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Long-term conservation of dormant buds of *Prunus dulcis* (Miller) D.A. Webb. using three different new cryotechniques

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Abstract

Winter dormant buds of Prunus dulcis were cryopreserved using either two-step freezing, encapsulation-dehydration or vitrification. A two-step freezing cryoprotocol preceded by desiccation to 18 to 28% moisture content was developed. Recovery conditions, including dark incubation and rehydration in sterile moist moss grass for different durations, led to higher survival. For encapsulation-dehydration, alginate beads containing descaled bud 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. Treatment of alginate beads with 0.75 M sucrose was more effective in promoting re-growth of explants after immersion in liquid nitrogen. For vitrification, descaled buds were directly immersed for 20, 40, 60, 90 or 120 min in a vitrification solution (PVS_2). Re-growth of explants was also observed following vitrification and this reached 50% with increasing duration of the PVS_2 treatment from 20 to 90 min. Overall, the highest frequency of explant re-growth was obtained when explants were subjected to two step freezing followed by encapsulation-dehydration in the presence of 0.75 M along with 24 h sucrose dehydration pre-treatment and followed by desiccation for 5 h in laminar air flow.

Keywords: almond, dormant buds, alginate beads, cryopreservation, PVS_2, re-growth

Introduction

Plant biodiversity is essential for classical and modern plant breeding programmes and provides a source of compounds to the pharmaceutical, food and crop protection industries. Preservation in field collections is risky, as valuable germplasm can be lost because of pests, diseases, adverse weather conditions and 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) is now a suitable option for long-term storage [2, 3, 4].
Almond (*Prunus dulcis* (Miller) D.A. Webb., of family *Rosaceae*, is a large genus of deciduous or evergreen trees and shrubs, distributed chiefly in the temperate regions of the Northern hemisphere and is valued for its edible fruits or seeds or for ornamental flowers. The

cultivated sweet almond is diploid and now classified as *P. dulcis* (Miller) D.A. Webb. with *P. amygdalus* as a synonym. With its origin traced to Central to South Western Asia [5]. It is one of the most important nut crops of temperate hilly regions of India, commercially grown in Jammu and Kashmir, Himachal Pradesh and Uttarakhand [6]. It is important to conserve this vast genetically unique and important diversity for long-term using cost effective methods. In the Himalayas especially in the Khasi and Jaintia hills, almonds are being used locally as rootstocks for commercial stone fruits. Rich diversity of seedling populations which are highly variable is found in Kashmir (J&K) and Himachal Pradesh. Several cultivars developed in India and also those introduced as exotic collections e.g Prianyaj and Primorskij from Russia, White Brandis from Australia and others like Merced, Nonpareil, California, Paper Shell, Ne Plus Ultra now present good choice to farmers for improved cultivation. Almond is cultivated on an area of 21,300 ha with an annual production of 15,620 mt. and the productivity is 0.73 t/ha [7].

Tissue culture with its distinct advantages is used for short-term preservation [8] but it does not serve for long-term preservation. Hence, cryopreservation only economically viable method is adopted for long-term preservation. Thus, cryopreservation ensures genetic stability of the almond germplasm besides requiring only limited space and protecting material from contamination. *Prunus* seeds have been reported to show poor germination due to low seed viability, seed dormancy and pathogens. They are desiccation tolerant but do not store well at subzero temperature and have short life. Based on this seed storage behavior, seed conservation is difficult due to several factors [9]. The accessions which are not responding to dehydration and slow freezing protocols have cryopreserved based on the new cryopreservation techniques like encapsulation-dehydration, vitrification, encapsulation-vitrification, pregrowth dehydration, droplet freezing methods [10, 11, 12, 13, 14]. The encapsulation-dehydration procedure, originally described for cryopreservation of Solanum shoot tips [15, 16]. Shoot tips of almond have been cryopreserved using vitrification technique [17, 18, 19] and encapsulation dehydration and vitrification [20]. There are no reports so far for cryopreservation of dormant buds using two-step freezing and other cryotechniques.

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

Materials and Methods

Winter dormant buds of *Prunus dulcis* (Miller) D.A. Webb 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^{o}C temperature and used up for experimentation within 25 days of harvest.

Two-step freezing

Determination of Moisture Content [21]

The moisture content (MC %) of fresh and desiccated dormant buds was determined by drying at 103±2^{o}C in an oven for 17 hours. About five dormant buds were taken randomly in duplicates and moisture content calculated using the following formula:

$$MC\ (\%) = \frac{B - C}{B - A} \times 100 \qquad MC\ (\%) = \frac{\text{Loss of weight}}{\text{Initial weight}} \times 100$$

Two-step freezing

Nodal explants (2.0-2.5cm long, one dormant bud attached on nodal segment) were cut with the help of sketier carefully and kept in steel container in charged silica gel at -10^{o}C for 4-5 days for desiccation. These desiccated buds were then used for cryopreservation in liquid nitrogen at -196^{o}C using two-step freezing protocol [10]. The two-step freezing was achieved by sequentially lowering the temperature at -5^{o}C/day up to terminal temperature of -30^{o}C before plunging in liquid nitrogen at -196^{o}C. The desiccated buds were sealed in heat shrinkable tubes with birch plugs in both ends. These tubes were shifted sequentially at 5^{o}C, -5^{o}C, -10^{o}C, -15^{o}C, -20^{o}C and -25^{o}C, and -30^{o}C keeping at each of the temperature for a minimum of 48 hrs. The tubes were held at -30^{o}C for 72 hrs and then directly plunged in the liquid nitrogen at -196^{o}C.

Thawing and rehydration

Cryopreserved nodal sections were thawed by slow thawing. These nodal sections were placed at 5^{o}C for 48 hour for thawing. Nodal sections were rehydrated in sterile moist peat vermiculite for 7 days at 5^{o}C. The viability of fresh and cryopreserved dormant buds of almond 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

The buds were excised from the rehydrated nodal sections and 4 to 5 outer scales were removed using microscope. The explants were washed with Tween 20 for 15 minutes. Tween 20 was rinsed off with running tap water. These buds were then surface sterilized with 0.1% mercuric chloride for 9 minutes, rinsed three times with autoclaved distilled water (5 min each). The sterilized buds were cultured on WPM medium [22] with 2% sucrose (w/v) solidified with 0.6% agar. WPM medium was supplemented with 1 mgl^{-1} BAP for bud sprouting. The cultures raised from cryopreserved buds were maintained in culture room in dark for 7 days followed by 3 days in diffused light. After 10 days of culturing, these cultures were exposed to normal culture room light intensity (3000 lux/ 36 µ mol $^{-1}$ sec^{2}). The sprouted buds were sub-cultured on the fresh WPM medium supplemented with 1.0 mgl^{-1} BAP for elongation. The elongated plants were further sub-cultured and transferred to multiplication medium (MS [23] + 0.5 mgl^{-1} BAP) and finally transferred to rooting medium (half MS + 0.1mgl^{-1} IBA).

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 pre-culture 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 petri dishes. These buds were then incubated overnight at culture room.

Osmotic Loading treatment

The pre-cultured 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^{o}C temperature 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 minutes 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°C 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°C.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) pre-cultured in semisolid pre-culture 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 100mM $CaCl_2$ in a 250 ml sterile beaker to form beads (calcium-alginate beads). The beads, each containing 1 explant were held in the $CaCl_2$ solution for 20 min to polymerize properly.

Pre-culture and dehydration

The beads measuring 4 to 5 mm in diameter were removed from the $CaCl_2$ solution and pre-cultured in liquid MS medium supplemented with various concentrations of sucrose (0.3, 0.5, 0.75, 1.0M) at an approximate volume of 50 ml in a 250 ml conical flask for 48 hours while placed on rotary shaker at room temperature on 100 rpm. Following pre-culture, 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 hours 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 **[21]**.

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°C in the dark for 7 days. After dark incubation these cultures were shifted in diffused light for 3 days. After 10 days of culture these cultures were exposed to normal culture room light intensity (3000 lux/ 36 µ mol $^{-1}$ sec^2).

Results

Cryopreservation of dormant buds of almond by Two-step freezing

Moisture content (MC)

The MC of fresh buds ranged from 36.59% to 53.01%. More than seventy five percent of the cultivars showed MC above 50%. Highest MC (53.01%) was found in Makhdoom cultivar, while lowest was 36.59% in Merced. After desiccation on charged silica gel the moisture content got reduced within the range of 20.18 to 27.43% which was optimal MC for cryopreservation of dormant buds of almond cultivars (Table 1).

Table 1 Viability percentage and moisture content of different cultivars of almond through TTC and *in vitro* culturing before and after cryopreservation following by Two-step freezing

Cultivar Name	Moisture content (%)		Viability (%)				
			by TTC method		by *In vitro* Method		
	Fresh	After desiccation	Fresh	After Cryo	Fresh	After desiccation	After Cryo
California Paper shell	50.08 (±0.97)	25.17 (±0.65)	80.00 (±4.71)	33.33 (±2.72)	75.00 (± 2.36)	0.00 (± 0.00)	0.00 (± 0.00)
Almond-IXL	48.01 (±0.83)	24.91 (±0.49)	90.00 (± 2.72)	40.00 (± 2.36)	83.33 (± 4.71)	40.00 (± 2.36)	16.67 (± 2.72)
Makhdoom	53.01 (±0.92)	26.20 (±0.29)	100.00 (±0.00)	33.33 (±2.72)	90.00 (± 2.72)	40.00 (± 2.36)	0.00 (± 0.00)
Merced	36.59 (±0.44)	25.99 (±0.28)	100.00 (±0.00)	50.00 (± 4.71)	100.00 (± 0.00)	50.00 (± 4.71)	33.33 (± 2.72)
NonPareil	48.78 (±0.83)	22.19 (±0.35)	50.00 (± 4.71)	20.00 (±4.71)	37.50 (± 2.36)	33.33 (± 2.72)	0.00 (± 0.00)
Pranyaj	50.31 (±0.96)	27.43 (±0.77)	100.00 (±0.00)	50.00 (± 4.71)	71.42 (± 4.71)	40.00 (± 2.36)	25.00 (± 2.36)
Primorskij	52.10 (±0.98)	20.18 (±0.36)	100.00 (±0.00)	33.33 (±2.72)	80.00 (± 4.71)	50.00 (± 4.71)	20.00 (± 4.71)
Shalimar	52.85 (±0.47)	23.95 (±0.43)	60.00 (±2.72)	25.00 (± 2.36)	50.00 (± 4.71)	33.33 (± 2.72)	0.00 (± 0.00)
Waris	40.30 (±0.57)	26.03 (±0.28)	60.00 (±2.72)	40.00 (±2.36)	42.85 (± 5.60)	33.33 (± 2.72)	16.67 (± 2.72)

Viability determination after TTC method

The viability of fresh buds through TTC test was found to ranged from 50 to 100%. Highest viability (100%) was obtained in Makhdoom, Merced, Pranyaj and Primorskij and lowest (50%) was found in NonPareil. The viability of cryopreserved buds ranged from 20 to 50%. Maximum viability (50%) was found in Merced and Pranyaj and minimum (20%) was observed in NonPareil (Table 1). Viable tissues had a bright red colour, while the dead tissues were greenish or dark brown due to oxidation process (Fig. 1).

Figure 1. Viability determination of dormant buds of almond using TTC method [A] Non-viable showing green colour and [B] Viable showing red colour

Recovery after *in vitro* culturing followed by two-step freezing

The regeneration after cryopreservation followed by two-step freezing was tested through *in vitro* culturing (Table 1). The viability of fresh buds ranged from 37.50 to 100%. Highest viability (100%) was obtained Merced and lowest (37.50%) was found in NonPareil. The viability of desiccated buds before cryopreservation ranged from 33.33 to 50%. Highest viability (50%) was obtained in Merced and Primorskij and lowest (33.33%) was found in NonPareil, Shalimar and Waris after desiccation. California Paper Shell buds were non-viable. The recovery percentage of cryopreserved buds ranged from 16.67 to 33.33%. Maximum viability (33.33%) was found in Merced and minimum (16.67%) was observed in Almond-IXL and Waris. Cultivars California Paper Shell, Makhdoom, NonPareil, and Shalimar were non-viable after cryopreservation (Table 1). The plantlets obtained *in vitro* from cryostored dormant buds showed direct regrowth without any intervening callus. Initiation of growth was apparent within 25 days of *in vitro* inoculation in almost all the cultivars and plantlets elongated after 60 days. Rooting was observed after 100 days in some of the cultivars (Fig. 2 A, B, C and D).

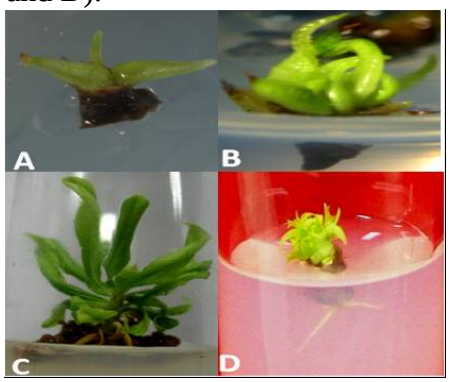

Fig 2. *In vitro* recovery of dormant buds of *P. dulcis* after cryopreservation using 2-step freezing [A] Shoot induction after 25 days, [B] proliferation after 40 days, [C] shoot elongation after 60 days and [F] rooting after 100 days of culturing

Vitrification

Effect of PVS_2 treatment on survival and regeneration of dormant buds

Dormant buds of almond exposed to PVS_2 for 40 min. survival was 6.67%. PVS_2 exposure at 25°C for 90 min. produced maximum frequency of survival and regeneration (16.67% and 10% respectively). However, exposure of 120 min. showed a steep decline in survival 6.67% (Table 2). The buds sprouted on recovery growth medium (WPM + 1.0mgl^{-1} BAP) (Fig. 3A) and elongated after 9 weeks of culture (Fig. 3B and 3C).

Table 2 *P. dulcis* (Merced). 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

Treatments*	Before Cryo [-LN]		After Cryo [+LN]	
	Survival** (%) (± SE)	Regeneration** (%) (± SE)	Survival** (%) (± SE)	Regeneration** (%) (± SE)
PMV	66.67 (± 2.72)	50.00 (± 4.71)	0.00 (± 0.00)	0.00 (± 0.00)
LS	60.00 (± 4.71)	50.00 (± 4.71)	0.00 (± 0.00)	0.00 (± 0.00)
PVS_2-20	60.00 (± 4.71)	45.00 (± 2.72)	0.00 (± 0.00)	0.00 (± 0.00)
PVS_2-40	50.00 (± 4.71)	45.00 (± 2.72)	6.67 (± 3.60)	0.00 (± 0.00)
PVS_2-60	50.00 (± 4.71)	40.00 (± 4.71)	10.00 (± 4.71)	6.67 (± 3.60)
PVS_2-90	50.00 (± 4.71)	40.00 (± 4.71)	16.67 (± 4.71)	10.00 (± 4.71)
PVS_2-120	40.00 (± 4.71)	20.00 (± 4.71)	6.67 (± 3.60)	0.00 (± 0.00)

*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.

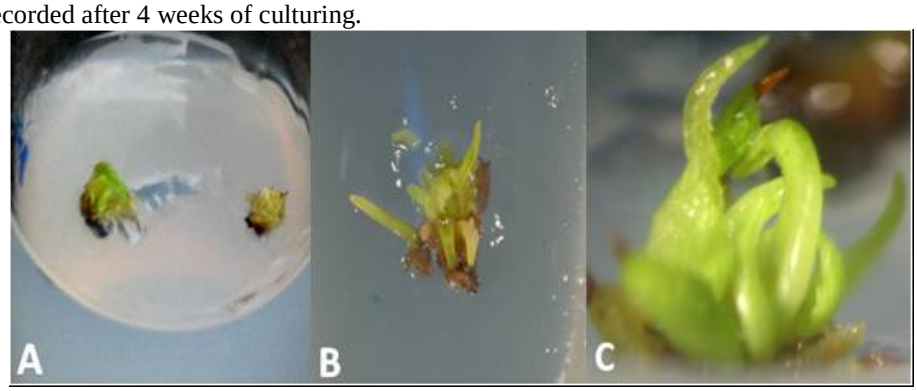

Fig 3 *P. dulcis* (Merced). Recovery growth of dormant buds after cryopreservation using vitrification technique, [A] Sprouted dormant bud after 5 week, [B] elongated bud after 9 weeks and [C] multiple shoots after 10 weeks of culture

Encapsulation-dehydration

Cryopreserved encapsulated dormant buds of almond precultured with 0.75 M sucrose solution for 24 hours produced maximum survival and regeneration frequency (25% and 10%, respectively) after 5 hour desiccation (Fig. 4). The survival and regeneration frequency decreased with increasing dehydration duration after 5 hour. Pre-culture of encapsulation dormant buds with solution of higher sucrose concentration (1.0M) followed by 5 hour dehydration followed by LN exposure produced lower survival (6.67%) in comparison to dormant buds treated with 0.75 M sucrose. Pre-culture with 0.3M sucrose (10%) produced similar results. A similar trend was recorded with non-frozen encapsulated dormant buds (Table 3). MC of the beads reduced with increasing the desiccation duration (0-6 hours) after pre-culture with 0.30, 0.50, 0.75 and 1.00 M sucrose. The results indicate that very high (>28%) or very low (<18%) moisture content was detrimental for recovery after freezing and a critical moisture content produced optimum survival and regeneration after cryopreservation (Table 3). The buds sprouted on recovery growth medium (WPM + 1.0mgl^{-1} BAP) from successfully encapsulated dormant buds (cryopreserved) after 4 weeks of culture (Fig. 4). Regeneration of 6-10% was observed only in frozen encapsulated buds which were subjected to dehydration in sucrose of 0.5 and 0.75M followed by 5h desiccation. However, full plantlets could not be obtained.

Table 3 *P. dulcis* (Merced). Effect of different sucrose concentration after 24 hours pretreatment and duration of desiccation on survival and regeneration of encapsulated dormant buds with [+LN] and without cryopreservation [-LN]

Sucrose conc.*	Desiccation duration (hours) [-LN]					
	4		5		6	
	Survival** (%) (± SE)	Regeneration** (%) (± SE)	Survival** (%) (± SE)	Regeneration** (%) (± SE)	Survival** (%) (± SE)	Regeneration** (%) (± SE)
0.30M	66.67 (± 2.72)	25.00 (± 4.71)	60.00 (± 4.71)	20.00 (± 2.72)	50.00 (± 4.71)	25.00 (± 4.71)
0.50M	60.00 (± 2.72)	25.00 (± 2.72)	50.00 (± 2.72)	33.33 (± 2.72)	40.00 (± 4.71)	25.00 (± 4.71)
0.75M	60.00 (± 2.72)	30.00 (± 4.71)	50.00 (± 4.71)	33.33 (± 2.72)	40.00 (± 2.72)	20.00 (± 2.72)
1.00M	45.00 (± 2.72)	10.00 (± 2.72)	40 (± 2.72)	20.00 (± 4.71)	33.33 (± 2.72)	6.67 (± 4.71)
	[+LN]					
0.30M	0.00 (± 0.00)	0.00 (± 0.00)	10.00 (± 2.72)	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)

MC*** (%)	34.45 (± 0.09)		27.95 (± 0.07)		17.08 (± 0.15)	
0.50M	0.00 (± 0.00)	0.00 (± 0.00)	16.67 (± 4.71)	6.67 (± 4.71)	0.00 (± 0.00)	0.00 (± 0.00)
MC*** (%)	29.55 (± 0.58)		24.99 (± 0.93)		16.02 (± 0.32)	
0.75M	20.00 (± 4.71)	6.67 (± 2.72)	25.00 (± 2.36)	10.00 (± 4.71)	16.67 (± 3.60)	0.00 (± 0.00)
MC*** (%)	27.05 (± 0.21)		22.83 (± 0.68)		17.03 (± 0.18)	
1.00M	0.00 (± 0.00)	0.00 (± 0.00)	6.67 (± 3.6)	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)
MC*** (%)	20.56 (± 0.56)		17.09 (± 0.50)		13.73 (± 0.12)	

*The encapsulated dormant buds were pretreated with the above listed sucrose solutions for 24 hours and dehydrated for 4-6 hours 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.

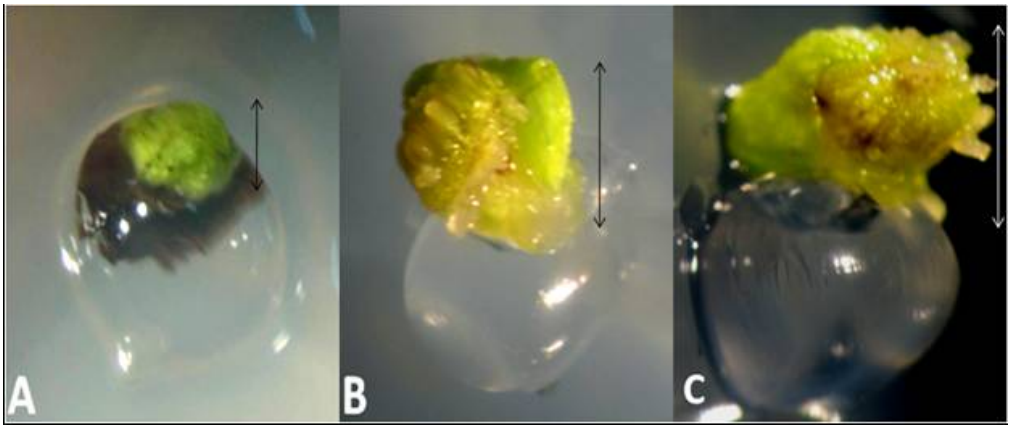

Fig 4. *P. dulcis* (Merced). Recovery growth of cryopreserved dormant buds using encapsulation-dehydration technique [A] Encapsulated dormant bud initiating recovery growth after 4 weeks on recovery growth medium, [B] after 5 weeks and [C] 8 weeks

Discussion

Higher percentage (upto 100%) of viability except in some accessions was obtained through TTC method. Non-frozen buds clearly showed red coloured in all woody species. However, the buds cryopreserved after dehydration followed by two-step freezing showed maximum viability as compared to direct freezing in all the plant species. These results were similar to that reported by some other researchers [24, 25]. Dormant buds having higher moisture content did not show recovery. It is essential to critically dehydrate the tissues to avoid lethal intracellular freezing prior to rapid cooling to produce high level of survival after cryopreservation [26]. In this study, the cultivars having moisture content above 28% were failed to show growth. In the present investigation, culture conditions such as rehydration, dark incubation to get good survival after cryopreservation were slightly modified, which resulted in higher recovery.

In vitrification process, PVS_2 exposure at 25^{o}C for 90 minutes produced maximum frequency of survival and regeneration. Dormant buds exposed to PVS_2 for 20 min and 120 min showed a steep decline in survival and regeneration. Post-thaw survival frequency was highest when

the dormant buds were treated with PVS_2 for 90 min at 25^0C [17, 20, 27, 28, 29, 30]. However, the post-thaw recovery drastically decreased after a long exposure of PVS_2.
Moisture content (MC) of the beads has a very significant role after a particular treatment. A combination of sucrose pre-culture along with air desiccation resulting in moisture content of 18-25% produced appreciably high survival and regeneration frequency. Cryopreserved encapsulated dormant buds of almond precultured with 0.75 M sucrose solution produced maximum survival and regeneration frequency after 5 hour desiccation. The survival and regeneration frequency decreased with increasing dehydration duration after 5 hour. Similar trend was observed by Al-Ababneh et al. [20]. The present study showed regeneration without any intermediary callus phase. Direct regeneration *P. dulcis* is very relevant because it would be true to type, which is an important aspect to be considered when protocol for long-term conservation is developed.

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