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Storage behavior and cryopreservation studies in Indian rough lemon (*Citrus jambhiri*): a promising rootstock for long-term conservation

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Abstract: The study of the seed storage behavior and cryopreservation of embryonic axes was attempted in *Citrus jambhiri* genotypes using air desiccation-freezing, vitrification, and encapsulation-dehydration methods. Seeds of this species revealed nonorthodox seed storage behavior with moderate seed longevity and sensitivity to desiccation and freezing. Successful cryopreservation was achieved with all three methods using embryonic axes; however, the recovery growth percentage significantly differed among these methods. Cryopreservation was efficient for the embryonic axes desiccated to 13%–15% moisture content. Freezing of embryonic axes at this moisture content in liquid nitrogen showed a good recovery rate of 40%–50%. In the vitrification method, embryonic axes subjected to PVS2 treatment for 40 min had the highest recovery rate of 25% after cryopreservation. In the encapsulation-dehydration method, encapsulated axes precultured on 0.5 M sucrose medium followed by 6 h of desiccation had the highest recovery growth of 30%–60% after cryopreservation. Out of the three methods employed, the encapsulation-dehydration method gave the best recovery values for embryonic axes. High regeneration values of embryos in comparison to embryonic axes prompted us to undertake large-scale cryobanking of *C. jambhiri* using embryos (seeds without seed coats) and more than 100 accessions of *C. jambhiri* have been cryostored in the national cryobank. This would further serve the purpose of conserving nucellar embryos with zygotic embryos.

Key words: Air desiccation, rough lemon, embryonic axes, encapsulation-dehydration, vitrification

1. Introduction

Citrus is one of the most important and widely grown fruit crops in the world. The genus *Citrus* comprises many economically important species including *C. reticulata*, *C. sinensis*, and *C. limon*. Citrus occupies the second position in terms of area (1064 ha) and the third position in terms of production (9.94 t) of fruit crops in India (http://www.nhb.gov.in). Northeastern India is thought to be the area of origin of several *Citrus* species and abundant diversity of various citrus types is available in the northeastern and northwestern states. India has an enormous diversity of *Citrus* genetic resources, both cultivated and wild.

Rough lemon (*Citrus jambhiri* L.) is one of the indigenous species of probable hybrid origin. It is thought to have originated in India and is found throughout the country up to an altitude of 1000 m. An array of forms of "jambhiri" are commonly met with in Assam and also in other parts of India. The varieties of jambhiri occurring in Assam are clearly divisible into two distinct subgroups on the basis of acidity and sweetness of the pulp vesicles. The acidic fruits, which include the well-known rough lemon, constitute the normal group and are well distributed all over the province up to an altitude of 1524 m. The sweet form is very rare and is usually grown as a novelty in homestead gardens of the plain districts of the province (Bhattacharya and Dutta, 1956). In India, rough lemon has largely been used as a rootstock for commercial plantations of citrus; it is one of the major rootstock for mandarin, oranges, kinnow, etc. Trees on these rootstocks are vigorous, high-yielding, and tristeza- and drought-tolerant (Sonkar et al., 2002). It has good adaptability for light sandy soils and is also fairly tolerant to salts. This species constitutes a very important diverse germplasm to be employed for future rootstock breeding programs, especially for abiotic stress tolerance traits. Therefore, activities including collection, conservation, characterization, and assessment of genetic diversity within this important rootstock *Citrus* species are of prime interest. Indeed, to overcome the limiting effects of biotic and abiotic constraints and improve yield and fruit quality, the use of grafted plants on adequate and adapted rootstocks in modern citrus cropping systems is required (Lamine and Mliki, 2015).

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Genetic improvement of a species depends on safe conservation and efficient utilization of the indigenous genetic diversity available. Many of the *Citrus* species are found growing in their natural habitats in a wild or semiwild state (Malik et al., 2013). In view of the rapid destruction of these natural habitats and biotic and abiotic threats foreseen in the field gene banks, there is an urgent need to conserve the vast genetic resources of *Citrus* for effective utilization in improvement of existing varieties and rootstocks through conventional and biotechnological methods. Ex situ conservation strategies have to be implemented for the effective conservation of citrus genetic resources. Long-term seed storage serves as a safe and relatively inexpensive method of ex situ conservation of plant genetic resources. Storage in the form of seeds in gene banks and cryobanks has been the most preferred method of genetic conservation because of easy handling and accessibility. Seeds of many *Citrus* species show nonorthodox storage behavior and therefore cannot be stored using conventional –20 °C storage methods. In this case, cryopreservation is the only available alternative for long-term conservation of *Citrus* species. Cryopreservation is the long-term conservation of viable biological tissues at ultralow temperatures of –160 to –196 °C. It offers long-term storage capability, maximal stability of phenotypic and genotypic behavior of stored germplasms, and minimal storage space and maintenance requirements (Engelmann et al., 1994).

Embryonic axes, being an organized structure isolated from seeds, have been shown to be an excellent explant for *Citrus* cryopreservation. Indeed, embryonic axes of *C. aurantifolia*, *C. halimii*, *C. madurensis*, *P. trifoliata*, *C. sinensis*, *C. macroptera*, and *C. hystrix* survived storage in liquid nitrogen and germinated after thawing. Successful cryopreservation of embryos of *C. sinensis* using an air-desiccation protocol has been reported and successful cryopreservation of embryonic axes of *C. sinensis* by vitrification method has been reported (Lambardi et al., 2004). The present study was undertaken for the cryopreservation of embryonic axes of *Citrus jambhiri* genotypes using three different approaches: air-desiccation, vitrification, and encapsulation-dehydration.

2. Materials and methods

Three accessions of rough lemon collected from the field gene bank of the National Research Centre for Citrus, Nagpur, were used for cryoexperiments (Table 1; Figures 1A and 1B). A cryopreservation technique was attempted using embryonic axes. For obtaining embryonic axes, the seed coat was removed from the whole seed and the seed surface was decontaminated with 1.0% sodium hypochlorite for 10 min followed by washing with sterilized deionized water three times. The concentration of the disinfectant and the duration of the treatment was altered as required. Embryonic axes were carefully excised from whole seeds and spread over sterile filter paper discs. While excising embryonic axes, the time taken to handle a number of samples should be minimized so that there are no major differences in moisture contents between axes.

Table 1. *C. jambhiri* accessions used for cryoexperiments.

Accession no.	IC no.	Common name	State
IPS-7	IC 311339	Jatti Khatti	Rajasthan
IPS-120	IC 285361	Soh Jhalia	Meghalaya
IPS-85	IC 311500	Jambhiri	Maharashtra

Cryopreservation of embryonic axes was undertaken using three cryotechniques: air desiccation followed by fast freezing, encapsulation-dehydration, and vitrification. In all three techniques moisture content determination was done by gravimetric method (low constant temperature oven method) by drying at 103 ± 2 °C for 17 h (ISTA, 1985). Five axes per accession were taken for calculating moisture content. MC% was expressed on a fresh weight basis. The moisture content was calculated by using the following formula:

Moisture content (%) = B – C × 100 / B – A,
where A = Wt. of empty weighing bottle,
B = Wt. of bottle + fresh axes,
C = Wt. of bottle + oven-dried axes.

2.1. Air desiccation

Excised embryonic axes were kept in batches of 20–25 in the sterile air flow of a laminar flow cabinet immediately after excision. Axes were then desiccated for 1 to 6 h; the desiccation time depended on the size of the axes and the initial and desired moisture content levels. After each desiccation interval, moisture content and viability of embryonic axes were determined. Desiccated embryonic axes were sealed in 1.0-mL polypropylene cryovials and fast-frozen by direct plunging in liquid nitrogen (LN). After a minimum of 24 h of storage, the cryovials were rapidly thawed in a water bath at 38 °C for 5 min and axes were cultured in vitro on two different media within 30 min of retrieval. MS Medium A (Murashige and Skoog, 1962) contained macro- and micronutrients, vitamins, 2 g/L iron, and activated charcoal supplemented with 1 mg/L each of 6-benzylaminopurine (BAP) and naphthalene acetic acid (NAA). In Medium B charcoal was omitted and it was supplemented with 0.1 mg/L each of BAP and NAA. Cultures were maintained at 25 ± 2 °C with a 16-h photoperiod under a light intensity of 35 $\mu Em^{-2} s^{-1}$.

2.2. Encapsulation-dehydration

In this method, alginate beads were used to encapsulate embryonic axes followed by partial desiccation before

Figure 1. *C. jambhiri*: A- Natural habitat, B- fruits; recovery growth after cryotreatment using C- air desiccation, D- vitrification, and E- encapsulation dehydration of embryonic axes. Scale bars in Panels A, B = 2.5 cm; Panels C–E = 1.5 cm.

plunging in LN. For the encapsulation-dehydration process, aseptically excised embryonic axes were encapsulated in alginate beads by first suspending them in calcium-free MS basal liquid medium containing 3% (w/v) Na-alginate. Drops of this solution, each containing one axis, were dispensed with a pipette into MS basal liquid medium supplemented with 100 mM calcium chloride. Beads were solidified by 15 min of incubation at 25 °C with occasional stirring and were then precultured on a rotary shaker in liquid MS medium supplemented with sucrose (0.3, 0.5, and 0.75 M) at 100 rpm for 20 or 40 h. The beads were then removed from the liquid medium and dehydrated at room temperature for 6 h in a laminar flow cabinet. The moisture content of the beads was determined by drying at 103 ± 2 °C for 17 h. Beads were finally enclosed in 1.0-mL cryovials and fast-frozen in LN. The cryovials were thawed at room temperature for 15 min and the beads were cultured in vitro. Experiments were repeated with 10–20 axes per treatment.

2.3. Vitrification

This procedure involves the preculture of explants on media enriched with cryoprotectants, followed by treatment with cryoprotectants in vitrification solutions followed by fast freezing. For vitrification experiments, aseptically excised embryonic axes were precultured on basal MS medium supplemented with 0.3 M sucrose and 2 M glycerol for 16–24 h. Embryonic axes in batches of 15–25 were then transferred to 1.0-mL sterile cryovials and

treated with loading solution (0.4 M sucrose, 2 M glycerol in basal MS medium) for 20 min at 25 °C. The loading solution was replaced by plant vitrification solution 2 (PVS2; 30% (w/v) glycerol, 15 % (w/v) ethylene glycol, 15 % (w/v) dimethyl sulfoxide, 0.3 M sucrose) for 20, 40, and 60 min at 25 °C. Axes were then frozen by fast freezing in LN. Frozen cryovials were thawed after a minimum of 24 h of storage by 5 min of immersion in a water bath at 38 ± 1 °C, following which the PVS2 solution was replaced with unloading solution for 20 min. The axes were then germinated in vitro. Experiments were repeated with 15–20 embryonic axes per treatment.

2.4. Statistical analysis

In each experiment, 25 explants were used per treatment and each experiment was repeated three times. Standard errors (SEs) of the arithmetic means were calculated for each treatment. The data were analyzed by analysis of variance (ANOVA) in SPSS 15.0 Windows (SPSS Inc., Chicago, IL, USA). Significant differences between means were assessed using Duncan's multiple range test at $P \leq 0.05$ (Gomez and Gomez, 1976).

3. Results and discussion

3.1. Seed longevity and storage behavior

In the genotype IPS-7, seeds with original 100% viability and 12.52% desiccated moisture content were stored at four different temperatures and tested after different storage periods. By 30 days of storage at room temperature (RT) they retained only 66.67% viability, and at 5 °C they retained 64.46% viability. However, these seeds showed higher viabilities after storage for same period at –20 °C (73.89%) and –196 °C (83.33%). After 60 days of storage at RT, 5 °C, and –20 °C, the viability gradually declined to 26.67%, 43.33%, and 53.33%, respectively, and at –196 °C temperature storage 76.67% viability was noted. After 90 days of storage at RT, 5 °C, and –20 °C, the viability further declined gradually to 10%, 23.33%, and 43.33%, respectively. After 120 days of storage seeds at RT lost viability completely, while for those stored at 5 °C and –20 °C, little viability was observed. Cryostored seeds were found to retain 75% viability for 120 days (Table 2).

In IPS-85, seeds with 90% viability and 13.81% desiccated moisture content were stored at four different temperatures and tested after different storage periods. By 30 days of storage at RT they retained only 53.33% viability, and at 5 °C they retained 56.67% viability. However, these seeds showed higher viabilities after storage for same period at –20 °C (76.67%) and –196 °C (90.00%). After 60 days of storage at RT, 5 °C, and –20 °C, the viability gradually declined to 25.00%, 46.67%, and 56.67%, respectively, and 86.67% viability was noted at –196 °C. After 90 days of storage at RT, 5 °C, and –20 °C, the viability further declined gradually to 6.67%, 16.67%, and 26.67%, respectively, but it remained same at –196 °C (86.67%). After 120 days of storage at RT seeds lost viability, while for those stored at 5 °C and –20 °C negligible viability (3.33%) was observed. Cryostored seeds were found to retain 85% viability for 120 days (Table 2). Overall results showed that –196 °C (LN) storage temperature is better for long-term survival and no decrement of viability after 30, 60, 90, and 120 days was observed as compared to other storage temperatures, i.e. room temperature, 5 °C, and –20 °C.

Ascertaining seed storage behavior of a cultivar or species is important before adopting any ex situ conservation approach. The success of ex situ conservation depends on the longevity of seeds under storage and the ability to generate whole explants/plantlets after retrieval

Table 2. Viability of *C. jambhiri* accessions at different storage temperatures and different durations.

Accession	MC%		Storage temp.	Viability (%) after storage			
	Fresh	DC		30 days	60 days	90 days	120 days
IPS-7	45.64 (±0.37)	12.52 (±0.17)	RT 5 °C –20 °C –196 °C	66.67^{b} (±2.72) 64.46^{c} (±1.81) 73.89^{b} (±3.18) 83.33^{a} (±1.36)	26.67^{c} (±1.36) 43.33^{b} (±2.72) 53.33^{b} (±5.44) 76.67^{a} (±3.60)	10.00^{b} (±4.71) 23.33^{c} (±3.60) 43.33^{b} (±5.44) 76.67^{a} (±5.44)	00.00 (±0.00) 00.00 (±0.00) 06.67^{b} (±5.44) 75.00^{a} (±2.36)
IPS-85	48.74 (±0.15)	13.81 (±0.11)	RT 5 °C –20 °C –196 °C	53.33^{b} (±2.27) 56.67^{b} (±7.20) 76.67^{a} (±2.72) 90.00^{a} (±1.36)	25.00^{c} (±4.08) 46.67^{b} (±2.72) 56.67^{b} (±7.20) 86.67^{a} (±5.44)	06.67^{d} (±2.72) 16.67^{b} (±3.60) 26.67^{b} (±1.36) 86.67^{a} (±2.72)	0.00 (±0.00) 03.33^{b} (±2.72) 03.33^{b} (2.72) 85.00^{a} (±2.36)

Values in parentheses indicate the standard errors of means. Means with the same superscripted letters in a column showing viability before and after liquid nitrogen exposure do not significantly differ ($P \leq 0.05$) based on Duncan's test.

from storage conditions. Seed longevity (i.e. the period of survival) varies greatly among species. It may also vary among accessions within a species because of differences in genotype and provenance. Seeds of *Citrus* are classified into an intermediate category, i.e. these seeds with large size and high moisture content at the time of shedding are variably desiccation- and freezing-sensitive (Hong and Ellis, 1996; Ellis et al., 2007). Substantial loss of seed viability in the three accessions of *C. jambhiri* at ambient, 5 °C, –20 °C, and –196 °C temperatures within 30–60 days of storage confirms the desiccation- and freezing-sensitive nature of seeds of these genotypes. Sharp decline in the viability of seeds at ambient temperature within 30 days confirms the short longevity of seeds of *C. jambhiri*. These studies confirm the nonorthodox seed storage behavior of this species with more behavior towards recalcitrance. Plant growth is demonstrated in Figures 1C–1E.

3.2. Air desiccation

The moisture contents and viability percentages of embryonic axes of three *C. jambhiri* genotypes, namely IPS-7, IPS-85, and IPS-120, following desiccation under various time factors with and without cryopreservation are given in Table 3. The initial moisture content was as high as 45.64%, 48.74%, and 51.22% in IPS-7, IPS-85, and IPS-120, respectively. With increasing duration of desiccation, the moisture content of the axes declined steadily. Since the recovery growth of fresh, desiccated, and frozen axes was comparable on both culture media, Medium A and Medium B, observations using both are reported. Reduction in moisture content of axes was, however, accompanied by a reduction in the survival rate (Table 3).

The initial viability values in freshly excised embryonic axes were about 90%, 100%, and 100% for IPS-7, IPS-85, and IPS-120, respectively. The viability of axes declined steadily with increasing duration of desiccation (Table 3). In the genotype IPS-7, the viability remained as high as 85% at 26.64% moisture content and declined to 30% at 8.06% moisture content. It was seen that viability fell drastically after 4 h of desiccation at moisture contents below 12.13%. Similar observations were recorded in the case of the other two accessions, in which the viability remained highest (80%) at 29.64% moisture content and was 100% at 39.56% moisture content, declining to 20% at 9.03% and 50% at 9.11% moisture content.

In IPS-7, cryopreservation was efficient for the axes desiccated to 14.27% and lower moisture contents. Freezing of embryonic axes at 14.27% moisture level in LN at –196 °C showed a moderate recovery rate (40%), whereas embryonic axes possessing moisture contents above 26.64% and below 12.13% lost viability completely when exposed to LN (Table 3). In IPS-85, embryonic axes desiccated to 15.64% moisture content showed maximum recovery of 33.33% after LN exposure, whereas axes possessing moisture content above 29.64% and below 13.21% completely lost viability after LN exposure (Table 3). Similar was the case with genotype IPS-120, in which axes possessing 13.55% moisture content retained the maximum viability of 45%, while above and below that moisture content there was a drastic reduction in viability (Figure 1C).

Excised embryonic axes were preferred for cryopreservation in this species indicating nonorthodox seed storage behavior, which is more inclined towards recalcitrance. Small size and organized structure, independent identity, and appreciable proportion of meristematic tissues with high morphogenetic potential makes embryonic axes a more preferred explant in such species. Embryonic axes have the ability to produce vigorous plantlets by careful manipulation of postfreezing conditions and represent good material for cryopreservation in *Citrus* (Chaudhury and Malik, 1999). Cryopreservation techniques have found application in the long-term conservation of *Citrus* germplasm using seeds, embryonic axes, and somatic embryos. Viability percentages of 50% to 100% have been achieved using air desiccation methods in various *Citrus* species (Cho et al., 1992; Radhamani and Chandel, 1992; Normah and Serimal, 1995; Malik and Chaudhury, 2006; Malik et al., 2015).

3.3. Encapsulation-dehydration

Fresh encapsulated embryonic axes of genotypes IPS-7, IPS-85, and IPS-120 showed high viability of 100%, 100%, and 80%, respectively, when cultured in vitro (Table 4). Encapsulated axes following 40 h of preculture treatment in various sucrose concentrations of 0.3 M, 0.5 M, and 0.75 M had moisture contents of 82.06%, 74.77%, and 66.67%, respectively, for IPS-7; 91.7%, 89.36%, and 86.08% for IPS-85; and 84%, 82.97%, and 80.27% for IPS-120. Furthermore, 6 h of air desiccation reduced the moisture contents to less than 22% in all the accessions, but this was accompanied by a decline in viability. Preculturing beads on 0.5 M sucrose followed by 6 h of air desiccation gave maximum survival of axes before and after cryopreservation (Figure 1E) in IPS-7 and IPS-85, but for accession IPS-120 successful cryopreservation with a highest viability of 66.66% and 50%, respectively, was achieved for axes precultured for 40 h in 0.3 M sucrose preculture media, followed by 6 h of desiccation (Table 4). Successful cryorecovery has been achieved in *C. grandis*, *C. paradisi*, and *C. karna* using encapsulation-dehydration techniques (Malik et al., 2012a, 2012b).

3.4. Vitrification

Preculture of embryonic axes for 24 h in preculture medium retained only 50%–75% viability in IPS-7, IPS-85, and IPS-120. Treatment with loading solution for 20 min without cryopreservation retained 50%, 45%, and

Table 3. Survival of embryonic axes of IPS-7, IPS-85, and IPS-120 after different periods of air desiccation and LN exposure.

Desiccation duration (h)	IPS-7			IPS-85			IPS-120		
	Moisture content (%)	Viability before LN (%)	Viability after LN (%)	Moisture content (%)	Viability before LN (%)	Viability after LN (%)	Moisture content (%)	Viability before LN (%)	Viability after LN (%)
0	45.64 (±0.37)^{a}	90.00 (±5.77)^{a}	0.00 (±0.00)	48.74 (±0.15)^{a}	100.00 (±0.00)^{a}	0.00 (±0.00)	51.22 (±0.13)^{a}	100.00 (±0.00)^{a}	0.00 (±0.00)
1	26.64 (±0.03)^{b}	85.00 (±2.89)^{a}	10.00 (±5.77)^{b}	29.64 (±0.21)^{b}	80.00 (±2.89)^{b}	20.00 (±5.77)^{ab}	39.56 (±0.25)^{b}	100.00 (±0.00)^{a}	0.00 (±0.00)
2	17.66 (±0.61)^{c}	70.00 (±2.89)^{b}	30.00 (±5.77)^{a}	18.86 (±0.08)^{c}	50.00 (±5.77)^{c}	20.00 (±5.77)^{ab}	27.64 (±0.21)^{c}	75.00 (±2.89)^{b}	20.00 (±5.77)^{c}
3	14.27 (±0.16)^{d}	60.00 (±2.89)^{bc}	40.00 (±5.77)^{a}	15.64 (±0.21)^{d}	50.00 (±5.77)^{c}	33.33 (±3.33)^{a}	15.86 (±0.08)^{d}	66.67 (±3.33)^{b}	33.33 (±3.33)^{b}
4	12.13 (±0.08)^{e}	50.00 (±2.89)^{c}	40.00 (±5.77)^{a}	13.21 (±0.12)^{e}	33.33 (±3.33)^{d}	33.33 (±3.33)^{a}	13.55 (±0.26)^{e}	66.67 (±3.33)^{b}	45.00 (±2.89)^{a}
5	10.08 (±0.53)^{f}	30.00 (±5.77)^{d}	0.00 (±0.00)	10.48 (±0.30)^{f}	20.00 (±5.77)^{e}	10.00 (±5.77)^{bc}	10.48 (±0.28)^{f}	50.00 (±5.77)^{c}	41.67 (±1.67)^{ab}
6	8.06 (±0.04)^{g}	30.00 (±5.77)^{d}	0.00 (±0.00)	9.03 (±0.02)^{g}	20.00 (±5.77)^{e}	0.00 (±0.00)	9.11 (±0.64)^{g}	50.00 (±5.77)^{c}	0.00 (±0.00)

Values in parentheses indicate the standard errors of means. Means with the same superscripted letters in a column showing viability before and after liquid nitrogen exposure do not significantly differ ($P \leq 0.05$) based on Duncan's test.

Table 4. Survival of embryonic axes of IPS-7, IPS-85, and IPS-120 after encapsulation-dehydration.

Treatment	MC (%) fresh	MC(%) after 6 h of desiccation	Viability before LN (%)	Viability after LN (%)
IPS-7				
PME	---	----	100 (±0.00)[a]	0.00 (±0.13)
0.3 M	82.06 (±1.19)[a]	18.67 (±0.04)[a]	60.00(±5.77)[c]	40.00 (±5.77)[b]
0.5 M	74.77 (±4.41)[a]	17.21 (±0.12)[b]	73.33 (±3.33)[b]	60.00 (±5.77)[a]
0.75 M	66.67 (±3.33)[b]	15.43 (±0.83)[c]	60.00(±2.89)[c]	33.33 (±3.33)[b]
IPS-85				
PME	---	----	100.00 (±0.00)[a]	0
0.3 M	91.70 (±0.98)[a]	22.42 (±0.33)[a]	75.00 (±2.89)[b]	33.33 (±3.33)[b]
0.5 M	89.36 (±0.37)[b]	18.67 (±0.39)[b]	75.00 (±2.89)[b]	50.00 (±5.77)[a]
0.75 M	86.08 (±0.62)[c]	15.27 (±0.42)[c]	60.00 (±5.77)[c]	40.00 (±5.77)[ab]
IPS-120				
PME	---	----	80.00 (±5.77)[a]	0
0.3 M	84.00 (±0.25)[a]	16.74 (±0.15)[a]	66.66 (±3.33)[a]	50.00 (±5.77)[a]
0.5 M	82.97 (±0.21)[b]	15.08 (±0.53)[b]	33.33 (±3.33)[b]	33.33 (±3.33)[ab]
0.75 M	80.27 (±0.42)[c]	13.07 (±0.61)[c]	33.33 (±3.33)[b]	20.00 (±5.77)[b]

Values in parentheses indicate the standard errors of means. Means with the same superscripted letters in a column showing viability before and after liquid nitrogen exposure do not significantly differ ($P \leq 0.05$) based on Duncan's test.

75% viability respectively in the three accessions, but after cryopreservation there was no regrowth. Exposure of axes to PVS2 for 20, 40, and 60 min without LN exposure gave 33.33%, 33.33%, and 0% viability for IPS-7, while the same treatment after LN exposure showed 16.67%, 20%, and 0% viability respectively in IPS-7. In IPS-85, PVS2 treatment for 20, 40, and 60 min without LN exposure showed 33.33%, 50%, and 0% viability, while the same treatment after LN exposure gave 0%, 25%, and 0% viability, respectively (Table 5). For IPS-120, PVS2 treatment for 20, 40, and 60 min without LN exposure showed 20%, 33.33%, and 25% viability, while the same treatment after LN exposure gave 20%, 20%, and 0% viability, respectively. Thus, in the case of all three accessions, PVS2 treatment for 40 min followed by cryopreservation in LN was optimal for axes survival (Figure 1D). However, earlier studies reported that successful cryorecovery after vitrification was achieved in *C. grandis*, *C. paradisi*, *C. karna*, *C. latipes*, *C. macroptera*, and *C. indica* using axes and cell suspensions (Malik et al., 2012a, 2012b). *C. jambhiri* genotypes indicated nonorthodox seed storage behavior and showed moderate longevity. Seeds are desiccation- and freezing-sensitive, which further confirms the presence of recalcitrance in seeds of the species. All three methods, namely air desiccation-freezing, vitrification, and encapsulation-dehydration, were successfully applied with moderate to good recovery of embryonic axes leading to regeneration of healthy plantlets. Cryopreservation was efficient for the axes desiccated to 13%–15% moisture content. Freezing of embryonic axes at this moisture content in LN showed a good recovery rate of 40%–50%. In the vitrification method, embryonic axes subjected to PVS2 treatment for 40 min had the highest recovery rate of 25% after cryopreservation. In the encapsulation-dehydration method, encapsulated axes precultured on 0.5 M sucrose medium followed by 6 h of desiccation recorded the highest recovery growth of 30%–60% after cryopreservation. Out of the three methods employed, the encapsulation-dehydration method gave the best recovery values, but air desiccation-freezing is recommended for the routine cryostorage of germplasm, particularly as it represents a simple and practical method that eliminates the need for cryotoxic chemicals.

3.5. Conclusion

In the present study, seed longevity and seed storage behavior of different accessions of *C. jambhiri* have been investigated. Based on results of longevity experiments with the selected genotypes, it was apparent that seeds have a very short life and a significant decline in viability occurred by the first 30 days. Desiccation of seeds to

Table 5. Survival of embryonic axes of IPS-7, IPS-85, and IPS-120 after different periods of air desiccation and LN exposure.

Desiccation duration (h)	IPS-7		IPS-85		IPS-120	
	Viability before LN (%)	Viability after LN (%)	Viability before LN (%)	Viability after LN (%)	Viability before LN (%)	Viability after LN (%)
PMV	50.00 (±5.77)[a]	0.00 (±0.00)	60.00 (±5.77)[a]	0.00 (±0.00)	75.00 (±2.89)[a]	0.00 (±0.00)
Loading solution	50.00 (±5.77)[a]	0.00 (±0.00)	45.00 (±2.89)[bc]	0.00 (±0.00)	75.00 (±2.89)[a]	0.00 (±0.00)
PVS2 (20 min)	33.33 (±3.33)[b]	16.67 (±3.33)[a]	33.33 (±3.33)[c]	0.00 (±0.00)	20.00 (±5.77)[c]	20.00 (±5.77)[a]
PVS2 (40 min)	33.33 (±3.33)[b]	20.00 (±5.77)[a]	50.00 (±5.77)[ab]	25.00 (±2.89)[a]	33.33 (±3.33)[b]	20.00 (±5.77)[a]
PVS2 (60 min)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	0.00 (±0.00)	25.00 (±2.89)[bc]	0.00 (±0.00)

Values in parentheses indicate the standard errors of means. Means with the same superscripted letters in a column showing viability before and after liquid nitrogen exposure do not significantly differ ($P \leq 0.05$) based on Duncan's test.

moisture contents of 12%–14% in *C. jambhiri* led to decline in viability by various degrees, indicating the desiccation-sensitive nature of the seeds. After LN exposure of these desiccated embryos, a further decline in viability was noticed in a few of the cultivars, which indicated sensitivity to freezing but at various levels within the genotypes of *C. jambhiri*. Overall the results showed that –196 °C storage temperature is most suitable for long-term conservation as there is very little decrement of viability after 30, 60, 90, and 120 days in comparison to other storage temperatures, i.e. room temperature, 5 °C, and –20 °C.

All three methods, namely air desiccation-freezing, vitrification, and encapsulation-dehydration, were successfully applied with good recovery of embryonic axes leading to regeneration of healthy plantlets. Cryopreservation was efficient for the axes desiccated to 13%–15% moisture content. Freezing of embryonic axes at this moisture content in LN showed a good recovery rate of 40%–50%. In the vitrification method, embryonic axes subjected to PVS2 treatment for 40 min had the highest recovery rate of 25% after cryopreservation. In the encapsulation-dehydration method, encapsulated axes precultured on 0.5 M sucrose medium followed by 6 h of desiccation had the highest recovery growth of 30%–60% after cryopreservation. Out of the three methods employed, the encapsulation-dehydration method gave the best recovery values. More than 100 accessions of *C. jambhiri* have been cryostored in the National Cryogenebank (NBPGR, New Delhi, India) with large-scale cryobanking using embryos (seeds without seed coats) due to high regeneration values in comparison to embryonic axes. This would further serve the purpose of conserving nucellar embryos with zygotic embryos.

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