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LOW-TEMPERATURE STORAGE AND CRYOPRESERVATION OF GRAPEFRUIT (*Citrus paradisi* Macfad.) SEEDS

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Abstract

BACKGROUND: Grapefruit is an economically important fruit worldwide, but our knowledge of its seed biology is rather poor. **OBJECTIVE:** The present study aimed to develop techniques for banking and cryopreservation of grapefruit seeds. **MATERIALS AND METHODS:** Grapefruit seeds with the exotesta removed were used. Seeds were desiccated to three moisture levels between 5-9% and stored at 15ºC, 4ºC and -20ºC for up to 24, months to investigate seed lifespan in conventional seed bank. Meanwhile seeds desiccated by silica gel or saturated salt solution and embryonic axes by flash drying were employed to develop cryopreservation protocols. **RESULTS:** It was confirmed that grapefruit seeds have some intermediate properties, being able to withstand removal of type II water up to 7% MC, but sensitive to -20°C storage. For cryopreservation, the excised embryonic axes had a wider moisture window between 5% and 15%, with a maximum past-thaw emergence of 95%, while seeds survived only with a maximum past-thaw emergence of 50% or 70% from a much narrow moisture window. **CONCLUSION:** In contrast to previous reports on another type II seed, coffee, we found that citrus seeds desiccated by silica gel had better post-thaw viability than those subjected to equilibrium desiccation with saturated salt solutions. Further investigation is required to elucidate the mechanisms that contribute to variable cryopreservation tolerance.

Keywords: intermediate seeds, desiccation tolerance, seed storage, equilibrium desiccation, saturated salt solutions, tropical plant germplasm conservation.

INTRODUCTION

Grapefruit (*Citrus paradisi* Macfad.), having high nutritional value and unique taste, is one of the major citrus fruits produced in the world. Grapefruit as a spontaneous hybrid may have originated from the Caribbean and was introduced into Florida in 1837 (7, 17). However, some scholars believe that grapefruit may be native to northeastern India (9) or South China (20). Now this fruit has been widely cultivated in subtropical and tropical areas, with America, Philippines, Israel, South Africa, Brazil, Argentina and Australia being the main producers in the world (7). Grapefruit has many cultivars, which can be conveniently classified into two categories by pulp colour: white pigmented cultivars, such as 'Marsh'; and red pigmented cultivars, such as 'Star Ruby' and 'Henderson' (19). These cultivars are usually conserved as living plants in field genebanks, at the risk of disease, pest, fire, drought and human damage. So it is necessary and urgent to apply seed banking techniques to these grapefruit genetic resources as complementary conservation methods.

However, *Citrus* genus has a high frequency of intermediate and recalcitrant seeds

(13). Previous studies reported that grapefruit seeds survive desiccation to 15.3% (1) and 14.1% MC (15). Citrus seed has an average oil content as high as 41.4% (13). Based on these facts, Liu et al. (13) suggested that they were possibly intermediate in storage response, although this is not certain for all species in the genus. Therefore more investigations are needed.

In this study, grapefruit seeds at three moisture levels were stored at three temperatures to investigate the lifespan under conventional seed bank conditions. Meanwhile whole seeds and embryonic axes were dehydrated and frozen to develop effective cryopreservation protocols for long-term conservation of this species.

MATERIALS AND METHODS

Plant material

Xiaotianyou, one of the widely-grown cultivars of grapefruit in China, with a yellowish-white sarcocarp, was used in this study. Mature fruits were harvested from trees growing in Menglun, Mengla, Xishuangbanna (21°41'N, 101°25'E; altitude 580 m) in November of 2010 and 2012. After extraction, the seeds were air-dried at ambient condition for 1-2 days and then the exotesta was removed, as it is known to inhibit germination of *Citrus* seeds (12, 16). Hereafter, these partially decoated seeds are referred to as seeds.

Initial viability, seed weight and moisture content were determined immediately using the methods described below. The rest of the seeds were stored in polyethylene bags at 15ºC for up to 5 days prior to use in the experiments.

Determination of seed moisture content

According to the ISTA (11) recommendations for oily seeds, 17 h at 103 ± 2ºC in a ventilated oven was used to remove seed water. Moisture content was expressed on a percentage fresh weight basis. Eight replicates of single seeds or five embryonic axes were used for each of these determinations.

Seed viability assessment

Prior to sowing, all seeds withdrawn from the desiccation, freezing or storage treatments were pre-humidified for 24 h at 30ºC to prevent imbibition damage. This was accomplished by placing seeds over distilled water in hermetically sealed sandwich boxes. Then six 100-mm Petri dishes, each containing 20 seeds sown on 1% plain agar, were placed in a 30ºC incubator and germination was monitored once a week for 3 months. Those seeds that formed normal seedlings were scored as 'emergence'. A crush test was employed to the ungerminated seeds before finishing the germination test, with soft seeds being judged as being inviable.

Seed desiccation and storage in conventional seed bank

Seeds harvested in 2010 were used in this experiment. They were firstly dried in a drying room (kept at 15ºC and 50% RH) for one week and further dried by silica gel to 9.6%, 7.4% and 5.6% MC. After desiccation, seed viability was determined. The rest of the seeds at each moisture level were divided into 21 subsamples, for storage at 15°C, 4°C or -20°C in hermetically sealed aluminium foil laminate bags. They were withdrawn from low temperatures for viability determination after storage for 1 week, 1, 3, 6, 9, 12 and 24 months.

Seed desiccation and cryopreservation

These experiments were conducted in 2010 and 2012, respectively, and two methods were used to desiccate these seeds. The equilibrium desiccation (ED) experiment followed the methods of Hor et al. [2005 (10)]. Seeds were equilibrated at 25ºC for 4 or 5 weeks in enclosed chambers over one of ten relative humidities from 8% to 92%, provided by saturated salt solutions of KOH, K-acetate, K_2CO_3, NH_4NO_3, NaCl, NH_4Cl, $(NH_4)_2SO_4$, KCl, $BaCl_2$ or KNO_3 (10, 26). Seeds were sampled once a week after the third week to check for changes in seed moisture content. For fast desiccation (FD), seeds were put into sandwich boxes, each carrying 130 seeds, over 10–50 g silica gel for 1–5 d to reach 9 target MCs between 3-20%. Samples were taken regularly to determine moisture contents.

After desiccation, 120 seeds at each moisture level were used as desiccation controls; the other seeds contained in a 100-mL polypropylene tube were pre-cooled for 2 h at -20ºC in a domestic refrigerator and then immersed in liquid nitrogen rapidly (18). After 24 h cryostorage, they were removed and rewarmed at ambient conditions and assessed for viability.

Embryonic axes dehydration and cryopreservation

The top 2 mm of cotyledon containing the embryonic axes (thereafter referred as

embryonic axes) were cut under aseptic conditions from fresh seeds after surface-sterilization with 75% ethanol for 90 s and 0.1% $HgCl_2$ for 20 min, followed by rinsing 5 times with sterilized de-ionized water. Desiccation followed the methods of Wen and Song (24). These embryonic axes were placed in small containers (20 mm in diameter) made of aluminum foil, which were placed over activated silica gel for 0.5-12 h within a closed desiccator at ambient temperature.

After desiccation, 40 embryonic axes at each moisture level were used as desiccation-controls, and another 40 embryonic axes were cryopreserved. This was accomplished by direct LN immersion of 2-mL cryovials each containing 10 embryonic axes. After cryostorage for one week, these cryovials were taken out from the liquid nitrogen tank and rapidly thawed by immediate plunging into 40ºC sterile water for 60 s (2).

A modified Murashige and Skoog (MS) medium was employed to assess viability of grapefruit embryonic axes. This medium comprised of basal MS medium modified by the addition of 0.17 g L^{-1} NaH_2PO_4, plus 30 g L^{-1} sucrose, 0.2 mg L^{-1} 2-naphthyl acetic acid (NAA) and 0.1 mg L^{-1} 6-benzylaminopurine (BAP), and solidified with 7 g L^{-1} agar, which was dispensed into 55-mm diameter Petri dishes after autoclaving at 121ºC for 15 min. Every 5 embryonic axes were cultured in a Petri dish. All Petri dishes were wrapped with parafilm and maintained at 25ºC in an air-conditioned room, with 40 - 50% RH and a photoperiod of 14 h light (66 μmol m^{-2} s^{-1}) and 10 h dark. Growth of embryonic axes was examined once every 5 d for 6 months. 'Emergence' was scored as the percentage of embryonic axes showing root and shoot formation during this period; 'survival' was scored as the percentage of embryos showing any visible elongation (25).

Thirty parallel samples withdrawn from each treatment were used to analyze electrolyte leakage, with a test tube containing 5 embryonic axes in 20-mL de-ionized water as a replicate. The conductivity of the bathing solution was measured with a conductivity meter (DDS-307, Shanghai Precision and Scientific Instrument Co., Shanghai, China) after incubation for 0, 2, 5 and 24 h. The tubes were then heated in a boiling bath for 30 min to destroy the plasma membranes and the total conductivity (C_{total}) was measured after the solution had cooled (23). The relative integrity of plasma membranes was expressed as the percentage electrolyte leakage according to the following formula:

$$\text{Electrolyte leakage (\%)} = C_i / C_{total} \times 100\%$$

where, C_i = the conductivity at time i and C_{total} = the total conductivity; each value is an average from six replicates (23).

Statistical analyses

The data were expressed as mean ± SE of six (for seed germination and electrolyte leakage) or eight (for embryonic axis culture) replicates. Using SPSS version 19.0 for Windows, data were subjected to analysis of variance (ANOVA) and means were compared by the Kruskal-Wallis test, with percentage data transferred into their arcsine before these statistical analysis.

RESULTS

Desiccation and cryopreservation of whole seeds

Seeds equilibrating over saturated salt solutions required a period of time to reach their equilibrium moisture contents, depending on the relative humidity the salt solutions provided. The higher the relative humidity, the longer period was required. For grapefruit seeds, no further substantial changes in moisture contents were detected after 4-weeks' treatment (Fig. 1); thus it was thought that equilibrium had been reached by then. On the resultant water sorption, 81% RH corresponded to 8.4% seed MC and 22.5% RH to 3.5% seed MC. Roughly this is in the range for sorption zone (type) II water.

Grapefruit seeds exhibited variable desiccation tolerance depending on seed lots and desiccation methods used. Seeds rapidly dehydrated by silica gel showed higher desiccation tolerance than those by saturated salt solutions, and seeds harvested in 2012 showed higher desiccation tolerance than those in 2010 (Fig. 2A, B). Roughly, under silica-drying condition grapefruit seeds had emergence values above 90% when their moisture content fell from an value of 40% to 7%; further desiccation to 5% significantly decreased emergence (Fig. 2B, Tab. 1). Seeds harvested in 2012 had 90% emergence after desiccation to 6% MC over salt solutions, but those harvested in 2010 had only <70% emergence (Fig. 2A). Probit analysis indicated that WC_{85}, the critical moisture content for 85% seed viability, was 6.5% and 6.8% for FD and ED seeds,

respectively, in 2010, and 2.5% and 4.4% in 2012.

After freezing, seedlings emerged from seeds desiccated to a narrow moisture range (Fig. 2, 3A). No seeds survived cryo-exposure with > 20% MC. Also, both the post-thaw emergence and width of this moisture range varied between seed lots and desiccation methods used, with the maximum post-thaw emergence ranging from 29% to 70%. But the optimum moisture content for the maximum post-thaw emergence was around 7% whatever seed lots or desiccation methods were used. Among these trials the best cryopreservation results came from seeds harvested in 2012 and seeds desiccated by silica gel. Obviously, seeds harvested in 2012 gave higher post-thaw emergence than those harvested in 2010, and seeds desiccated by silica gel prior to freezing gave higher post-thaw emergence than those desiccated over saturated salt solutions. Two-way ANOVA found significant differences in the maximum postthaw emergence in these experiments in relation to both seedlots and desiccation methods ($F_{seedlot}$=5.296**, $F_{desiccation\ method}$=13.018**, $F_{seedlot \times desiccation\ method}$=0.311).

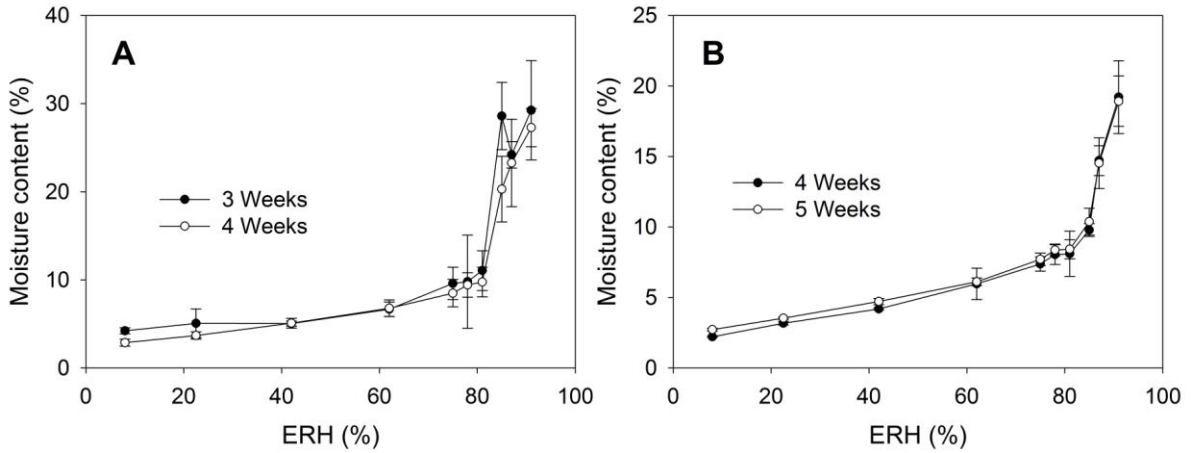

Figure 1. The moisture contents of grapefruit seeds equilibrated over saturated salt solution for 3 to 5 weeks. Experiments were conducted in 2010 (A) and 2012 (B), respectively. All values are means ± SE of 8 replicates.

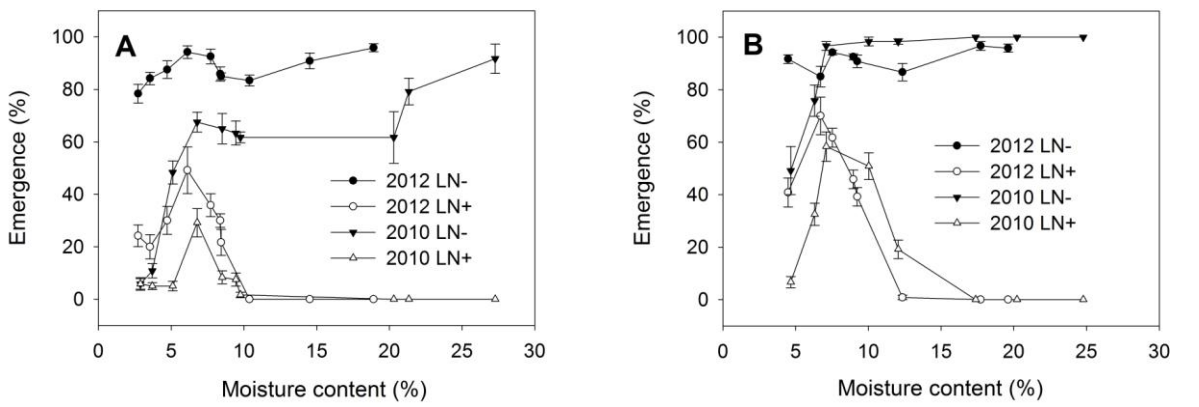

Figure 2. The emergence of equilibration desiccated and/or frozen seeds (A) and the emergence of rapidly desiccated and/or frozen seeds (B). Experiments were conducted in 2010 and 2012, respectively. Seeds were dehydrated to the moisture contents (MC) indicated over saturated salt (A) or silica gel (B) and frozen or not in liquid nitrogen (LN), pre-humidified prior to incubation on 1% agar at 30°C. Normal seedling formation was scored as 'emergence' and all values are means ± SE of 6 replicates of 20 seeds.

Storage of seeds in conventional seed bank

After desiccation to three moisture levels of 9.6%, 7.4% and 5.6%, these seeds were stored at 15ºC, 4ºC and -20ºC, respectively. Their storage lifespan was short in a conventional seed bank. Viability was >90% after desiccation, but one-week's storage resulted in obvious viability loss no matter what temperature the seeds were stored at. The maximum emergence percentages were 65.8%, 71.7% and 49.2% after one-week storage at 15ºC, 4ºC and -20ºC, respectively. They lost viability gradually as time went, but obviously more rapidly at 15°C and -20°C than at 4°C (Fig. 4, Tab. 2). About one third of seeds survived 12-months' storage at 4°C, at this temperature seeds at 5.6% MC kept higher viability than those at higher moisture levels, but few seeds lasted 24 months whatever their moisture contents (Fig. 4B). Grapefruit seeds were more sensitive to sub-zero temperatures. Only one third of seeds at 7.4% MC germinated after 3-months' storage at -20ºC. This sensitivity to sub-zero temperatures was most severe when seed moisture was higher, as one-week's storage at -20ºC killed almost all seeds at 9.6% MC (Fig. 4C). Furthermore, grapefruit seeds also cannot be stored at a higher, above-zero temperature. About 20-50% seeds germinated after 3-months' storage at 15ºC and no seeds survived 6-months' storage at this temperature whatever their moisture contents (Fig. 4A).

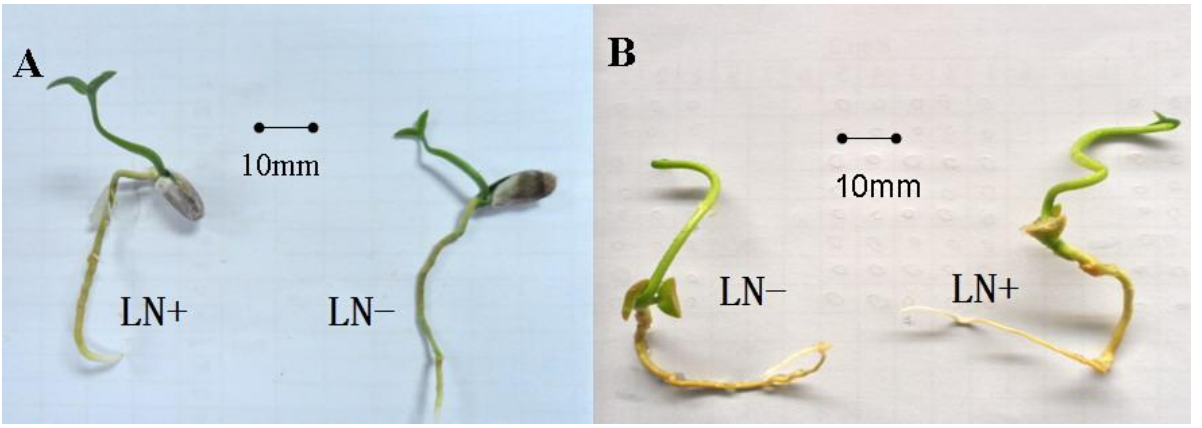

Figure 3. Seedlings regenerating from desiccated and/or frozen seeds (A) and desiccated and/or frozen embryonic axes (B).

Table 1. One-way ANOVA of frozen and desiccated-only (control) seeds (K=Kruskal-Wallis test, those significant with P values < 0.05 were marked with an asterisk and those with P values < 0.01 with two asterisks).

Desiccation method	Year	Frozen or not	Statistical analysis	DF	Chi-square	Sig.
ED	2010	LN+	K	9	36.217	0.000**
	2010	LN-	K	9	42.547	0.000**
	2012	LN+	K	9	43.576	0.000**
	2012	LN-	K	9	25.25	0.003**
FD	2010	LN+	K	8	50.431	0.000**
	2010	LN-	K	8	42.126	0.000**
	2012	LN+	K	7	41.291	0.000**
	2012	LN-	K	7	14.992	0.036*

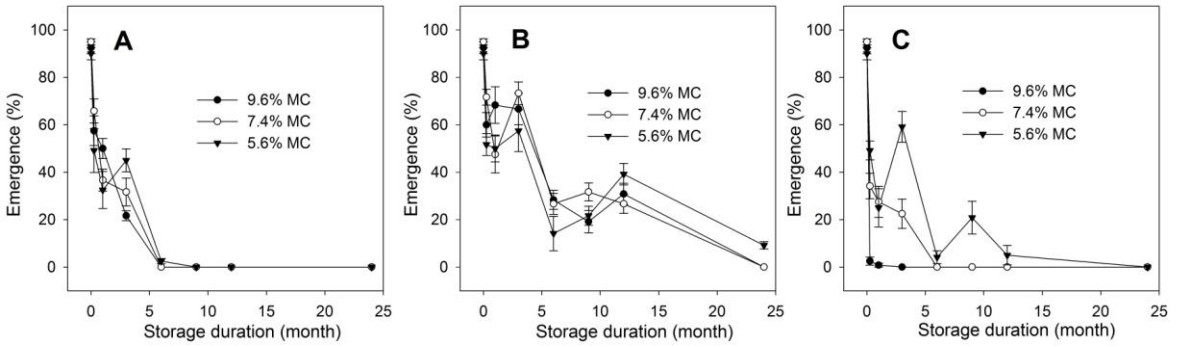

Figure 4. Changes in the emergence of grapefruit seeds stored at 15°C (A), 4°C (B) and -20°C (C). Seeds collected in 2010 were dehydrated to three different moisture contents and stored for different time indicated, pre-humidified prior to incubation on 1% agar at 30°C. Normal seedling formation was scored as 'emergence' and all values were means ± SE of 6 replicates of 20 seeds.

Desiccation and cryopreservation of embryonic axes

When flash dried, grapefruit embryonic axes lost moisture rapidly in the first 2 h, and then very slowly. A 12 h equilibration period resulted in axes with MCs between 2% and 55% (Fig. 5). As expected, flash-desiccated embryonic axes exhibited much higher desiccation tolerance than whole seeds, such that only the 9 h desiccation treatment reduced emergence significantly and there was a 30% reduction in emergence after 12 h desiccation (Fig. 5; for desiccation treatment, Chi-square = 36.542, $p < 0.01$; for desiccation and freezing treatment, Chi-square = 61.102, $p < 0.01$).

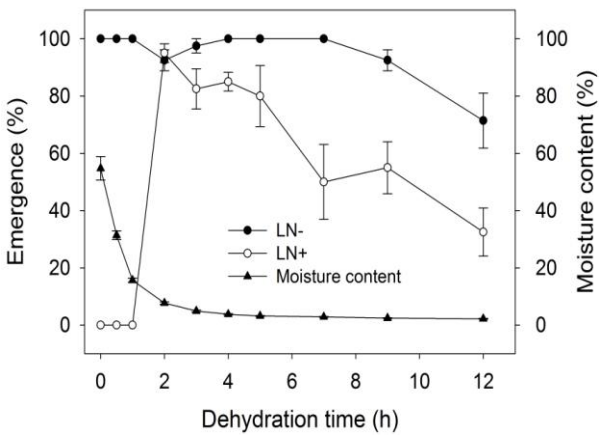

Figure 5. Grapefruit seeds' embryonic axes emergence in response to dehydration and ultra-low temperature. Embryonic axes excised from disinfected seeds were immediately dehydrated over activated silica gel to the indicated moisture contents, frozen or not, and then cultured on medium. Normal seedlings are scored as 'emergence' and all values are means ± SE of 8 replicates of 5 embryo axes.

Axes with > 15% MC failed to recover growth after freezing, but seedlings emerged from frozen embryonic axes when desiccated no less than 2 h (Fig. 5, 3B). The maximum post-thaw emergence of 95% for axes was achieved at 7.7% MC, corresponding to 2 h drying period, which was close to the optimum moisture content for the cryopreservation of whole seeds (Fig. 2). Further desiccation reduced post-thaw emergence remarkably. There was a considerable difference in emergence between frozen and non-frozen control for samples with moisture content below 3%, particularly when desiccation lasted for up to 7 h (Fig. 5).

These changes in axis viability may be associated with damage to the cellular membrane during desiccation and freezing (Fig. 6). Electrolyte leakage of desiccated-only embryonic axes increased linearly as desiccation period was extended to 5 h; further drying did not markedly increase leakage, with values being no larger than those of frozen embryonic axes, except the 0 h, non-dried material when the leakage values were low. For frozen axes, their electrolyte leakage decreased as MC was reduced, but increased after 3 h desiccation. The

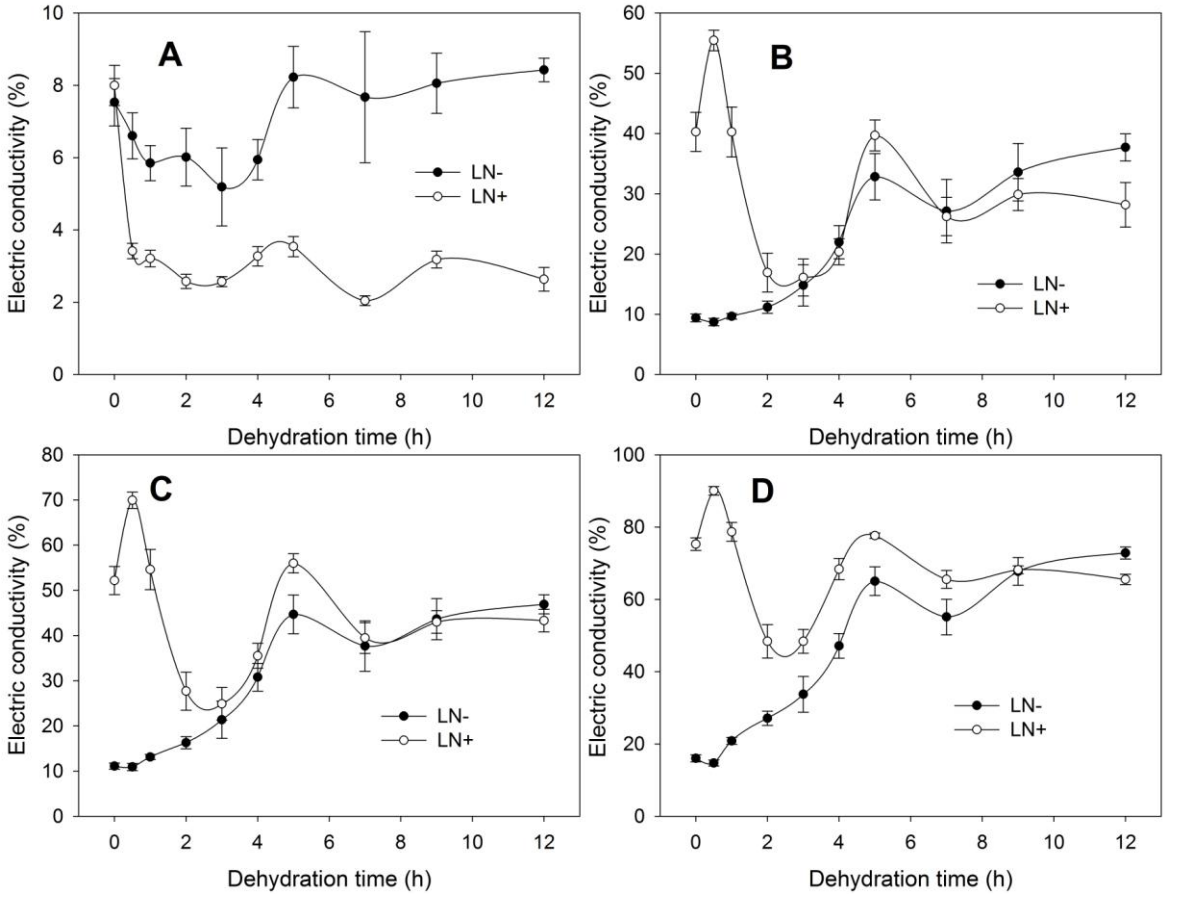

Figure 6. The electric conductivity of grapefruit embryonic axes after desiccation and /or freezing, read after incubation for 0 h (A), 2 h (B), 5 h (C) and 24 h (D). All values are means ± SE of 6 replicates.

minimum electrolyte leakage corresponded to 7.7% MC, at which frozen axes had maximum post-thaw emergence (Fig. 5), suggesting that desiccation and freezing stresses impacted on both electrolyte leakage and embryonic axes viability (Fig. 6).

DISCUSSION

Compared to previous reports (1, 15), we detected much higher desiccation tolerance in grapefruit seeds, which could be desiccated to 7% MC without viability loss in this study. Based on the water sorption isotherms constructed, it was found that these seeds can withstand removal of some sorption zone type II water. Furthermore, grapefruit seeds can not be stored in conventional seed banks for long-term conservation, as they are sensitive to low temperatures, especially subzero temperatures, and their longevity is short, no longer than 24 months. These results confirm that grapefruit produces intermediate-like seeds and suggest that it is necessary to develop cryopreservation protocols for grapefruit seed / embryo conservation.

Meanwhile, we achieved a much higher post-thaw emergence for grapefruit seeds. Graiver et al. (8) reported a maximum post-thaw emergence of approximately 30% for grapefruit seeds, close to the value of the seeds harvested in 2010 and desiccated by saturated salt solutions prior to freezing in this study. But using the same cryopreservation protocol we got a maximum post-thaw emergence of approximately 49% in 2012, and this value was 70% when seeds desiccated by silica gel before freezing (Fig. 2).

Based on research on coffee seeds, Ellis et al. (5, 6) defined intermediate seeds and suggested three features of this seed category: having relatively high levels of desiccation tolerance compared to other non-orthodox seeds, but not the very high desiccation tolerances of orthodox seeds; being sensitive to low temperature, especially subzero temperatures;

and exhibiting inconsistency in desiccation tolerance because of seed provenance, maturity, etc. All of these were true for grapefruit seeds in the present study. We support the categorization of intermediate seeds from orthodox and recalcitrant seeds, because this will provide a guide for short and medium-term storage of seeds of this category. Recently Walters et al. (21) estimated that 10 to 15% of angiosperm species produce intermediate seeds. Considering that there are likely to be many intermediate-seeded species in Xishuangbanna, we have installed a 4ºC storage room and a drying room control at 15ºC and 50% RH in our seed bank facility.

Long-term conservation of intermediate seeds depends on the development of cryopreservation protocols. Three protocols were developed and assessed in this study. Among them, cryopreservation of embryonic axes achieved the best post-thaw results, with a much wider moisture window for post-thaw axes emergence and a maximum emergence as high as 95%. This accorded with previously reported data on pomelo (14, 22). In cryopreservation of intermediate seeds, cryopreservation of embryos / embryonic axes usually gives better results than that of whole seeds, as the small size of the targeted materials may benefit from flash drying, rapid freezing and faster thawing. But compared with the cryopreservation of whole seeds, this protocol also has disadvantages, such as requirement of aseptic conditions, consumption of time and labour, and complexity in growth recovery. So, it is important to improve viability of post-thaw whole seeds. For this, the seed moisture content prior to freezing is a critical factor to determine survival or death of post-thaw seeds. Dussert et al. (3, 4) employed a set of saturated salt solutions to equilibrate seeds into discrete moisture contents. This equilibrium desiccation, which usually lasts for a few weeks, can ensure seeds reach the target eRH very evenly. In contrast to previous study on coffee, we found that fast desiccation of seeds by silica gel facilitated cryopreservation more than equilibrium desiccation by saturated salt solutions in this study. Similar results are being generated from cryopreservation of pomelo seeds (data unpublished). Why different species' seed / embryos vary in response to rapid drying and equilibrium desiccation requires further elucidation of the mechanisms of stress and tolerance.

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Table 2. Effects of storage duration, storage temperature and seed moisture content on emergence of grapefruit seeds.

Source*	Type III Sum of Squares	DF	Mean Square	F	Sig.
A	221365.198	7	31623.6	555.372	0.000
B	31208.892	2	15604.446	274.044	0.000
C	1265.574	2	632.787	11.113	0.000
A*B	19037.235	14	1359.802	23.881	0.000
A*C	2703.129	14	193.081	3.391	0.000
B*C	4092.48	4	1023.12	17.968	0.000
A*B*C	18514.777	28	661.242	11.613	0.000

* A: Storage duration; B: Storage temperature; C: Seed moisture content.

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