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# CRITICAL MOISTURE CONTENT WINDOWS DIFFER FOR THE CRYOPRESERVATION OF POMELO (*Citrus grandis*) SEEDS AND EMBRYONIC AXES

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## Abstract

Cryopreservation was attempted using a partial dehydration and freezing protocol on pomelo (*Citrus grandis*) seeds of ten cultivars and embryonic axes of one cultivar collected from Xishuangbanna. Although seeds of all ten cultivars could be dehydrated safely to 10% moisture content, further dehydration impaired seed viability with critical moisture contents ranging from 9% to 7%. Complete seedling regenerated from seeds frozen in a moisture window between 5-9%, and maximum seedling recovery varied between 22-86%. Cryopreservation of Jiajieyou cv. embryonic axes had a moisture window between 3-20%, much wider compared to whole seed cryopreservation, and maximum post-thaw emergence of 93% was achieved at 13% moisture content. Emergence differed only slightly from survival when whole seeds were cryopreserved, but for the embryonic axis cryopreservation at low moisture contents resulted in much lower emergence than survival. Possible causes of intraspecific variation in cryotolerance in pomelo seeds is discussed.

**Keywords:** *Citrus grandis*; cryotolerance; desiccation tolerance; intermediate seeds; intraspecific variation

## INTRODUCTION

Pomelo is a commercially important tropical and subtropical fruit crop, characterized by a large fruit size, unique taste and nutritional value. It is now planted worldwide and the largest planting area and production are in China. Meanwhile, China is also thought to be the centre of origin and main distribution area for pomelo, with a long history of planting and the richest mix of cultivars. The first record of pomelo planting is the ancient Chinese book titled YUGONG from the Xia Dynasty more than 3000 years ago. It is thought that some cultivars have a

planting history as long as hundreds and thousands years. For example, Shatianyou, originating from Shatian Town in Guangxi, was recorded in the book titled BINZHOU ZHI written in DC 1585 (Min Dynasty), and Huazhou Juhong can be traced to the Liang Dynasty, about 1500 years ago. Although there are more than 200 pomelo cultivars recorded in China today, only a few are grown widely. A few cultivars, such as Wendan, Shatian and Huyou, account for 80% of the total planting area. Most cultivars, especially the traditional ones, are represented by only very limited individuals growing around farmers' houses in remote mountain villages, with an urgent need to conserve variability of pomelo germplasm *ex situ*.

The seed conservation of pomelo germplasm is rather problematic, for pomelo produces intermediate seeds (20), which can not be conserved long-term in conventional seedbanks in which seeds are stored at very low moisture contents and at –20°C. At present, the collection of pomelo genetic resources are conventionally maintained as living trees in field genebanks, such as China's National Citrus Germplasm Repository in Chongqing, or kept as *in vitro* vegetative clones in retarded-growth storage, such as National Indoor Conservation Center of Virus-free Germplasm of Fruit Crops in Wuhan, China. Only cryopreservation can promise long-term germplasm conservation for intermediate seeds (21, 30). Though there are some reports on cryopreservation of *Citrus* species, such as *Citrus aurantium* (2), *Citrus aurantifolia* (6), *Citrus madurensis* (7), *Citrus limon* (24) and Australian wild *Citrus* species (19), this technique has been rarely applied to *Citrus grandis*. Hor *et al*. (21) reported a post-thaw viability as low as 20% for Malaysian pomelo seeds, indicating the need for considerable improvement in the cryopreservation of this species. In this study on material from Xishuangbanna, Southwest China cryopreservation was successfully applied to both whole seeds and embryonic axes and revealed different critical moisture content windows for maximum survival.

## MATERIALS AND METHOD

*Plant material*

Pomelo [*Citrus grandis* (burm.) Merr.] can be distinguished into various cultivars according to their fruit shape, taste and mesocarp colour. Fruits of ten cultivars were collected for this study from trees growing in Xishuangbanna, Southwest China in December, 2008. After harvest, seeds were extracted, air-dried for 1-2 days and then their testa was removed, as previous studies reported that the testa inhibited germination in *Citrus* seeds after drying and/or freezing (22, 24).

Initial viability and moisture content was determined immediately using the methods described below. Seed weights were measured on ten replicates of 50 individuals. Thirty seeds were sampled randomly from each cultivar for dimension measurement.

*Moisture content determination*

Moisture contents of whole seeds and embryonic axes were determined gravimetrically after oven drying at 103 ± 2°C for 17 h and 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*

One hundred and twenty or 100 seeds (for Yuenanyou only), with 20 seeds each sown in a 100 mm diameter Petri dish, were incubated at 30 ± 1°C in a temperature-controlled incubator.

Seeds were pre-humidified in water-saturated air at 35°C for 24 h, achieved by spreading the seeds in a smaller dish placed in a larger dish containing water. Seed germination was monitored once a week for up to three months. Seeds germinated to ≥ 5 mm radicle protrusion were scored as having survived treatment (% survival), and those having normal root and shoot formation scored as showing seedling emergence (% emergence). Before finishing the germination test a crush test was employed to reveal the proportion of ungerminated seeds that were soft and thus nonviable.

*Seed desiccation and cryopreservation*

Seeds were dehydrated to 7-10% moisture content by placing them in monolayer under 45-65% relative humidity (in a drying room controlled by a dehumidifier) and 15°C, and further dehydrated to 3-5% moisture content under activated silica gel. During dehydration samples were regularly withdrawn for moisture content determination, viability assessment and cryopreservation. For cryopreservation, seeds were put into 100 ml polypropylene tubes, hung over liquid nitrogen on a cord for 1 h before immersion in liquid nitrogen. After 24 h cryostorage, they were removed and rewarmed at ambient conditions and then pre-humidified and germinated as described above.

In a pilot experiment using Jiajieyou cv., seeds were regularly sampled for cryopreservation across a wide range of moisture contents. It was found that pomelo seeds can be safely desiccated to c.10% moisture content, and post-thaw recovery growth was achieved from frozen seeds only when seed moisture content was reduced to 12%. So cryopreservation and viability assessment for the other nine cultivars began only after their moisture contents fell below c. 20%.

*Embryonic axis isolation, cryopreservation and viability assessment*

Newly harvested seeds were cleaned, surface-sterilized with 75% ethanol for 90 seconds, 0.1% $HgCl_2$ for 20 minutes and then rinsed 5 times with sterilized de-ionized water prior to removing the embryonic axes under aseptic conditions. The isolated embryonic axes were placed in small containers (20 mm in diameter) made of aluminum foil, and then the containers were placed over activated silica gel for 1-12 h within a closed desiccator at ambient temperature (32).

Half of embryonic axes at each moisture level were cryopreserved, and the other half was used as dried-only control. For cryopreservation, embryos were put into 2 ml cryovials and then plunged into liquid nitrogen. After one week's storage at -196°C, the cryovials were taken out and immersed in 40°C sterilized water for 60 seconds to rapidly thaw the embryos, which were then cultured on sterile medium. Control embryos were directly cultured on medium to assess viability after desiccation alone.

For viability assessment, embryos were cultured on a modified Murashige and Skoog (MS) medium, which comprised 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. The medium was dispensed into 55 mm diameter Petri dishes after autoclaving at 121ºC for 15 minutes. All cultures were maintained at 24 ± 2ºC, with 40 - 50% relative humidity and a photoperiod of 14 h light (66 μmol $m^{-2}$ $s^{-1}$) and 10 h dark for eight months. They were examined regularly. 'Emergence' was

scored as the percentage of embryos showing root and shoot formation during this period; 'survival' was scored as the percentage of embryos showing any visible elongation. 'Survival' therefore includes 'emergence'.

## RESULTS

*Seed variation from different cultivars*

The seeds used in this study contained a rich intraspecific variation (Table 1). Cultivars differed from each other in seed weight and size; Yunnanyou had the largest seed dry weight (50 seeds weighed 11.7 g), almost double that of Mansailong. Seed lengths ranged from 15.4 mm to 12.7 mm, with Jiajieyou seeds being the longest. Yunnanyou seeds were also the widest and Shatianyou seeds the thinnest. Although all seeds were extracted from full mature fruits, initial moisture contents varied from 28 to 42%. Nonetheless, all seedlots had high viability. For example, Mansailong, Shatianyou and Yuenanyou had initial emergence values of 86%, 87.5% and 91%, respectively.

**Table 1**. Initial data for the seeds from the ten pomelo cultivars investigated

| Cultivar | Fresh weight (g/50 seeds) | Dry weight | Initial MC (%) | Seed length (mm) | Seed width (mm) | Seed depth (mm) |
|---|---|---|---|---|---|---|
| Jiajieyou | 14.8491±0.0977 | 9.0569 | 39.01±1.47 | 15.414±0.253 | 8.373±0.176 | 4.433±0.055 |
| Feizhouyou | 15.2143±0.0759 | 8.9477 | 41.19±1.13 | 14.540±0.246 | 8.571±0.184 | 4.622±0.078 |
| Menglunhong | 15.3774±0.0660 | 10.4257 | 32.21±0.96 | 13.712±0.178 | 8.991±0.128 | 4.523±0.072 |
| Baerpao | 11.4479±0.0728 | 7.6514 | 33.16±0.94 | 12.592±0.202 | 7.923±0.121 | 4.415±0.045 |
| Suanpaoguo | 14.3291±0.1119 | 9.0869 | 36.58±1.04 | 13.582±0.192 | 8.835±0.195 | 4.461±0.050 |
| Yunnanyou | 17.7628±0.1814 | 11.6807 | 34.24±1.05 | 14.297±0.212 | 9.785±0.164 | 4.679±0.098 |
| Mansailong | 10.4973±0.0860 | 6.6047 | 37.08±1.04 | 12.661±0.184 | 7.765±0.156 | 4.038±0.041 |
| Thaiyou | 15.0730±0.1339 | 10.8335 | 28.13±0.81 | 13.901±0.173 | 9.130±0.157 | 4.599±0.057 |
| Shatianyou | 10.4147±0.0569 | 7.4884 | 28.10±1.60 | 14.016±0.193 | 7.332±0.144 | 3.700±0.060 |
| Yuenanyou | 15.0679±0.1820 | 10.2886 | 31.72±1.74 | 14.079±0.161 | 9.635±0.250 | 4.413±0.088 |

Note: (1) Fresh weights of 50 seeds were measured using testa-removed seeds and expressed as means ± SE of 10 replicates, and dry weights of 50 seeds were calculated using their fresh weights and moisture contents; (2) Moisture contents were expressed as means ± SE of 8 replicates of single seeds. Seed dimensions represent means ± SE of 30 seeds.

*Desiccation and cryopreservation of whole seeds*

For all cultivars, dehydration to 10% moisture content did not impair seed viability, but intraspecific variation in desiccation tolerance was demonstrated when further dehydration was employed. Critical seed moisture contents below which viability was reduced was around 9.0% for Suanpaoguo, Shatianyou and Feizhouyou, about 8.0% for Menglunhong and Yuenanyou and 7.0% for Jiajieyou, Baerpao and Yunnanyou. Thaiyou retained more than 80% viability after dehydration to a moisture content of c.3.5%. However, seed viability at this moisture level was only 15% for Yuenanyou and Feizhouyou, 40% for Jiajieyou and Mansailong, and 60% for Shatianyou (Fig. 1).

All cultivars exhibited a seed moisture window between c. 5-9% that allowed survival after cryo-exposure (Fig. 1, Fig. 2a). The range varied a little with cultivar, and the cryopreservation results distinctly differed from each other. The maximum viability of post-thaw seeds ranged from 22% to 86%. Among them the highest was 86% from Baerpao, next was 80% for Jiajieyou and 77% for Shatianyou, then 68% for Yunnanyou, 67% for Suanpaoguo, 63% for Mansailong and 60% for Thaiyou, followed by 53% for Menglunhong, 35% for Feizhouyou and lastly, 22% for Yuenanyou.

We recorded the difference in viability between frozen seeds and their non-frozen controls when seeds reached their maximum post-thaw viability. This gap was very small for Jiajieyou and Baerpao, but very large for Yuenanyou and Feizhouyou. Thaiyou, which demonstrated the highest desiccation tolerance in this study, still had a moderate gap between maximum post-thaw viability and its non-frozen control (Fig. 1). Importantly, almost all seeds surviving dehydration and/or freezing developed into complete seedlings, so the survival and emergence values for whole seed cryopreservation only varied a little (Fig. 1).

*Desiccation and cryopreservation of embryonic axes*

Isolated embryonic axes from Jiajieyou seeds were employed in this study and complete seedlings were regenerated from frozen samples (Fig. 2b, Fig. 3). Compared to whole seeds, isolated embryonic axes exhibited higher desiccation tolerance; dehydration to 7% made no difference to viability, but further dehydration to 3.5% slightly impaired survival and greatly depressed emergence to 22% (Fig. 3a, b). Within the range 5-20% moisture content more than 60% embryonic axes survived the freeze-thaw cycle. A maximum post-thaw emergence of 93% was achieved after 3-hour's dehydration to 13% moisture content, a value that was very close to the emergence of the non-frozen control, and a little higher than the maximum postthaw viability obtained from whole seed cryopreservation of this cultivar.

Jiajieyou had a wider moisture window for survival of cryopreservation when embryonic axes were used such that the upper moisture limit extended to a much higher moisture level compared to when whole seeds were cryopreserved. The maximum post-thaw emergence of 93% for axes was achieved at 13% moisture content, which was beyond the moisture range allowing whole seeds to survive cryopreservation. For all samples with moisture contents below 5%, both frozen and their non-frozen control, there was a large difference between survival and emergence (Fig. 3a, b). Meanwhile, it was found that many low-moisture embryonic axes that survived freezing developed shoots but not roots, with only incomplete seedling regeneration (Fig. 2b). Dehydration below 13% moisture content was detrimental to root growth, and many seedlings developed small roots only. Complete failure in root development was mostly found for axes dried for 9 hours or more. We calculated the ratio of samples with root development in relation to the total samples scored as surviving for both freezing and non-freezing treatment. The resultant curve (Fig. 3c) was very similar to the emergence curve (Fig. 3a), suggesting that failure in root development was a main contributor to emergence values being lower than those of survival.

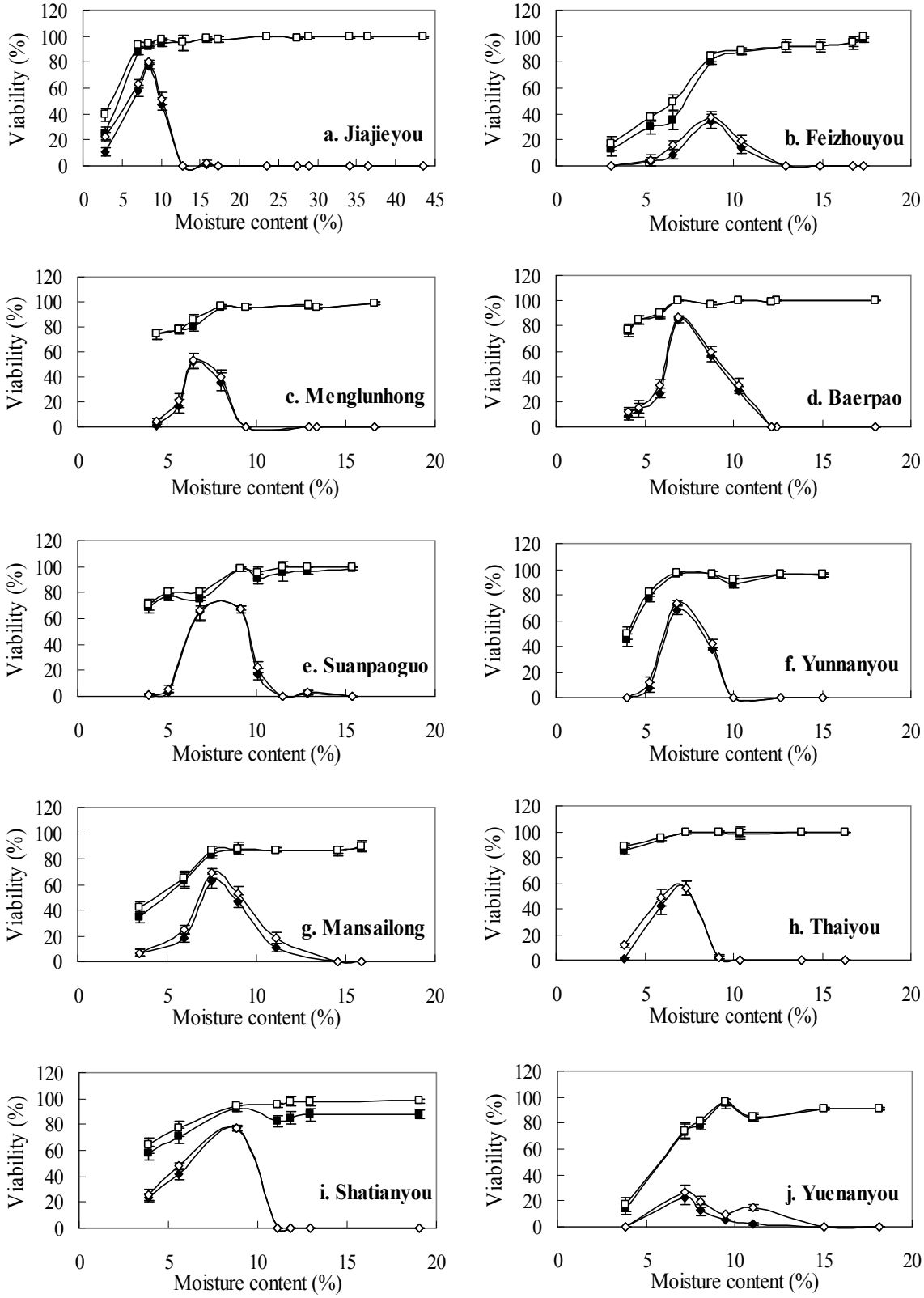



**Figure 1.** The emergence (closed symbols) and survival (open symbols) for frozen seeds (diamonds) or the nonfrozen controls (squares) of ten pomelo cultivars from Xishuangbanna, SW China. Whole seeds were dehydrated to the moisture contents indicated and frozen or not, pre-humidified prior to incubation on 1% agar at 30 °C. Those forming normal seedlings were scored as 'emergence', and those having ≥5 mm radicle protrusion as 'survival'. All values are means ± SE of 6 or 5 (for Yuenanyou only) replicates of 20 seeds.

This study using a partial dehydration protocol successfully accomplished cryopreservation of ten pomelo cultivars from Xishuangbanna, Southwest China. Clear intraspecific variation in desiccation tolerance and cryotolerance was revealed, and emergence between 22-86% was achieved from frozen seeds. Eight of the ten cultivars investigated had maximum post-thaw emergences above 50% and Jiajieyou embryonic axes had up to 93% post-thaw emergence. Hopefully, these findings provide a useful protocol for long-term storage of pomelo genetic resources, at least those cultivars from Xishuangbanna.

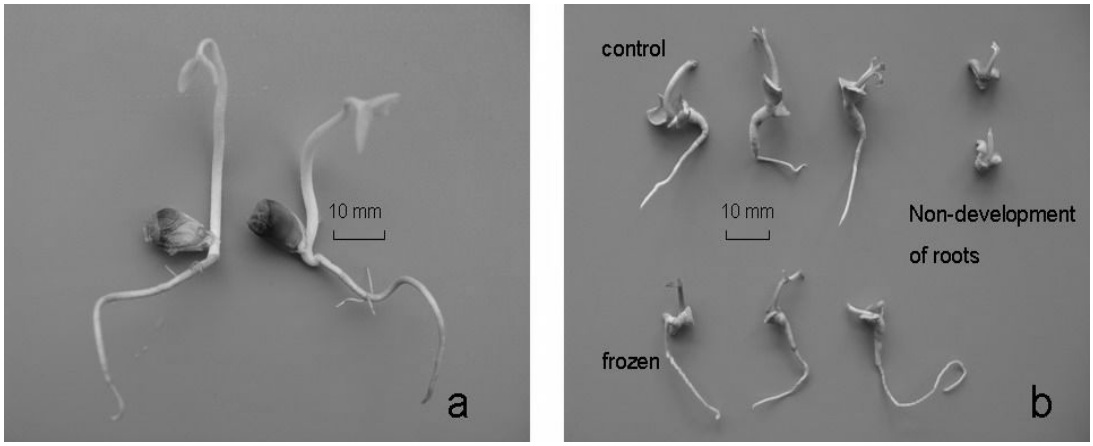
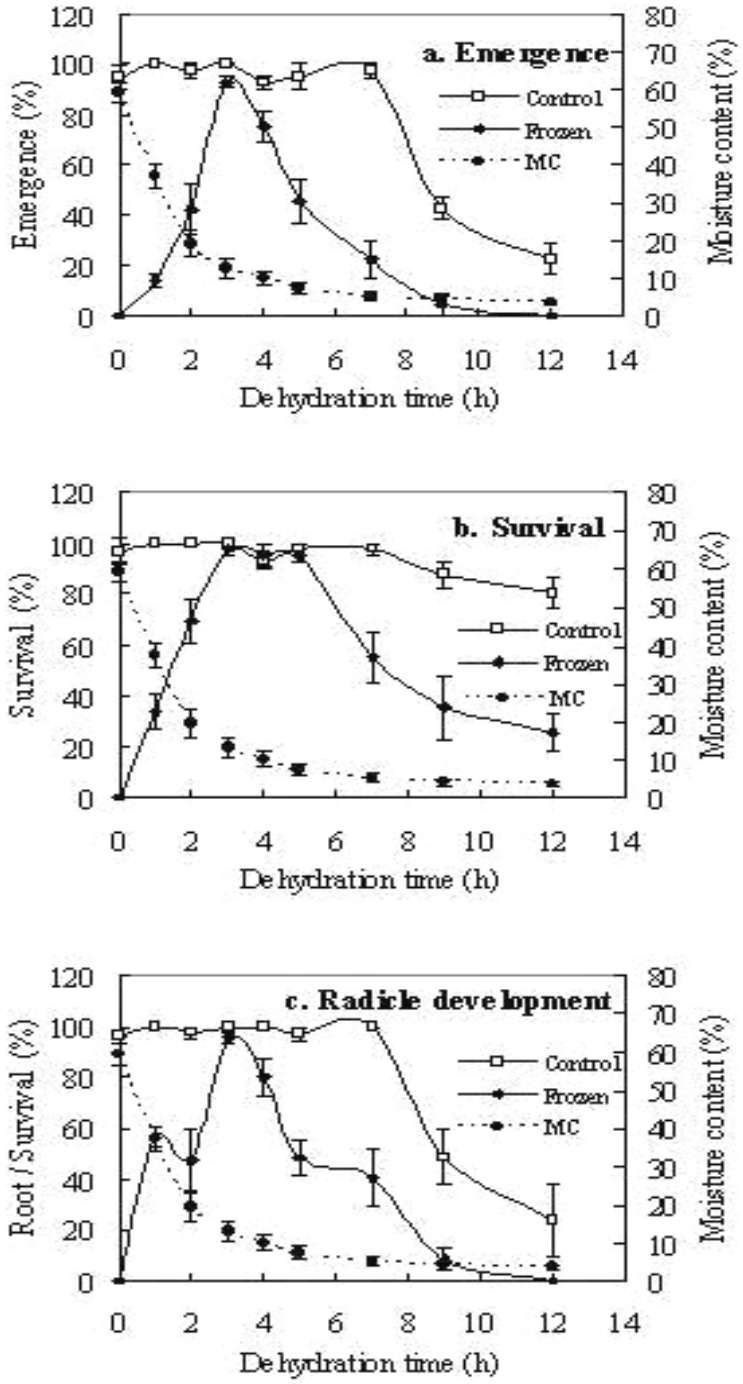



**Figure 2**. Seedlings regenerating from frozen seeds (a) and dehydrated and frozen embryonic axes (b) of Jiajieyou cv. Note the abnormal seedlings in the upper right in (b), which recovered from embryonic axes after 9-12 h desiccation and freezing or not; these developed shoots but no roots.

## DISCUSSION

Honjo and Nakagawa (20) and Hor *et al*. (21) reported that pomelo seeds are sensitive to desiccation and low temperatures. This study confirmed that pomelo produces intermediate seeds, or Type II seeds as Pritchard (26) defined. Compared to previous reports, this study revealed a rich variation in desiccation tolerance and cryotolerance among cultivars, and different critical moisture content windows for maximum survival between whole seeds and embryonic axes. In our study, pomelo seeds tolerated desiccation to moisture contents of 6-9%, depending on cultivar. This value is clearly lower than the lowest safe moisture content between 10-12% reported previously (20). Variation in cryotolerance is another aspect of pomelo seeds. At the cryopreservation moisture window, some cultivars such as Jiajieyou and Baerpao had only a slight difference in viability between frozen seeds and their dried-only control, while Feizhouyou and Yuenanyou demonstrated fall in viability after freezing, similar

**Figure 3**. Pomelo embryonic axis response to dehydration and/or freezing in Jiajieyou cv. (a) emergence, (b) survival and (c) radicle development ratio. Embryonic axes excised from fresh seeds were immediately dehydrated over activated silica gel to the indicated moisture contents, frozen or not, and then cultured on medium. Those embryos forming both root and shoot were scored as 'emergence', and those embryos showing any visible elongation were scored as



to the that recorded by Hor *et al*. (21). Cryopreservation is the only technique available for long-term storage of intermediate seeds (12, 21, 26). Both whole seeds and isolated embryos/embryonic axes can be used in the cryopreservation of intermediate seeds, such as oil palm (14, 18) and coffee (1, 10, 25). Compared to whole seeds, a much higher survival level is often obtained from frozen embryos or embryonic axes as more rapid drying, freezing and thawing generally enhances survival of cryopreservation (18). For the same reasons embryos or embryonic axes may have wider critical moisture content windows than whole seeds during cryopreservation as demonstrated by this study. This indicates a potential for improving cryopreservation of desiccation-sensitive seeds. Usually, controlled freezing is required during whole seed cryopreservation of intermediate species while sterile manipulation is needed during isolated embryo or embryonic axis cryopreservation. Thus each approach provides different challenges. As for pomelo seeds from Xishuangbanna, whole seed cryopreservation is recommended for most cultivars because of its high recovery rate and easy manipulation, while embryonic axis cryopreservation is worth trying for Yuenanyou and Feizhouyou cultivars, as a relative improvement in post-thaw emergence can be expected.

We report much better cryopreservation results for pomelo than Hor *et al*. (21) with post-thaw emergences between 50-86% from whole seeds for eight of the ten cultivars investigated here. It is not unusual to observe differences in cryopreservation results between studies on the same species (15), e.g. *Coffea arabica* (5, 9, 10, 11, 13) and *Ribes nigrum* and *R. aureum* (27). The possible factors contributing to this variation might relate to different seed provenances. For example, *Acer pseudoplatanus* fruit origin across Europe had different desiccation tolerance and cryotolerance (8). Similar variability has also been observed for *Coffea arabica* (29). A recent study can provide some explanation for this phenomenon that seeds with different geographic gradients may have a difference in the proportion of saturated fatty acid, leading to differences in mean onset temperature for lipid melting (19). Another cause is intraspecific variation contained in different varieties and cultivars, putatively originating from their genetic background. For example, maximum recovery percentages of 50% and 85% were reported for two cultivars of hazelnut when the embryonic axes were cryopreserved (16). In the case of coconut zygotic embryos, experiments on four different varieties led to recoveries between 33 and 93% (4, 33). In our study, emergence from frozen seeds was 22%, 35%, 63% and 86% for four cultivars Yuenanyou, Feizhouyou, Mansailong and Baerpao, respectively, demonstrating evident inter-cultivar variation in cryotolerance. It is also possible that the seed used between studies may have different maturities. Goveia *et al.* (17) suggested that development status is a critical factor when selecting recalcitrant seed axes for cryopreservation, e.g. jackfruit embryonic axes (23, 28). Similarly, coconut embryos excised from seeds before full maturity produced the best cryopreservation results (3, 4). Moreover, Wen and Song described changes of cryotolerance with seed development in orthodox maize and recalcitrant Chinese fan palm (31, 32). For the current study, we harvested fruits from trees in December 2008 when they were fully mature. However, use of immature seeds can not be precluded if the fruits come from commercial sources because pomelo is usually harvested early for human use, i.e. from end of August in Xishuangbanna. Finally, the cryopresrvation technique may be an important contributory factor for survival. There have been significant improvements to cryopreservation protocols over the last 30 years, such that

cryopreservation on *Coffea arabica* has moved from regular failure to success (1, 5, 9, 10, 11, 13).

From this study and that by Honjo and Nakagawa (20) and Hor et al. (21), it can be confirmed that pomelo seeds contain obvious intraspecific variation in desiccation tolerance and cryotolerance. It is therefore necessary to establish cryopreservation protocols on a variety/cultivar basis, paying particular attention to the maturity of the seed. Further experiments are needed to investigate the casual factors for intraspecific variation in cryopreservation success in pomelo and to improve the cryopreservation results for cultivar Yuenanyou and Feizhouyou.

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## REFERENCES

1. Abdelnour-Esquivel A, Villalobos V & Engelmann F (1992) *CryoLetters* **13**, 297-302.
2. Al-Ababneh SS, Karam NS & Shibli RA (2002) In vitro *Cellular and Developmental Biology-Plant* **38**, 602-607
3. Assy-Bah B & Engelmann F (1992) *CryoLetters* **13**, 67-74
4. Assy-Bah B & Engelmann F (1992) *CryoLetters* **13**, 117-126
5. Becwar MR, Stanwood PC & Leonhardt KW (1983) *Journal of the American Society for Horticultural Science* **108**, 613-618
6. Cho EG, Noor NM, Kim HH, Rao VR & Engelmann F (2002) *CryoLetters* **23**, 309-316
7. Cho EG, Hor YL, Kim HH, Rao VR & Engelmann F (2002) *CryoLetters* **23**, 325-332
8. Daws MI & Pritchard HW (2008) *CryoLetters* **29**, 189–198, 527
9. Dussert S, Chabrillange N, Engelmann F, Anthony F & Hamon S (1997) *CryoLetters* **18**, 269-276
10. Dussert S, Chabrillange N, Engelmann F, Anthony F, Louarn J & Hamon S (1998) *Seed Science Research* **8**, 9-15
11. Dussert S, Chabrillange N, Vasquez N, Engelmann F, Anthony F, Guyot A & Hamon S (2000) *CryoLetters* **21**, 47-52
12. Dussert S, Davey MW, Laffargue A, Doulbeau S, Swennen R & Etienne H (2006) *Physiologia Plantarum* **127**, 192-204
13. Dussert S & Engelmann F (2006) *CryoLetters* **27**, 169-178
14. Engelmann F, Chabrillange N, Dussert S & Duval Y (1995) *Seed Science Research* **5**, 81-86
15. Engelmann F (2004) In Vitro *Cellular and Developmental Biology-Plant* **40**, 427-433.
16. Gonzalez-Benito ME & Perez C (1994) *CryoLetters* **15**, 41-46
17. Goveia M, Kioko JI & Berjak P (2004) *Seed Science Research* **14**, 241-248
18. Grout BWW, Shelton K & Pritchard HW (1983) *Annals of Botany* **52**, 381-384
19. Hamilton KN, Ashmore SE & Pritchard HW (2009) *CryoLetters* **30**, 268-279

20. Honjo H & Nakagawa Y (1978) in *Long Term Preservation of Favourable Germplasm in Arboreal Crops*, (eds) T Akihama & K Nakajima, Ministry of Agriculture and Forestry, Tokyo, Japan, pp. 31-35.
21. Hor YL, Kim YJ, Ugap A, Chabrillange N, Sinniah UR, Engelmann F & Dussert S (2005) *Annals of Botany* **95**,1153-1161
22. King MW & Roberts EH (1980) *Annals of Botany* **45**, 489-492
23. Krishnapillay B (2000) in *Conservation of Plant Genetic Resources* in vitro. *Volume 2: Application and Limitation*, (ed.) Razdan MK, Science Publishers, Inc., pp. 139-166
24. Mumford PM & Grout BWW (1979) *Seed Science and Technology* **7**, 407-410
25. Normah MN & Vengadasalam M (1992) *CryoLetters* **13**, 199-208
26. Pritchard HW (2004) in Ex situ *Plant Conservation. Supporting Species Survival in the Wild*, (eds) EO Guerrant, K Havens & M Maunder, Island Press, Washington, D. C., pp. 139-161.
27. Reed BM, Dumet D, Denoma JM &Benson EE (2001) *Biodiversity and Conservation* **10**, 939-949
28. Thammasiri K (1999) *CryoLetters* **20**, 21-28
29. Vasquez N, Salazar K, Anthony F, Chabrillange N, Engelmann F & Dussert S (2005) *Seed Science and Technology* **33**, 293-301
30. Walters C, Wheeler L & Stanwood PC (2004) *Cryobiology* **48**, 229-244.
31. Wen B & Song SQ (2007) *CryoLetters* **28**, 109-118
32. Wen B & Song SQ (2007) *CryoLetters* **28**, 291-302
33. Withers LA & Engelmann F (1998) in *Biotechnology in Agriculture*, (ed) Altman A, Marcel Dekker, New York, pp. 57-87

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