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# LONG-TERM, LARGE SCALE BANKING OF *Citrus* SPECIES EMBRYOS: COMPARISONS BETWEEN CRYOPRESERVATION AND OTHER SEED BANKING TEMPERATURES

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## Abstract

The long-term, large scale application of embryo cryopreservation has been assessed rarely and comparisons of viability loss for partially dried material with conventional seed bank storage conditions infrequently made. Five citrus species were cryopreserved following air drying of embryos (seed minus the testa) and embryonic axes: rough lemon (*Citrus jambhiri*), pommelo (*C. grandis*), mandarin (*C. reticulata*), citron (*C. medica*) and kagzi lime (*C. aurantifolia*). Although drying rates to c. 10% moisture content (MC) were approximately 10-times faster for isolated axes compared to embryos, the optimum MCs for cryopreservation were generally similar within a species, varying from c. 10% (*C. jambhiri*) to c. 20% (*C. medica*). Nonetheless, the hydration window for cryopreservation of the axis was usually wider than for the embryo. For all species, embryo or axis survival after cryopreservation ranged from 65% to 96% (*C. medica* axes), producing normal healthy seedlings from embryos and plantlets from axes without intervening callus growth *in vitro*. Whilst partially dried embryos of all five species survived fully liquid nitrogen vapour storage for 120 days, viability loss was rapid at -20°C, 5°C and ambient temperature, with a maximum interpolated half-life across these temperatures of c. 80 days for *C. grandis* at 5°C. The developed cryopreservation protocols were applied routinely to cryobank 377 accessions of *Citrus* germplasm from field genebanks, farmer's orchards, semi-wild and wild sources. After an average of 6.3 to 8.4 years cryo-storage, between 69 and 81% of accessions per species retained ≥ 70% of the viability after desiccation. The results provide irrevocable evidence for the importance of cryopreservation for the banking of seeds of higher plants.

**Keywords**: Embryo axis, cryo-base collection, commodity species, crop wild relative, tropical fruit.

## INTRODUCTION

Citrus is one of the most important fruit crops of the world with many important types originating in Southeast Asia, Australia, the intervening islands between Asia and Australia and central Africa (36, 37). Rich genetic diversity, particularly in primitive cultivars and the wild genepool of allied genera are represented in Northeastern and parts of Northwestern

India (33). Genetic improvement in the species depends on safe conservation and efficient utilization of this indigenous genetic diversity (34). Management of citrus diversity in field genebanks has proved to be difficult, labour intensive and at risk from disease and die-back syndrome (30, 35). Therefore, alternative *ex situ* approaches for long-term conservation of citrus genetic diversity are needed. Moreover presence of high degree of polyembryony in many citrus species provides the opportunity to conserve the original genotype as seed despite high levels of heterozygosity in them. Leaving aside the commercial cultivars or clones of citrus most of the wild species and rootstocks are seed propagated. Also, there are no reports of seed transmitted viruses in citrus, which gives extra impetus for conserving *Citrus* germplasm in the form of seeds.

Cryopreservation following air desiccation, vitrification and encapsulation-dehydration, has been extensively used for the development of protocols for the long-term conservation of various *Citrus* species and allied genera, particularly *Poncirus*. Various explants (seeds, zygotic embryos, embryonic axes) have been cryopreserved for at least 17 species (3, 4, 11, 18, 28, 32). Citrus shoot apices (10), cell suspensions (9, 30, 31) and somatic embryos (20) have also been successfully cryopreserved.

Target MCs below which seeds should be dried to avoid ice formation on ultra-cooling have been defined in relation to the seed oil content for citrus and other species (14, 26). Generally, citrus seed oil content varies from 30 – 54% dry mass basis, e.g. 37 – 52% [four species (14)] and 30 – 54% [three species (12)]. On this basis, target moisture contents for cryopreservation in citrus should be c. 14% for a 40% oil content seed. This correlates well with recent results, with many citrus species seed being cryopreserved after drying to intermediate MCs of 10 to 15% (14, 16, 17). However, the critical hydration window for survival may be material-dependent, being wider for embryonic axes (3 to 20%) than embryos (5 to 9 %) in *C. grandis* (39).

In contrast to cryo-storage, seeds at such intermediate MCs may, however, be precluded from storage at conventional seed bank temperature (-20ºC) due to ice formation or cold stress. For example, seeds of *Warburgia salutaris* at 17% MC tolerated 3 months storage at -20ºC but not 12 months (5). The cause may have been the loss of the glassy state at -20ºC, enabling slow ice formation (27). Generally, plant cryopreservation assessments are made over the short-term only. For citrus seed, embryo and embryonic axis cryopreservation, liquid nitrogen treatments have included immersion [4 species (14)], storage for c. 1 h [*C. suhuiensis* (17); *C. sinensis* (30)], c. 24 h [*C. aurantifolia*, (4); four species (16); *C. grandis*, (39)] and about 2 days (11). However, evidence of survival of multiple accessions over many years is required if cryopreservation is to be adopted as the method of choice for long-term conservation. Efforts in this direction have been made; for example, the assessment of cryotolerance of *in vitro* shoot tips of 93 genotypes of pear (29), the 5-year storage at -135ºC of winter buds of 376 varieties of mulberry (21) and 28-year cryopreservation of shoot tips of strawberry and pea (1).

*C. reticulata, C. grandis, C. medica* and *C. aurantifolia* are commercially important fruit species and *C. jambhiri* a valuable rootstock, especially for sweet oranges in India, as it is highly drought tolerant. Due to their economic importance, the need has been identified for the establishment of a cryogenebank base collection for the long-term conservation of a sizeable portion of genetic variability (18, 32, 33). For the species studied here, published evidence indicates that four of them (*C. aurantifolia, C. grandis*, *C. reticulata* and *C. medica*) have seeds that are partially desiccation tolerant (intermediate or Type II), whilst *C. jambhiri* may be very desiccation sensitive (i.e. recalcitrant or Type III) (13, 19). In this study, multiple accessions of all five species have been partially dried as embryos or embryonic axes, cryopreserved and stored successfully for many years.

## MATERIALS AND METHODS

*Seed sources and germplasm collection*

Ripe fruits of *C. jambhiri*, *C. grandis, C. reticulata*, *C. medica* and *C. aurantifolia* were collected from field genebanks, farmer's orchards, homestead gardens and from natural habitats in various parts of India. Collections from single plants were given an indigenous collection number (IC number) and treated as individual accession. Fresh fruits of each accession were collected in cloth bags. The fruits were cut open to extract the seeds by peeling off the transparent membrane of closely-packed carpel segments and removing the mucilage. The seeds were stored briefly at 15°C until used for experimentation. The testa was removed just before the initiation of the drying and storage experiments to obtain the embryos. Embryonic axes were excised from the embryos after separating the cotyledons.

In vitro *culture*

The embryonic axes, fresh as well as after different treatments, were cultured on Murashige and Skoog (MS) macro- and micro-nutrients, vitamins, iron, 1 g $l^{-1}$ activated charcoal and 0.17 g $l^{-1}$ $NaH_2PO_4$ supplemented with 1 mg $l^{-1}$ each of 6-benzylaminopurine (BAP) and α-naphthalene acetic acid (NAA), as defined by Chin *et al.* (2). Cultures were maintained at 25 ± 2°C with a 16 h light/8 h dark photoperiod under a light intensity of 35 μE $m^{-2}$ $s^{-1}$. Growth was assessed after 3 weeks of culture.

*Air desiccation, longevity and freezing of embryos and embryonic axes*

Moisture content (MC) was determined in triplicate on fresh embryos and after desiccation for 18, 24, 42 and 60 h over sterile silica gel after the low constant temperature oven method, *i.e.* 103 ± 2°C for 17 h (15). Embryonic axes were excised following surface decontamination of the seeds with 0.1% mercuric chloride for 10 min, followed by washing thrice with sterilized deionised water. Isolated axes were spread over sterile filter paper discs in batches of 20 to 30 and dried in a laminar flow cabinet for 1 to 6 h with moisture content determined hourly.

Desiccated embryos and axes were sealed in 1.2 or 5 ml polypropylene cryovials. For routine assessment, the vials were plunged into liquid nitrogen (LN) and stored for a minimum of 24 h. Thereafter, vials were rewarmed rapidly in a water bath at 38°C for 5 min. Within 30 min of retrieval, axes were transferred to *in vitro* culture, while embryos were placed between two sheets of moistened paper in plastic Petri plates (11 cm diameter). Incubation was at 27 ± 2°C with 16/8 h light/dark photoperiod (light intensity as specified above). The extrusion of the shoot and/or root indicated germination of the embryo and isolated embryonic axis.

For the assessment of the temperature-dependency of survival, partially desiccated embryos were stored in 5 ml polypropylene cryovials and kept at room temperature (ambient), 5°C, -20°C and LN vapour and the viability of embryos determined after 15 days to 120 days storage using methods described above. The results were recorded for >10 accessions for each of the five species, with representative, averaged data presented here. As the application of linear regressions to the viability loss data was not appropriate on all occasions, the half-life of the embryos was interpolated from the survival curves.

*Cryobanking of germplasm*

For long-term storage, the embryo and embryonic axes were cryopreserved after air desiccation over silica gel for embryos and in the laminar airflow for axes, followed by fast cooling (plunged into LN) and then storage the LN vapour phase (c. –160°C to –180°C) in

1000 l capacity cryotanks (MVE model XLC1830). Ten cryovials each with 8-10 embryos and 10-15 axes for each accession were stored. Recovery growth was monitored at regular intervals over maximum periods of 8 to 10 years (averages 6.3 - 8.4 years) and the number of accessions falling within different viability ranges calculated as percentages of their respective dried controls.

## RESULTS

*Critical moisture contents for desiccation tolerance and cryopreservation*

Embryos and excised embryonic axes of five *Citrus* species varied in their initial MCs, from 27 to 36% and 30 to 39% for embryos and embryonic axes, respectively (Figs. 1 & 2). Initial viability was 95 to 100% for both embryos and axes. On desiccation, embryo and axis viability declined (Figs. 1 & 2). Whilst embryo MC for all species decreased to c. 10% by c. 40 h drying (Fig. 1), isolated axes took c. 5 h for the same level of desiccation (Fig. 2).

Embryos and embryonic axes of *Citrus jambhiri* proved to be the most desiccation sensitive, with complete viability loss by 6 and 8% MC respectively (Figs 1 & 2). For *C. medica, C. reticulata* and *C. aurantifolia* desiccation tolerance was much higher; at 9-12% embryo MC and c. 11% axis MC, viability was 50 to 75% and 70 to 95%, respectively. However, in all species 6 h drying to 5 – 8% MC reduced axis viability to about 50% in *C. grandis* and *C. reticulata* and 0% in the other three species (Fig. 2).

The optimum MC for *C. jambhiri* embryo cryopreservation was c. 11%, with 70% survival (Fig. 1). Further drying to 9 % MC and cryopreservation reduced survival to c. 40% (Fig. 1). In contrast, *C. jambhiri* axes desiccated to 11 to 18% MC survived LN exposure with viabilities of 55 to 63% (Fig. 2). Across all species, the critical MC hydration window for survival post-cryopreservation was slightly wider for the axes compared with the embryos (Figs. 1 & 2). For embryos, the optimum values were 11%, 17%, 15%, 20% and 16% MC for *C. jambhiri*, *C. grandis*, *C. reticulata*, *C. medica* and *C. aurantifolia*, respectively (Fig. 1); corresponding value ranges for the axes were 11 to 18%, 10 to 21%, 12 to 25%, 11 to 22% and 10 to 20% MC, respectively (Fig. 2). At these critical MCs, embryo and axis survival (growth) after cryopreservation ranged from 66 to 82 % and 54 to 96%, respectively. Below the critical MCs there was a relatively small decline in survival after LN exposure in all species compared to their desiccated controls; the amount varied between species (Figs 1 & 2).

Embryo and axes after different treatments - *i.e.* control, desiccated and cryopreserved - initiated growth within 4 to 5 days. Well-formed shoots and roots became apparent within 15 to 20 days, leading to healthy seedlings and normal plantlets. Irrespective of treatment, the growth of plantlets was normal with no intervening callus. However, stunted growth was observed in just under one fifth of plantlets from axes of *C. jambhiri* cryopreserved at 11% MC. The radicle and plumule grew normally over 5 to 6 days, but then growth was arrested and a swelling appeared at the base of the shoot. Gradually the small plantlets turned greenish-yellow and remained in the same state for 3 months after which the cultures degenerated.

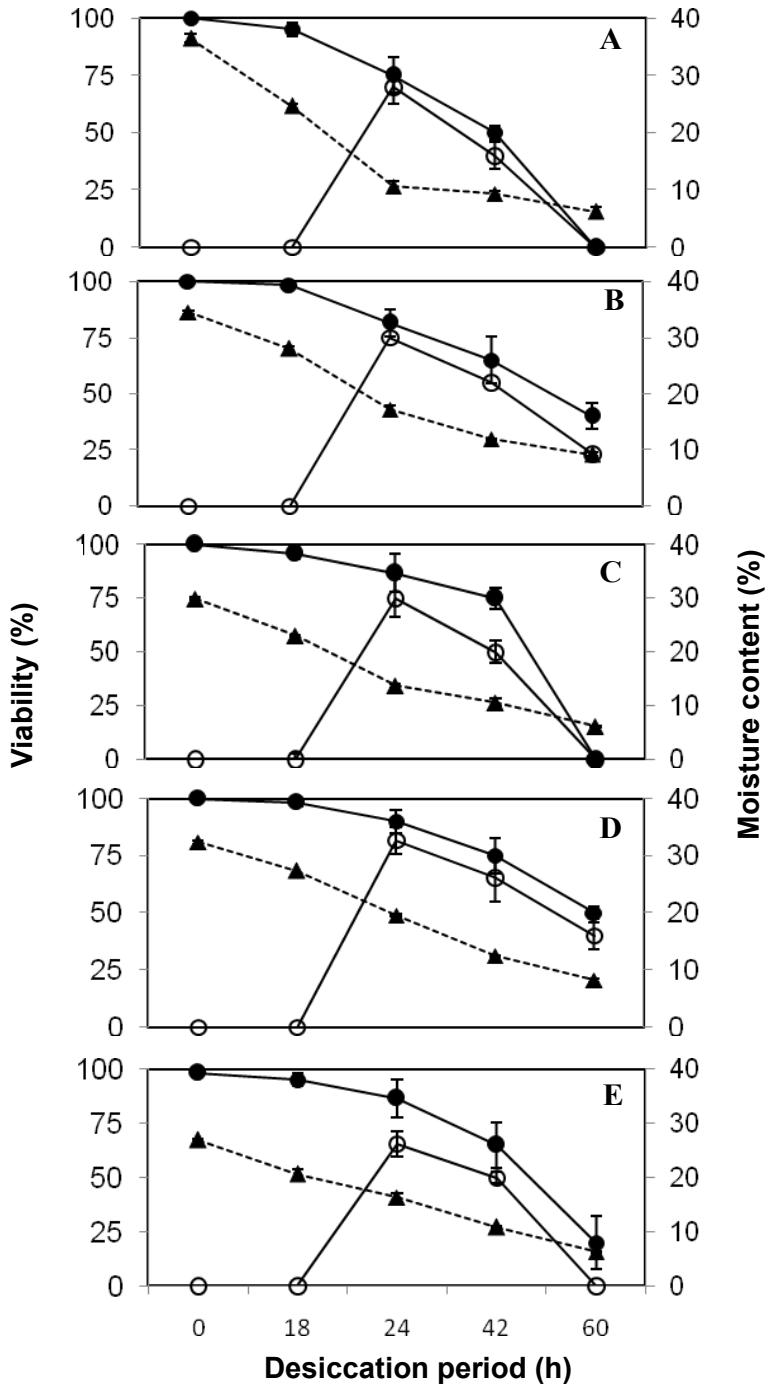


**Figure 1**. Effect of moisture content and desiccation period on embryo germination before and after LN exposure for *C. jambhiri* (A), *C. grandis* (B), *C. reticulata* (C), *C. medica* (D) and *C. aurantifolia* (E). Viability was determined before (●) and after (❍) cryopreservation at various moisture contents (▲). Values plotted are the mean of three replicates ± SE values.

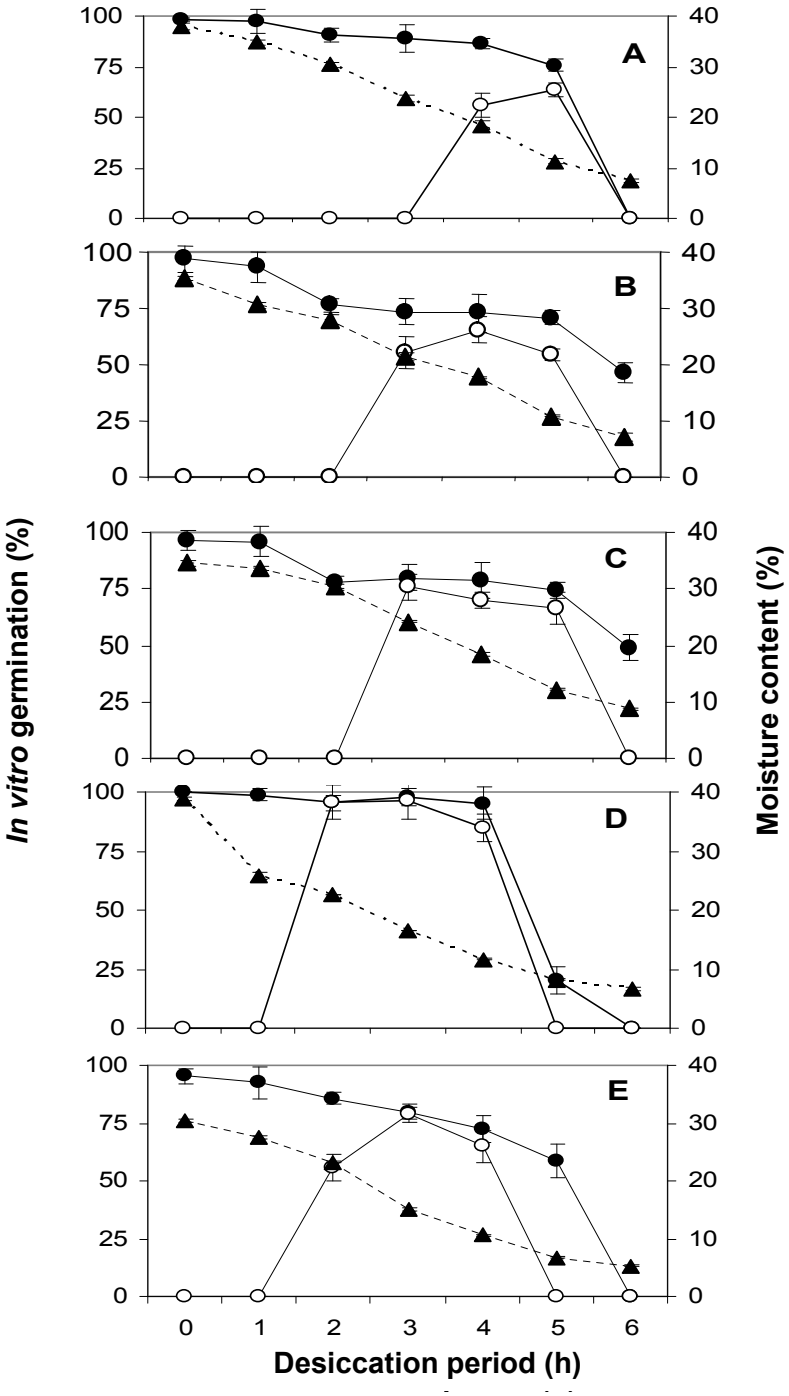


**Figure 2**. Effect of moisture content and desiccation period on viability (*in vitro* germination) of embryonic axes before and after LN exposure for *C. jambhiri* (A), *C. grandis* (B), *C. reticulata* (C), *C. medica* (D) and *C. aurantifolia* (E). Viability was determined before (●) and after (❍) cryopreservation at various moisture contents (▲). Values plotted are the mean of three replicates ± SE values.

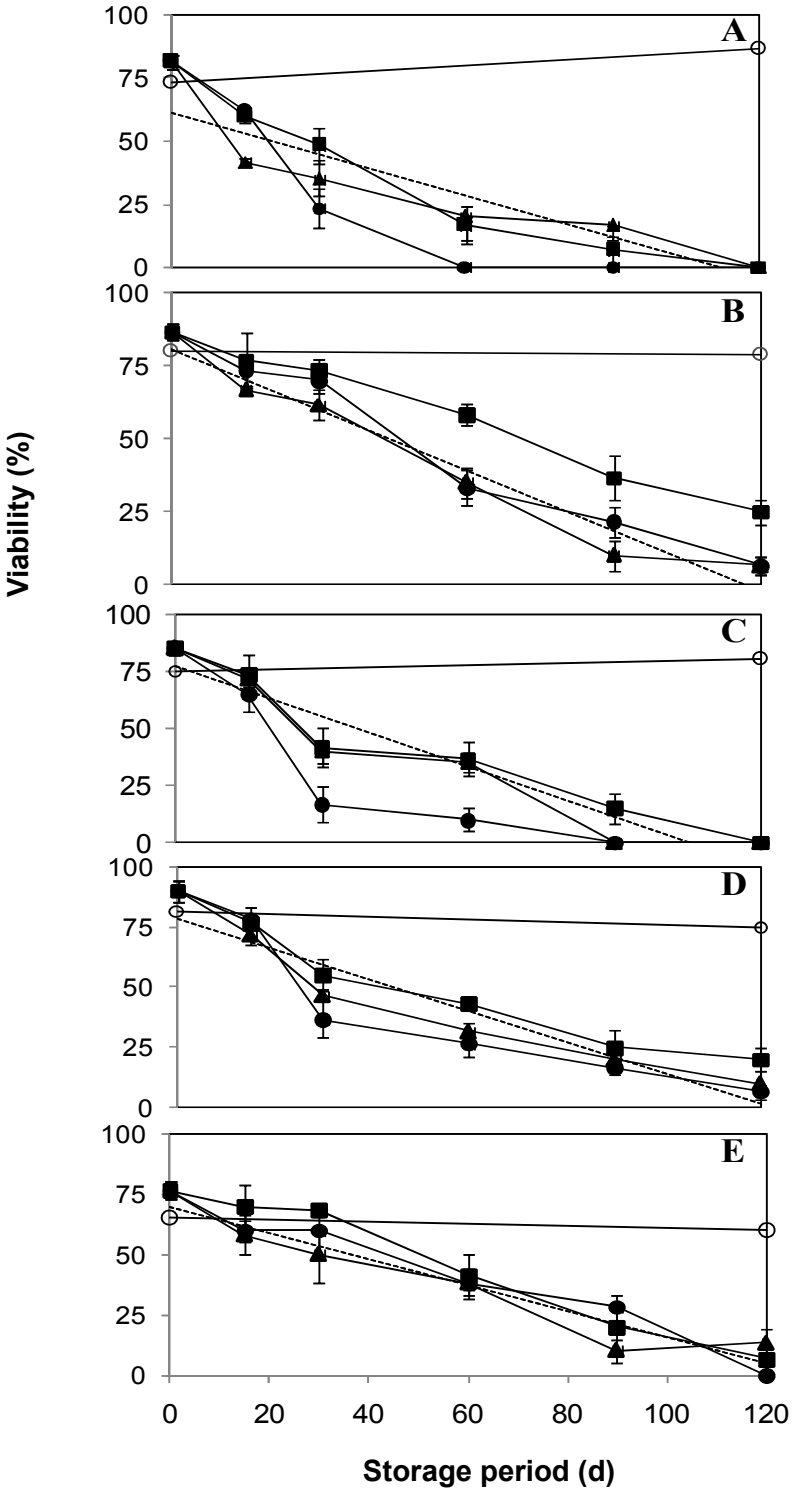


**Figure 3**. Effect of storage temperature on survival of partially dried (c. 10% MC) embryos of *C. jambhiri* (A), *C. grandis* (B), *C. reticulata* (C), *C. medica* (D) and *C. aurantifolia* (E). Viability was determined after storage at room temperature (●), 5°C (■) , -20°C (▲) and LN vapour (❍). Linear relations between viability and storage time at -20°C are shown as dashed lines. Values plotted are the mean of three replicates ± SE values.

*Longevity of embryos at various temperatures*

Embryo survival of all five species after partial drying and cryopreservation remained relatively unchanged after 120 d storage (Fig. 3). In contrast, survival at ambient temperature, 5°C and -20°C was limited. Irrespective of storage temperature between ambient and -20°C, a decrease in viability was evident by 15 to 30 d. At these temperatures, viability was 0% in *C. jambhiri* and *C. reticulata* by 120 d and ≤25% in the other three species by this time. Rapid viability loss at -20°C, 5°C and ambient temperature resulted in a maximum interpolated half-life across these temperatures of c. 80 days for *C. grandis* at 5°C.

*Long-term cryobanking*

Using the air desiccation-freezing protocol on embryos and axes, 377 diverse accessions/cultivars were cryobanked for up to 8 to 10 years (Table 1). Three hundred and sixteen accessions belonging to these five species were assessed for viability after average storage periods of 6.3 years for *C. reticulata* to 8.4 years for *C. medica* (Fig. 4). After this long-term storage, for each species 69 to 81% of the accessions retained ≥ 70 % of the original, post-drying viability.

**Table 1**. Present status of cryostored accessions of five *Citrus* species in liquid nitrogen vapour phase at NBPGR Cryogenebank.

| Species (common name) / embryony status | No. of access-ions | Indigenous and exotic cultivars | Explant |
|---|---|---|---|
| *C. aurantifolia* (lime) / polyembryonic | 58 | Kagzi lime, Assam lime, Sylhet lime, Kagzi Kalan and Coorg lime | Embryos |
| *C. grandis* (pommelo) / monoembryonic | 94 | Chakotra, White Flesh, Red Flesh, Trumph and Yama | Embryos and axes |
| *C. jambhiri* (rough lemon) / polyembryonic | 95 | Attor, Jatti-khatti, Gambhiri, Jullundhri-khatti, Florida Rough, Italian Rough, Mithi, Soh-myn-dong, Soh bitter, Hathi Nimbu, Soh-Julia, Gole Nimbu, Nimbu Tenga, Sinduri lemon, Hasu and Ganpen | Embryos and axes |
| *C. medica* (citron) / monoembryonic | 61 | Etrog Citron, Madkakkar, Tayum, The-ma-chi, Pati-Jora, Bira- Jora, Gandhraj, Pongam, Holong Tenga, Soh-mondong, and Jora Tenga | Embryos |
| *C. reticulata* (mandarin and mandarin hybrids) / polyembryonic | 69 | Nagpur orange, Local orange, Khasi Mandarin, Lahore Local, Laddu, Orange East India, Narangi, Kondanarum, Huzzara, Butwal, Cleopatra Mandarin, Billi Kitchli, King, Wilkins, Kinnow, Sikkim Mandarin and Feutal early | Embryos |

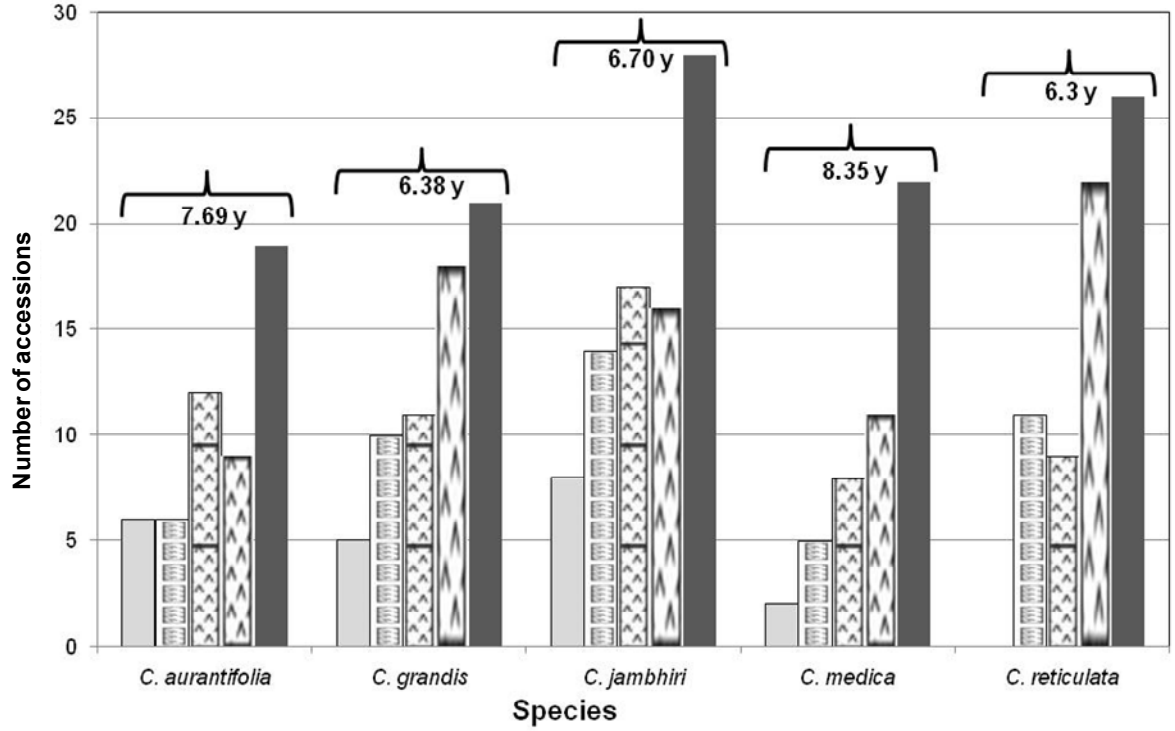


**Figure 4**. Viability ranges for 316 accessions of citrus embryos from five species cryopreserved in LN vapour ; average storage periods (in years) are indicated above each species data set. Within species data sets and from left to right, the viability ranges are 50-60, 60-70, 70-80, 80-90 and 90 – 100%, calculated as the percentages of their dried 'controls'. Please note that no samples were no lower than 50% viability.

## DISCUSSION

As with many other citrus, we observed that embryo drying gradually reduces germinability, when judged by germination tests on embryos (Fig. 1) or recovery of isolated axes *in vitro*. The mid-point for desiccation stress of around 15 – 20% embryo MC was slightly higher than that (c. 10 – 13% MC) observed for three accessions of the same species by Hor *et al*. (14). Moreover, the relative desiccation tolerance of the seed lots used here was considerably less than those of three Australian species (*C. australasica*, *C. inodora* and *C. garrawayi*), which produced radicles after drying to c. 3% MC (12). Amongst species, the greatest variance in survival of desiccation appears to be for *C. aurantifolia*, the embryos of which either tolerated drying to MCs equivalent to c. 10% RH (13) or retained only c. 40% germination after desiccation to 10% embryo MC. Similarly, *C. grandis* seed lots have been observed to have differing mid-points for desiccation sensitivity of 3 to 6% MC (39), 11% MC (14) and c. 15%. Such intra-species variation in desiccation tolerance can be a consequence of cumulative environmental conditions during seed development, particularly for trees, as shown previously for *Aesculus hippocastanum* and *Acer pseudoplatanus* (6, 7). For the latter species, such differences in stress tolerance also impacted on success levels post-cryopreservation, with only the most mature *Acer* fruits from southern Europe capable of surviving drying and short-term low temperature storage (8).

Survival of embryos post-cryopreservation was optimal after partial drying to 11 to 20% MC, depending on species. Target MCs below which seeds should be dried to avoid ice formation on ultra-cooling have been defined in relation to the seed oil content, for citrus and other species (14, 26). Citrus seeds have an oil content of c. 30 to 54% dry mass basis; e.g. 37 to 52% [four species (14)] and 30 to 54% [three species (12)]. Taking an average of 40% seed oil content, the target MC for citrus cryostorage should be c. 14%. Recent results support this perspective, with many citrus species seed, embryos and axes being successfully cryopreserved after partial drying to 10 to 15% MC (14, 16, 17).

Embryonic axes tended to survive drying to c. 10% MC, indicating a relatively higher level of desiccation tolerance than the whole embryo. However, the rate of drying of axes is 10-times higher compared to the whole embryo. Consequently, the exposure time to desiccation stress for isolated axes (a few hours) is potentially one order of magnitude less compared to axes dried within the whole seed. Rapid drying of recalcitrant (highly desiccation sensitive) seed axes has been shown also to enable at least short-term tolerance of low moisture contents, probably by reducing the deleterious effects of unregulated metabolism (24, 25). However, such rapid dehydration increases the risk of over-drying, which can result in stunted growth in some axes of *C. jambhiri* (this study) and non-development of roots in *C. grandis* (39).

Citrus axes have a relatively wide hydration window for cryo-survival, extending to higher MCs than that of whole embryos. Similarly, Wen *et al.* (39) showed for one of the species studied here, *C. grandis*, that optimum MCs for cryopreservation were 3 to 20% for the isolated embryonic axis compared to 5 to 9% for the embryo.

The application of cooling and warming to seed tissues that have been partially dried can result in further loss of viability. For example, c. 12 % MC embryos of *C. grandis* have been observed to retain less than half their viability following cooling / warming (14). However, for the five species studied here, there was evidence of only a small loss in viability as a result of the low temperature treatment, confirming that dehydration tolerance is a key component of low temperature survival (8, 24, 25, 27, 38).

There is an assumption that tissue that survives short-term drying and cryopreservation may have a near-indefinite lifespan. Recent studies have shown that measurable changes in germination of cryobanked seeds beyond 10 years storage are detectable (38). These were found to be affected by harvest year and pre-storage of seeds at higher temperatures. In the studies on shoot tips of pea and strawberry, variability was observed in survival after 28 years of cryobanking (1). This was attributed either to the heterogentity of the explants or physical injury suffered during isolation, freezing or rewarming. Variability in response between accessions in this study could also be due to operator-dependent application of the isolation and cryopreservation methodologies. Nonetheless, the vast majority of citrus germplasm cryo-stored for average times of 6.3 to 8.4 years was successfully regrown.

In contrast, survival of partially dried citrus material at higher temperatures (-20°C to ambient) is severely restricted. Causes of the rapid loss in embryo viability when partially-dried and stored may be attributed to numerous temperature-dependent processes. Under ambient conditions, accelerated embryo ageing may occur possibly as a result of cumulative desiccation stress (24, 25). At -20°C, the devitrification of the aqueous glass could enable ice formation (5, 27) or cold stress and this may be exacerbated by conformational changes in seed lipids (see 27). Based on differential scanning calorimetry, *C. reticulata* and *C. grandis* lipids have main melting peaks around -20°C and 0°C (14). As the seed lipids are mainly fluid by 5°C (14) and storage conditions are cool, ageing is anticipated to be slower than at ambient and -20°C. However, the evidence from our experiments is that citrus embryo longevity under partially dried conditions is relatively temperature independent over a storage

range of c. 40°C (from ambient to -20°C), suggesting a steady cumulative effect of desiccation stress.

High recovery growth of embryonic axes of *C. aurantifolia*, *C. halimii* and *C. madurensis* (22, 23) and in *C. macroptera* and *C. latipes* (18) after cryopreservation and similar results observed during the present study on five citrus species indicate the effectiveness of these techniques for long-term conservation. Polyembryonic seed species of *C. aurantifolia, C. jambhiri* and *C. reticulata*, carrying nucellar embryos genetically identical to the mother plant, present an added advantage for conservation. These findings are supported by the successful establishment of a citrus base collection in the cryogenebank at NBPGR. The facility offers safe storage for a substantial range of citrus genetic diversity in India, avoiding some of the challenges associated with field genebanks. The *ex situ* stored material serves as a resource for citrus crop improvement programmes.

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