at different ERH ranging between 11 and 95%; besides seed tissue desiccated on silica gel was also run. As can be seen in Fig. 4 the endothermic peak area close to 0 °C decreases as ERH diminishes. Similar behavior was observed for the other tested seeds.

The peak areas of the DSC thermograms (total enthalpy) were plotted as a function of the moisture content of the seed sample (determined gravimetrically). Figure 5 shows as an example the results obtained for *C. paradisi* and *C. sinensis*; it can be observed that at low humidity contents in the tissue, asymptotic values of enthalpy were reached. These ΔH values could be attributed to the presence of lipids in the seeds (20 J g^{-1} dry basis).

In order to validate this assumption, seed lipid extracts and seeds that were desiccated on silica gel, were analyzed by DSC to determine the endothermic lipid transition and to identify the position of the corresponding peaks. Figure 6 shows these thermograms for *C. sinensis* and also the thermal transition of the untreated seed. The endotherm of the silica gel dehydrated seed was similar to that of the lipid extract, therefore the melting transition of the lipids could be determined from the DSC curve; it must be noted that lipid and ice melting events are overlapped

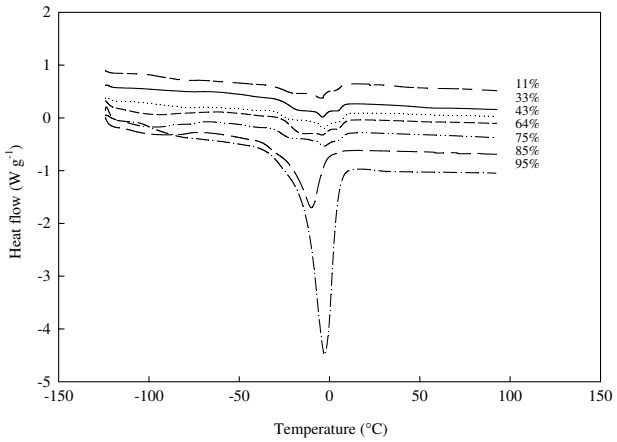

Figure 4. DSC heating thermograms of C. *sinensis* seed cotyledons that were desiccated at different ERH ranging between 11 and 95%. The water content (db) of each sample is also shown between brackets expressed as g H_2O g^{-1} db. ERH: 11%, [0.051] (- - - -); ERH: 33%, [0.058] (———); ERH: 43%, [0.059] (· · · ·); ERH: 64%, [0.086] (); ERH: 75%, [0.11] (- - - -); ERH: 85% [0.183] (– – –); ERH: 95%, [0.546] (–··–··–).

and to calculate the unfrozen water fraction, the lipid enthalpy must be substracted from the total enthalpy values.

The unfrozen water content (WC_u) was calculated as follows:

$$WC_u = WC - \left(\frac{\Delta H_T - \Delta H_L}{\lambda}\right)(1 + WC) \quad (5)$$

Table 4. Total lipid content (g kg^{-1} db), unfrozen water content (WC_u, g H_2O g^{-1} db) and optimum relative humidities for desiccation before cryopreservation in liquid nitrogen for the different *Citrus* seeds

Citrus species	Lipid content (g kg^{-1} db)	WC_u (g H_2O g^{-1} db)	ERH% *	Optimum ERH%
C. sinensis	336 (6)[a]	0.14	81	75–85
C. paradisi	364 (3)[b]	0.13	67	64–75
C. reticulata var. Criolla	435 (8)[c]	0.11	69	70–85
C. reticulata var. Dancy	412 (7)[c]	0.11	75	75–85

Standard deviations are indicated between parentheses.
* ERH% was calculated applying Equation 3 to WC_u values (second column).
[a,b,c] Means within columns without common superscript letters differ ($P < 0.05$) as calculated by the least significant difference test (LSD).

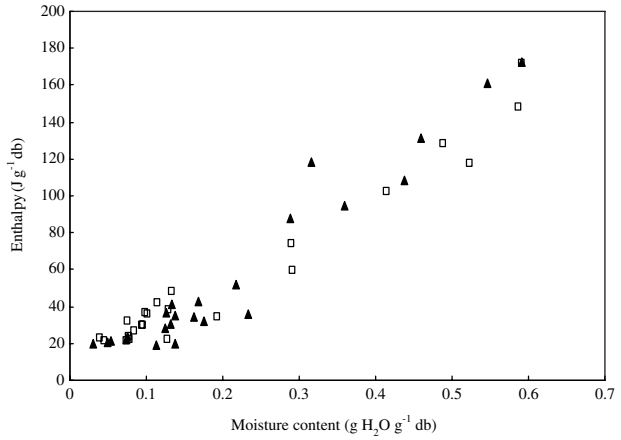

Figure 5. Total enthalpy measured by DSC as a function of the moisture content of the seed samples (determined gravimetrically) for *C. paradise* (▲) and C. *sinensis* (□).

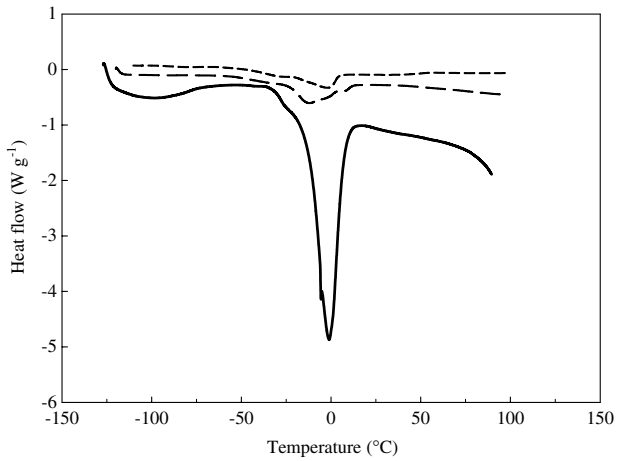

Figure 6. DSC heating thermograms of *Citrus sinensis* seed samples: (———) untreated, (– – –) seeds that were desiccated on silica gel, (- - - -) seed lipid extract.

where WC is the total water content (dry basis); ΔH_T is the the total enthalpy measured by DSC (J g^{-1} dry basis); λ is the latent heat of ice melting; ΔH_L is the lipid melting enthalpy (J g^{-1} dry basis).

Usually the enthalpy of pure water (333.9 kJ kg^{-1}) is used in Equation 5 according to the method proposed by Weast and Astle[24] Although it is known that the effective latent heat of ice melting decreases with increasing concentration of the solution; the amount of the decrease in latent heat depends on the solute. The effective latent heat of fusion in aqueous solutions can be calculated by considering the effects of freezing point depression and dilution heat in each aqueous solution;[25] exothermic or endothermic reactions occur when the solution is diluted with water due to melting of ice, and then the effective latent heat of fusion varies. However, this enthalpy variation is small in diluted solutions: for example in the case of NaCl solution as the concentration of solute increases from 0 to 5% w/w the enthalpy decreases only from 333.9 J kg^{-1} to 330 J kg^{-1} (lower than 1%). Therefore in Equation 5 it was assumed for the calculations of the unfrozen water fraction the enthalpy of pure water. Values obtained for WC_u are shown in Table 4.

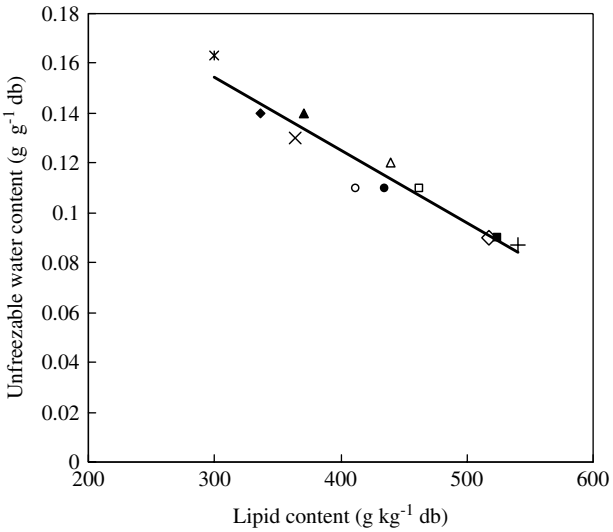

Figure 7. Relationship between the lipid content of the *Citrus* seeds and the unfrozen water content determined by DSC: (♦) *C. sinensis*, (×) *C. paradise*, (●) *C. reticulata* var. Criolla and (○) *C. reticulata* var. Dancy (present study); (■) *C. aurantifolia*, (▲) *C. grandis*, (□) *C. madurensis*, (◊) *C. reticulata* (Hor *et al.*[16]); (△) *C. australasica*, (+) *C. inodora* and (✱) *C. garrawayi* (Hamilton *et al.*[26]).

Figure 7 shows the relationship between the unfrozen water content and the lipid content of the *Citrus* seeds analyzed in the present work; data reported by Hor *et al.*[16] and Hamilton *et al.*[26] for different *Citrus* species were also included in the same plot. The obtained linear regression considering all the experimental data is given by the following equation:

$$WC_u = 0.243 - 0.00029 \times LC, \ (R^2 = 0.935) \quad (6)$$

where WC_u (g H_2O g^{-1} db) is the unfrozen water content of seed and LC is the seed lipid content (g kg^{-1} db).

Relationship between unfrozen water content in the seeds with LN tolerance

The unfrozen water contents (db) of the seeds (already determined by DSC) can be expressed as ERH% of the desiccation atmospheres using the sorption isotherms given by the D'arcy and Watt model (Equation 3). Introducing in the sorption curves of Fig. 2 the unfrozen water contents determined by DSC (WC_u), the corresponding ERH values were calculated using Equation 3 and are shown in Table 4. As can be observed, the calculated ERH values are close to the optimum ERH ranges determined by the germinability tests shown in the last column of Table 4. Therefore the optimum seed water content is that at which no more frozen water was detected, as determined by DSC analysis. The limit of dehydration previous to the LN treatment in intermediate *Citrus* species corresponds to the unfrozen water content in the seed, confirming that oily seed survival strictly depends on avoidance of intracellular ice formation.[16] The present results offer additional evidence that lipid-rich seeds do not withstand the presence of freezable water in their tissues during the cooling/thawing process.

The *Citrus* species studied in the present work shared a main common feature for the response of their seeds to LN exposure, which is that their optimal desiccation ERH ranged between 64 and 85%, corresponding to the higher values to *C. sinensis* and *C. reticulata* var. Criolla and var. Dancy and the lowest to *C. paradisi*.

CONCLUSIONS

In the present work the optimal moisture content hydration status for cryopreservation of *Citrus* seeds (*Citrus sinensis, Citrus paradisi, Citrus reticulata* var. Criolla and *Citrus reticulata* var. Dancy) were analyzed by establishing the relationships between equilibrium relative humidity (ERH%), seed water content, presence of frozen water (by differential scanning calorimetry) and germination percentage after immersion in LN.

For the desiccation proposed step the limit of hydration for *Citrus* seeds corresponds to the unfrozen water content in the tissue, confirming that seed survival strictly depended on avoidance of intracellular ice formation. *Citrus* seeds do not tolerate the presence of frozen water in their tissues during the cooling/thawing process.

The relationship between the lipid content of the seeds and their unfrozen water content was established and a good agreement with literature reported data was observed.

The controlled dehydration process before liquid nitrogen exposure constitutes a satisfactory method by which non-orthodox oily seeds withstand cryopreservation processes. From the obtained results, a simple and rapid methodology can be proposed to predict the tolerance of *Citrus* seeds to LN treatment that could avoid germinability tests and DSC measurements. It consists on the following stages: (1) Determination of the water sorption curve of the seed; (2) Measurement of the lipid content; (3) Determination of the unfrozen water (WC_u) content from the linear regression; and (4) Determination of the ERH% using the value of WC_u in the sorption isotherm; this ERH% is the optimum desiccation condition to obtain maximum viability after liquid nitrogen treatment. Therefore the proposed method gives a rapid and consistent evaluation of the possibility of cryopreserving whole seeds of these oily *Citrus* species.

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