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Development of an efficient cryoconservation protocol for Himalyan mulberry (*Morus laevigata* Wall. ex Brandis) using dormant axillary buds as explants

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The Himalayan mulberry, *Morus laevigata* Wall. ex Brandis, is a cold tolerant species found commonly in India from Himalayan foothill to Andaman islands, and it is known for its timber value, forage use, particularly as silkworm's feed. Preservation of germplasm, cryopreservation, particularly for long term storage, of such economically important plant species helps in breeding and development of new cultivars. In the present study, three cryotechniques *viz*., two-step freezing, encapsulation-dehydration and vitrification were attempted for cryopreservation of of *M. laevigata* using winter dormant buds as explants. A two-step freezing cryo procedure preceded by desiccation to 15-25% moisture content was developed. Recovery conditions, including dark incubation and rehydration in sterile moist moss grass for different durations, after cryopreservation led to higher survival when compared to untreated controls. For encapsulation-dehydration, alginate beads containing descaled buds were dehydrated for 1-3 days in various sucrose concentrations (0.3, 0.5, 0.75 or 1.0 M). Bead desiccation was performed using laminar air flow for either 1-6 h. For vitrification, descaled buds were directly immersed for 20, 40, 60, 90 or 120 min in a vitrification solution (PVS_2). Following encapsulation-dehydration, treatment of alginate beads with 0.75 M sucrose was more effective in promoting regrowth of explants after immersion in liquid nitrogen than in the presence of 0.75 M sucrose for 48 h. Regrowth of explants was also observed following vitrification which reached 50% with increasing duration of the PVS_2 treatment for 20-90 min. Overall, the highest frequency of explant regrowth was obtained when explants were subjected to encapsulation-dehydration. This is possibly the first attempt for cryopreservation of Himalayan mulberry adopting these three new cryotechniques.

Keywords: Alginate beads, Breeding, Conservation, Cryopreservation, Encapsulation-dehydration, PVS_2, Sericulture, Sucrose, Vitrification

Preservation of economically important plants solely by field collections is risky, as valuable germplasm can be lost due to pests, diseases and adverse weather conditions. Apart from this, the maintenance of collections is labour-intensive and costly[1]. Advances in biotechnology have generated new opportunities for genetic resources conservation and utilization and maintenance of plant materials at cryogenic temperatures (cryopreservation), suitable for long term storage[2-4].

Mulberry (*Morus* spp.), a primary host of silkworms (*Bombyx mori* L.), is exploited on a commercial scale for silk production. Preservation of germplasm of such economic plants, is important for not only for conservation but also development and maintenance of new cultivars[5]. Mulberry, in addition to its economic importance, has medicinal value as well. Stewart *et al*.[6]reported that the mature fruit contains significant amounts of resveratrol, a strong antioxidant and putative anticancer agent. Mulberry is also a rustic plant with a deep root apparatus. For this reason, it can grow at various altitudes, in lime-rich, dry and saline soils, in hot or cold areas, and consequently can be planted for reforestation of marginal zones[7,8]. The accessions which are not responding to dehydration and slow freezing protocols are cryopreserved based on the new cryopreservation techniques, such as encapsulation-dehydration, vitrification, encapsulation-vitrification, pregrowth dehydration, droplet freezing methods, etc.[9-11].

Cryopreservation protocols prevent formation of intracellular ice crystals, which can otherwise cause cell death and destruction of cell organelles during the freezing process, by inducing vitrification, an amorphous glassy state of water in cells, and also by applying dehydration and desiccation treatments and/or cryoprotectant combinations[12,13]. In the encapsulation-dehydration procedure, originally

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described for cryopreservation of Solanum shoot tips[14,15]. The conservation of mulberry (*Morus* spp.) biodiversity is a priority and large *ex situ* in field germplasm collections are present in China, Japan and India[16-18]. Cryopreservation of *Morus* spp. have been developed using winter buds[16,17] and *in vitro* grown shoot apices or buds in *M. alba* L. cv Florio[19] and *M. bombicys*[20,21].

In the present study, cryopreservation protocols were developed to conserve dormant buds of Himalayan mulberry using two-step freezing, vitrification and encapsulation-dehydration protocols.

Materials and Methods

Winter dormant buds of Himalayan mulberry (*M. laevigata*Wall. ex Brandis) in the form of twigs (60 cm long) were harvested from 1 year old lateral shoots of mature trees and immediately covered with wax at cut ends of the twigs for maintaining the moisture content. After receipt, the twigs were wrapped in polyethene bags and kept in refrigerator at 10-15°C and used up for experimentation within 25 days of harvest.

Determination of moisture content

The moisture content (MC %) of fresh and desiccated dormant buds was determined by drying at 103±2°C in an oven for 17 h following the International rules of seed testing rules, International Seed Testing Association, Switzerland[22]. About five dormant buds were taken randomly in duplicates and moisture content calculated using the following formula:

$$MC\ (\%) = \frac{B - C}{B - A} \times 100$$

$$MC\ (\%) = \frac{\text{Loss of weight}}{\text{Initial weight}} \times 100$$

Two-step freezing

Descaled buds of mulberry were tied in muslin cloth and put in charged silica gel for 4-7 h at ambient temperature (25+2°C) for desiccation. These desiccated buds were then used for cryopreservation in liquid nitrogen at −196°C using slow freezing protocol[9]. The desiccated buds were packed in 1.0 mL polypropylene cryovials. These vials were shifted sequentially at 5, −5, −10, −15, −20 and −25°C, and −30°C keeping at each of the temperatures for a minimum of 24 h. The cryovials were held at −30°C for 48 h and then directly plunged in the liquid nitrogen at −196°C.

Thawing and rehydration

Cryopreserved buds were thawed by slow thawing. The cryovials were placed in air at ambient temperature for 15 min. After thawing, buds were taken out and transferred in sterile moist moss grass for 2-4 h at room temperature (25+2°C) for rehydration. The viability of fresh, desiccated and cryopreserved dormant buds of was tested through TTC and *in vitro* culturing method.

Viability by TTC method

The longitudinal sections of the fresh and cryostored buds (with scales) were cut and transferred in 1% (w/v) TTC solution. These sections were incubated at room temperature in dark for overnight and observation was taken under the stereo microscope.

Viability by in vitro method

For recovery growth of the cryostored dormant buds, 1 to 2 outer scales of the rehydrated buds were further removed followed by washing with Tween 20 for 15 min. Tween 20 was rinsed off with running tap water. These buds were then surface sterilized with 0.1% mercuric chloride for 9 min, rinsed thrice with sterile water washes using autoclaved distilled water (5 min each). The sterilized buds were cultured on basal MS medium[22] with 3% sucrose (w/v) and solidified with 0.8% agar. MS medium was supplemented with 1 mgL^{-1} BAP initially for bud sprouting. The cultures raised from cryopreserved buds were maintained in culture room in dark for 7 days. After dark incubation these cultures were shifted in diffused light for 3 days. After 10 days of culturing, these cultures were exposed to normal culture room light intensity (3000 lux/ 36 μ mol^{-1} s^2). The sprouted buds were sub-cultured on the MS medium supplemented with 1.0 mgL^{-1} BAP and 0.2 mgL^{-1} GA_3 for elongation. The elongated plants were further subcultured and transferred to multiplication medium (0.5 mgL^{-1} BAP + 0.5 mgL^{-1} Kn + 0.1 mgL^{-1} IAA) and finally transferred to rooting medium (half strength of MS + 0.5mgL^{-1} IBA). The healthy rooted plants were transferred to pots.

Cryopreservation of dormant buds employing vitrification and encapsulation-dehydration techniques

Descaled dormant buds with intact apical dome were cryopreserved using modified vitrification and encapsulation-dehydration methods.

Vitrification

Isolation and preculture of dormant buds

The sterilised buds were transferred to a pre-culture medium for vitrification (PMV= MS + 0.3 M sucrose + 2M glycerol + 0.7% agar) in sterile Petridishes. These buds were then incubated overnight at culture room.

Osmotic loading treatment

The precultured buds were treated with 1 mL of osmotic loading solution (LS) containing 2M glycerol, 0.4 M sucrose in liquid MS medium (pH 5.8) placed in a 1.8 mL sterile cryovial for 20 min at 25±2℃ in laminar air flow.

Dehydration with PVS_2 and freezing

The loading solution in the cryovials was replaced with PVS_2 (MS + 30% (w/v) glycerol + 15% (w/v) ethylene glycol + 15% (w/v) DMSO + 0.4 M sucrose (pH 5.8) using a micro pipette. The buds were kept with PVS_2 for 20-120 min at room temperature. After dehydration with PVS_2, the dormant buds were transferred in fresh PVS_2 and cryovials were then plunged directly into liquid nitrogen (LN) for at least one hour.

Thawing and unloading treatment

The frozen vitrified buds were thawed rapidly by placing the cryovials in water bath at ±38℃ for 5 min with vigorous shaking. PVS_2 was replaced with unloading solution (US) (MS + 1.2M sucrose) and held for 20 min at ±25℃. Unloading solution was drained out with the help of a micropipette.

Plating and recovery growth

The buds were scooped out from the cryovial with the help of sterile forceps and were blotted on sterile filter paper. Vitrified buds were then cultured on semisolid recovery growth medium. The test tubes were sealed with parafilm and incubated under culture room conditions in dark for 7 days. After 7 days, test tubes were transferred to diffused light for 3 days and after ten days all test tubes were exposed to full light intensity in culture room.

Encapsulation-dehydration

Descaled buds (0.4-0.6 mm long) precultured in semisolid preculture medium for encapsulation (PME-MS + 0.7% agar) overnight were suspended in Ca^{++} free liquid medium supplemented with autoclaved 3% (w/v) sodium alginate in a screw cap vial. The suspended buds were dispensed drop-wise with a 1 mL micro pipette (Eppendorf) using broad mouth 1 mL tips in liquid MS medium supplemented with 100 mM $CaCl_2$ in a 250 mL sterile beaker to form beads (calcium-alginate beads). The beads, each containing one explant were held in the $CaCl_2$ solution for 20 min to polymerize properly.

Preculture and dehydration

The beads measuring 4-5 mm in diameter were removed from the $CaCl_2$ solution and precultured in liquid MS medium supplemented with various concentrations of sucrose (0.3, 0.5, 0.75 and 1.0 M) at an approximate volume of 50 mL in a 250 mL conical flask for 48 h while placed on rotary shaker at room temperature on 100 rpm. Following preculture, the beads were taken out and the superficial moisture was wiped off on a sterile filter paper. The beads were desiccated for 4, 5 and 6 h by placing on sterile filter paper under sterile air of a laminar airflow cabinet. After various preculture and dehydration treatments, the moisture content of the beads was measured on a fresh weight basis[22].

Freezing and thawing

Encapsulated and dehydrated buds (10 beads/ treatment) were transferred to autoclaved 1.8 mL cryovials and immersed directly into liquid nitrogen. The frozen encapsulated buds were thawed slowly by placing the cryovials at room temperature for 15 min in laminar air flow.

Recovery growth

The beads were transferred on to semisolid recovery growth medium and incubated at 25℃ in the dark for 7 days. After dark incubation these cultures were shifted in diffused light for 3 days. After 10 days of culturing, these cultures were exposed to normal culture room light intensity (3000 lux/36 μ mol^{-1} s^2).

The statistical analysis of the data was carried out using Duncan's multiple range test (DMRT) at the $P \leq 0.05$ level of probability to test the differences between the means using SPSS software.All the data on survival and regeneration (%) were analysed using one-way analysis of variance (ANOVA) in SPSS software for Windows (Release 15.0; SPSS Inc., Chicago, IL, USA) to measure the effect of various treatment of different cryotechniques.

Results and Discussion

Cryopreservation of dormant buds of mulberry by two-step freezing

The initial moisture content of fresh buds was 58.32%. After desiccation on charged silica gel for 6 h, the moisture content was reduced to 15.67-25.08%

which was optimal MC for cryopreservation. At this range of critical MC, buds survived after cryopreservation in most of the accessions. Non-frozen and cryopreserved buds after two-step freezing clearly showed red colour. However, those buds cryopreserved after dehydration followed by two-step freezing showed maximum viability as compared to direct freezing. The distinction between viable and non-viable tissues was clear. Viable tissues had a bright red colour, while the dead tissues were greenish or dark brown due to oxidation process (Fig. 1). Hence, TTC method showed a rough and quick estimate of the viability of fresh and cryostored germplasm. In cryopreservation technique, TTC can be applied for quick screening of protocols that are probably the most efficient. The colourless triphenyl tetrazolium chloride is infiltrated in the explants and then reduced by the enzymes in living plant cells (dehydrogenases) to yield triphenylformazan (reduced TTC)[24]. In our study, the brown colour caused by oxidation (freezing damage) sometimes looks similar like the red-coloured reduced TTC.

Buds of accessions having higher moisture did not show recovery irrespective of the method of freezing employed. It was considered essential to modify the culture conditions such as rehydration of dormant buds after cryopreservation and incubation under reduced light conditions to get good survival[16]. These optimized conditions gave fruitful recovery results in my study after cryopreservation following by two-step freezing protocol of dormant buds. In this study, the accessions having moisture content above 24% failed to show growth. Ice crystallization occurring due to high water content is detrimental[9]. The data generated on recovery of cryopreserved dormant buds of *M. laevigata* showed high growth values when predesiccated to moisture contents between 15 to 25%. *M. laevigata* showed desiccation sensitivity and were not easily amenable for high desiccation and freezing[16,17,24].

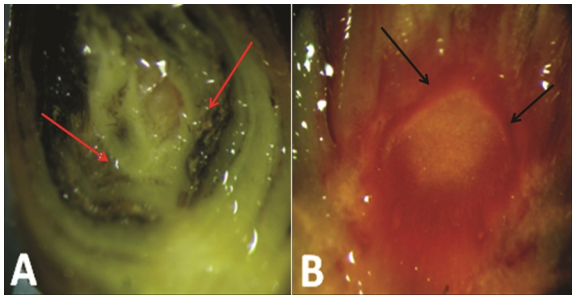

Fig. 1—Viability testing using TTC method of dormant buds of *M. laevigata* (A) non-viable showing green colour; and (B) viable buds showing red colour. [Arrows showing viable and non-viable buds]

Viability determination through *in vitro* culturing

The regeneration after cryopreservation followed by two-step freezing was tested through *in vitro* culturing. The viability of fresh buds was found up to 80%. The viability of desiccated buds before cryopreservation was 40% and after cryopreservation it was 33%. The plantlets obtained *in vitro* from cryostored dormant buds showed direct regrowth without any intervening callus (Fig. 2). Initiation of growth was apparent within 15-20 days of *invitro* inoculation. Within 25-30 days, the roots initiated attaining a length of 3-6 cm by 45-60 days. After 64 days of regrowth, all healthy plantlets with fully grown roots and fully opened leaves were transferred to soilrite (Fig. 2).

Vitrification

Viable dormant buds remained green after thawing and plating on recovery growth medium and initiated growth within 3-4 weeks. The surviving dormant buds showed green colouration as an indication of

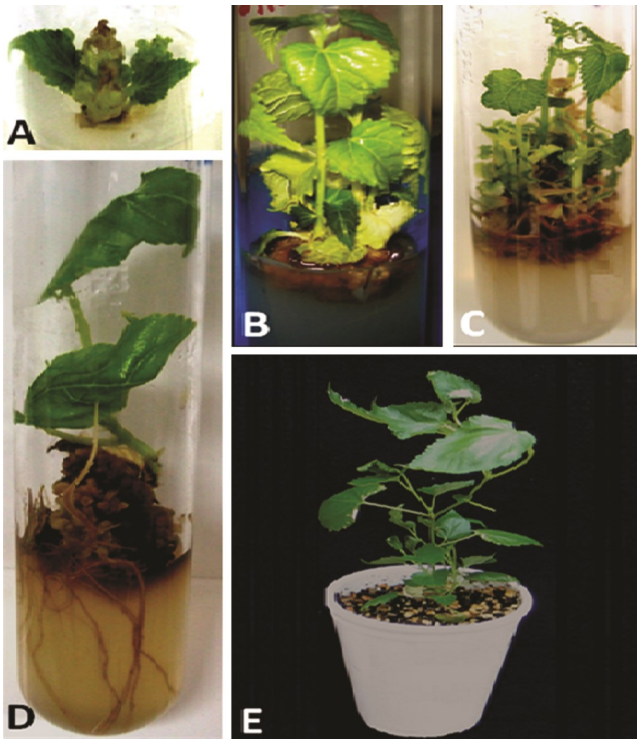

Fig. 2—Recovery of cryostored mulberry dormant buds of *M. laevigata* after two-step freezing (A) shoot initiation after 15 days of culturing; (B) elongated shoots; (C) clonal multiplication; (D) healthy rooted plantlets after 60 days; and (E) healthy plantlet in pot raised from buds subjected followed by two-step freezing.

survival. After 3 weeks of culturing, the thawed buds regenerated directly without intermediary callus phase.

Effect of PVS_2 treatment on survival and regeneration of dormant buds

Dormant budsexposed to PVS_2 for 20 min at 25℃, survival and regeneration frequency was 28.33 and 16.67%, respectively. However, exposure of 120 min showed a survival and regeneration frequency 31.67 and 16.67%, respectively. PVS_2 exposure at 25℃ for 90 min produced maximum frequency of survival and regeneration (58.89 and 50%, respectively) (Fig. 3 and Table 1).

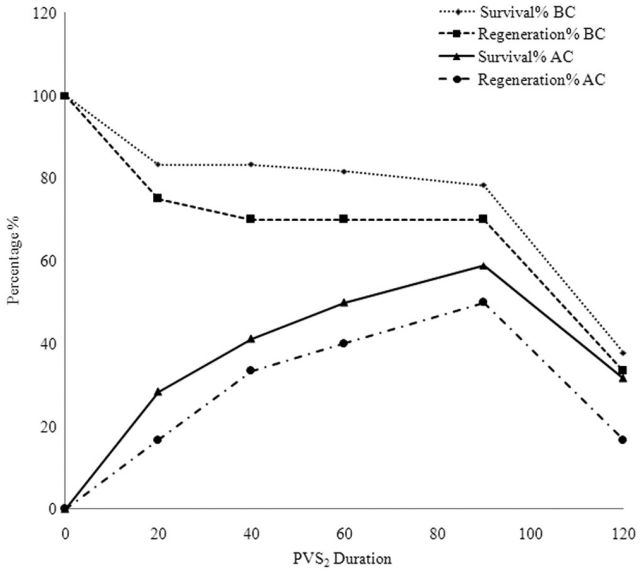

Fig. 3—Effect of duration of PVS_2 on survival and regeneration percentage.

Recovery growth

The buds sprouted on recovery growth medium (MS+ 1.0 mgL^{-1} BAP) (Fig. 4A) from vitrified dormant buds (LN exposed). These sprouts elongated on medium (MS + 1.0 mgL^{-1} BAP + 0.2 mgL^{-1} GA_3) (Fig. 4B). Out of these 88% cultures proliferated and gave multiple shoot on shoot multiplication medium (MS + 0.5 mgL^{-1} BAP + 0.5 mgL^{-1} Kn + 0.1 mgL^{-1} IAA) (Fig. 4C), while the rest turned black. The

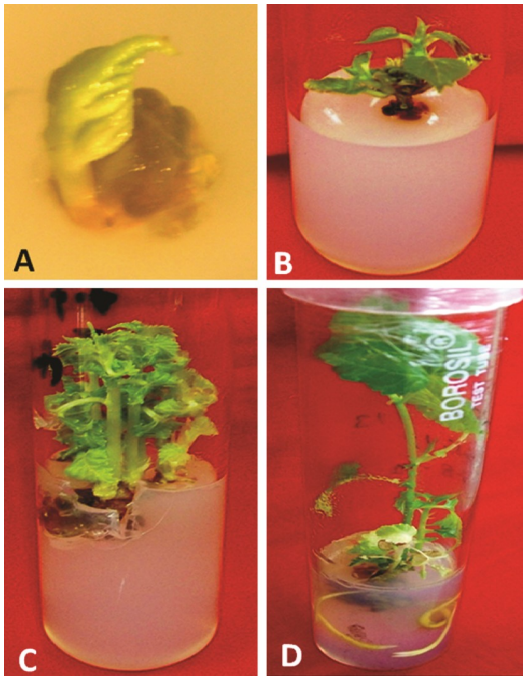

Fig. 4—Recovery growth in (MS+1mgL^{-1}BAP) of dormant buds after cryopreservation using vitrification technique, (A) sprouted dormant budafter 3 week, (B) elongated bud, (C) multiple shoots after 5 weeks of culturing and (D) fully grown rooted plantlets after 70 days.

Table 1—Effect of duration of PVS_2 treatment at 25°C on survival and regeneration of dormant buds with or without freezing to –196°C by vitrification in *Morus laevigata*.

Treatments*	Before Cryo [-LN]		After Cryo [+LN]	
	Survival** (%)(± SE)	Regeneration** (%)(± SE)	Survival** (%)(± SE)	Regeneration** (%)(± SE)
PCM	100 (± 0.00)^{a}	100 (± 0.00)^{a}	0.00 (± 0.00)	0.00 (± 0.00)
LS	96.67 (± 2.72)^{ab}	90.00 (± 2.36)^{b}	26.11 (± 2.36)^{d}	0.00 (± 0.00)
PVS_2-20	83.33 (± 5.44)^{b}	75.00 (± 2.36)^{c}	28.33(± 2.72)^{cd}	16.67 (± 4.71)^{d}
PVS_2-40	83.33 (± 5.44)^{b}	70.00 (± 4.71)^{c}	41.11 (± 4.71)^{b}	33.33 (± 2.72)^{bc}
PVS_2-60	81.67 (± 3.60)^{b}	70.00 (± 4.71)^{c}	50.00 (± 4.71)^{ab}	40.00 (± 4.71)^{b}
PVS_2-90	78.33 (± 2.36)^{bc}	70.00 (± 4.71)^{c}	58.89 (± 2.36)^{a}	50.00 (± 4.71)^{a}
PVS_2-120	37.78 (± 2.36)^{d}	33.33 (± 2.72)^{d}	31.67 (± 2.72)^{c}	16.67 (± 4.71)^{d}

[*All the dormant buds were pre-cultured in pre-culture medium for vitrification (PMV) (0.3 M Sucrose + 2 M Glycerol + 0.7% Agar in MS) and loaded with Loading solution (LS) (0.4 M Sucrose and 2 M Glycerol in MS) for 20 min, followed by dehydration with PVS2 at 25˚C for the different durations followed by freezing (+LN). After freezing in LN the dormant buds were unloaded with Unloading solution (US) (1.2 M Sucrose in MS) for 25 min at 25˚C. The dormant buds in treated control (-LN) were subjected to all treatment except freezing in LN. **All the values are the average of three replicates, ± standard error (SE) and the experiment was repeated thrice, data recorded after 4 weeks of culturing]

healthy rooted plants were obtained after 64 to 70 days of culturing (Fig. 4D). No morphological abnormalities were observed.

In the vitrification, survival generally was dependent on the duration of PVS_2 treatment. Exposure to vitrification solution for duration longer than the optimized time resulted in toxicity and injury prior to freezing. Although without PVS_2 dehydration no survival was observed after cryopreservation. In this study, post-thaw survival frequency was highest when the dormant buds were treated with PVS_2 for 90 min at 25℃. Similarly, high survival after freezing have been reported for cells, meristems of other plant species treated with chilled PVS_2[19,25,26]. In our investigations, loading treatment was effective after preculture of excised dormant buds to improve post-LN survival rates.

Encapsulation-dehydration

Maximum survival and regeneration frequency (75% and 66.67%, respectively) was obtained after 5 h desiccation when frozen encapsulated dormant buds precultured with 0.75 M sucrose solution for 48 h and minimum survival and regeneration frequency (45 and 25%, respectively) was found with 1.0 M sucrose solution for 48 h. The survival and regeneration frequency gradually decreased after 5 h of desiccation (Fig. 5). Similarly, with 0.3M sucrose, the maximum survival and regeneration frequency was 60 and 40%, respectively. Whereas, with 0.5 M sucrose, the maximum survival and regeneration frequency was 70 and 60%, respectively after 5 h of desiccation. Similar results were obtained with encapsulated dormant buds before cryopreservation. Highest regeneration frequency (80%) was found when encapsulated dormant buds were precultured with 0.75M sucrose followed by 5 h desiccation before cryopreservation which is comparable to regeneration frequency obtained from cryopreserved buds after the same treatment (66.67%) (Table 2).

Moisture content (MC) of the beads proved very significant in determining the fate of the frozen dormant buds after a particular treatment. MC of the beads reduced with increasing the desiccation duration (0-6 h) after preculture with 0.30, 0.50, 0.75 and 1.00 M sucrose. The survival and regeneration frequency decreased beyond 5 h of desiccation. The results indicate that very high (>25%) or very low (<18%) moisture content was detrimental for recovery after freezing and a critical moisture content produced optimum survival and regeneration after cryopreservation. A combination of sucrose preculture along with air desiccation resulting in moisture content of 18-25% produced appreciably high survival and regeneration frequency (Table 2).

Recovery growth

The buds sprouted on recovery growth medium (MS+ 1.0 mgL^{-1} BAP) from successfully encapsulated dormant buds (cryopreserved) and elongated on medium (MS + 1.0 mgL^{-1} BAP + 0.2 mgL^{-1} GA_3) after 6 weeks of culture (Fig. 6). No morphological abnormalities were observed.

In the encapsulation-dehydration technique, sucrose pretreatment has been reported to play a major role in the tolerance of apices to dehydration and further freezing in liquid nitrogen. Sucrose not only induces osmotic dehydration but is also absorbed by the cells, thus increasing the ability of intracellular solutes to vitrify and also by stabilizing membranes and proteins[27,28]. In some cases it has been reported that extraction of apices from the beads was necessary

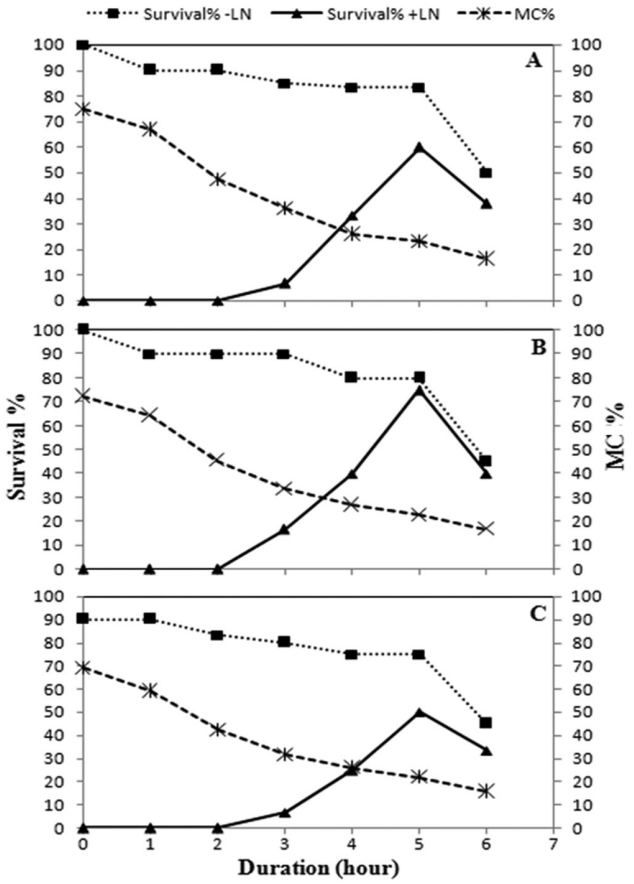

Fig. 5—Effect of sucrose concentrations (0.75 M) after (A) 24; (B) 48; and (C) 72 h pretreatment and of duration of desiccation on survival of encapsulated dormant buds of *M. laevigata* without (-LN) and with (+LN) cryopreservation.

Table 2—Effect of different sucrose concentrations after 48 hours pretreatment and of duration of desiccation on survival and regeneration of encapsulated dormant buds of *Morus laevigata* without [-LN] and with [+LN] cryopreservation

Sucrose conc.*	Desiccation duration (hours) [-LN]					
	4		5		6	
	Survival** (%) (± SE)	Regeneration** (%) (± SE)	Survival** (%) (± SE)	Regeneration** (%) (± SE)	Survival** (%) (± SE)	Regeneration** (%) (± SE)
0.30M	83.33 (± 5.44)^{a}	60.00 (± 4.71)^{b}	83.33 (± 5.44)^{a}	70.00 (± 4.71)^{b}	40.00 (± 4.71)^{c}	33.33 (± 2.72)^{c}
0.50M	83.33 (± 5.44)^{a}	75.00 (± 4.71)^{a}	80.00 (± 4.71)^{a}	75.00 (± 4.71)^{ab}	50.00 (± 4.71)^{b}	40.00 (± 4.71)^{bc}
0.75M	80.00 (± 4.71)^{a}	75.00 (± 4.71)^{a}	80.00 (± 4.71)^{a}	80.00 (± 4.71)^{a}	60.00 (± 4.71)^{a}	50.00 (± 4.71)^{a}
1.00M	70.00 (± 4.71)^{b}	60.00 (± 4.71)^{b}	66.67 (± 2.72)^{b}	60.00 (± 4.71)^{c}	40.00 (± 4.71)^{c}	33.33 (± 2.72)^{c}
	After Cryo [+LN]					
0.30M	16.67 (± 5.44)^{c}	6.67 (± 3.60)^{d}	60.00 (± 4.71)^{b}	40.00 (± 4.71)^{b}	20.00 (± 4.71)^{c}	8.33 (± 2.72)^{b}
MC*** (%)	29.64 (± 0.75)		25.94 (± 0.89)		18.62 (± 0.43)	
0.50M	25.00 (± 2.36)^{bc}	20.00 (± 4.71)^{bc}	70.00 (± 4.71)^{ab}	60.00 (± 4.71)^{a}	33.33 (± 2.72)^{bc}	30.00 (± 2.72)^{a}
MC*** (%)	27.79 (± 0.89)		23.69 (± 0.60)		17.03 (± 0.66)	
0.75M	40.00 (± 4.71)^{a}	33.33 (± 2.72)^{a}	75.00 (± 4.71)^{a}	66.67 (± 2.72)^{a}	40.00 (± 4.71)^{a}	33.33 (± 2.72)^{a}
MC*** (%)	26.98 (± 0.32)		22.60 (± 0.23)		16.78 (± 0.36)	
1.00M	33.33 (± 2.72)^{b}	16.67 (± 5.44)^{c}	45.00 (± 4.71)^{c}	25.00 (± 2.36)^{c}	20.00 (± 4.71)^{c}	10.00 (± 4.71)^{b}
MC*** (%)	24.19 (± 0.56)		19.16 (± 0.75)		14.68 (± 0.14)	

[*The encapsulated dormant buds were pretreated with the above listed sucrose solutions for 48 hours and dehydrated for 4-6 h in laminar airflow and frozen in LN. **All the values are the average of three replicates, ± standard deviation and the experiment was repeated thrice, data recorded after 6 weeks of culturing. ***Values in the parenthesis are moisture content percentage (MC %) of the beads after air desiccation in laminar airflow]

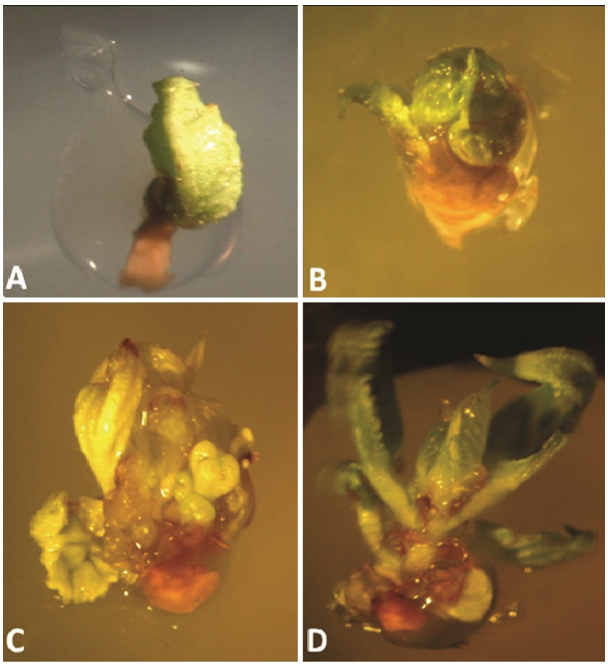

Fig. 6—Recovery growth of cryopreserved dormant buds using encapsulation-dehydration technique, (A) after 3 weeks on recovery growth medium; (B) after 4 weeks; (C) after 5 weeks; and (D) 6 weeks of plating.

for good growth[28,29] but in my study the beads provided no hindrance in the growth of the shoots. Pretreatment with high sucrose solution was necessary to induce tolerance to desiccation and cryopreservation. Buds appeared tolerant to high levels of sucrose (0.75M) as also reported for apices of grape, sugarcane, potato, coffee[28-30] as well as for white mulberry[19].

The results indicate that when the dehydration duration was lower than the optimal requirement, the treated control showed higher rate of survival and regeneration compared to that of dormant buds cooled to −196℃ as observed in vitrification. Alginate-encapsulated buds resumed growth slowly as compared to nonencapsulated ones. The encapsulated dormant buds pretreated in liquid MS medium supplemented with different sucrose concentrations showed difference in recovery response after freezing. Dehydration for less or more than 5 h showed lower in survival rates. Hence, 5 h was the optimum desiccation duration to obtain high survival and regeneration frequency when bead moisture contents were between 20 and 24%.

Conclusion

In this study, cryopreservation protocols were carried out, to conserve dormant buds of mulberry germplasm, employing two-step freezing, vitrification and encapsulation-dehydration techniques. All three cryotechniques i.e. two-step freezing, vitrification and encapsulation-dehydration, were effective for

cryopreservation. Two-step freezing was generally used for most of the accessions of mulberry for long term conservation. This method is simple and cost effective. After step-wise freezing, high viability, as good as controls was observed. Vitrification also produced marginally higher regeneration frequency as compared to encapsulation-dehydration techniques. This might be due to the fact that the cryoprotectant is in direct contact of the explants and hence, produced effective dehydration to minimize cryo injury during freezing. However, in the case of encapsulation-dehydration, the explants were encapsulated to provide protection from air dehydration and perhaps the dehydration could be obtained to an extent that would minimize injury during freezing. The growth was slow in encapsulation-dehydration because the explants had to initiate growth to break open the bead and grow out after cryopreservation. Vitrification involves toxic cryoprotectants and precise timing of treatment encapsulation-dehydration. However, it is time consuming but safe in the event of accidental thawing. As all the techniques have their own advantages and disadvantages, any of these three techniques can be used for cryopreservation. However, to be precise, vitrification and encapsulation techniques can be used for those species which have low survival after cryopreservation followed by two-step freezing. Overall, in this present study, the highest frequency of explant regrowth was obtained when explants were subjected to encapsulation-dehydration.

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