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Storage and high conversion frequency of encapsulated protocorm-like bodies of *Cymbidium devonianum* (orchid)

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SUMMARY
A method for the storage and high frequency conversion of *Cymbidium devonianum* protocorm-like bodies (PLBs) is reported. To study the effect of nutrient level on storage, PLBs were encapsulated in calcium alginate beads supplemented with 1.0×, 0.5×, 0.25×, or 0.125× Murashige and Skoog (MS) basal medium containing 0.3% (w/v) sucrose, without agar in the encapsulating matrix, and stored at room temperature (25º ± 2ºC) in the dark. Beads containing 0.25× MS were also kept at different temperatures (0ºC, 4ºC, 8ºC, or room temperature) in the dark to ascertain the optimal temperature for storage. One set of controls (i.e., non-encapsulated PLBs) was maintained for each treatment. The survival and subsequent percentage conversion values of PLBs were assessed at 30 d storage intervals after culturing on MS regeneration medium. Compared to the controls, all encapsulated PLBs showed improved storage at room temperature. In all treatments, non-encapsulated PLBs did not survive, but turned brown and died. Encapsulated PLBs in 0.25× MS medium could be stored for 90 d at room temperature without any significant loss in viability. However, a significant decrease in the survival percentage was recorded after longer storage times. Encapsulated PLBs containing 0.25× medium could be stored at 4ºC and 8ºC for 120 d and 180 d, respectively, without loss of viability. As storage times increased beyond 180 d, the survival percentage of encapsulated PLBs decreased. The conversion of encapsulated PLBs led to the emergence of regenerated plantlets. Initially, small green globular outgrowths from the PLBs were observed on the surface of the beads. These outgrowths multiplied to form clusters of PLBs which then regenerated into plantlets. In both studies, more prolonged storage of encapsulated PLBs increased the time interval for germination and plantlet regeneration on MS regeneration medium. Similarly, decreases in both basal MS strength in the matrix, and in storage temperature, resulted in an increase in the time required for germination and plantlet regeneration. Plantlets that regenerated from stored, encapsulated PLBs were hardened-off, and a high survival percentage (90%) was obtained in a glasshouse.

Cymbidiums are the most popular Spring-flowering garden plants. Their striking sprays of long-lasting blooms provide an ideal home or patio decoration and they occupy a prominent position in the global cut-flower trade. Northeast India is the richest phyto-geographical habitat for members of this genus because of the prevailing climate. *Cymbidium* plants grow satisfactorily under a mild climate; however, threats to biodiversity posed by the destruction of their natural habitats, notably by urbanisation, have led to the extinction of large numbers of *Cymbidium* species. Hence, we report work that would help in the conservation and storage of *C. devonianum* germplasm, a rare and threatened epiphytic orchid species of northeast India.

In vitro micropropagation techniques are useful for the propagation and maintenance of large numbers of threatened plant species (Dhar *et al*., 2000). However, genetic, physiological and/or biochemical (somaclonal) variations have often been observed during serial sub-culturing (Withers, 1991; Chen *et al*., 1998; Khoddamzadeh *et al*., 2010). To reduce such variations, and to save costs and time for the routine maintenance and transport of cultures, germplasm preservation through storage has been suggested (Henshaw, 1975; Ara *et al*., 2000). *In vitro* preservation of germplasm is possible through the encapsulation of protocorm-like bodies (PLBs), with the ultimate aim of producing artificial seed. This has been attempted for the propagation, conservation, and high frequency regeneration of several orchid species (Corrie and Tandon, 1993; Nayak *et al*., 1998; Datta *et al*., 1999; Martin, 2003; Saiprasad and Polisetty, 2003). However, reports on the production and storage of artificial seed of *Cymbidium* spp. are limited (Devi *et al*., 1998). Although there are a few reports on the cryopreservation of *Cymbidium* seed (Hirano *et al*., 2011), the short duration of preservation and generally poor retrieval of regenerative cultures were major drawbacks (Benson, 1999; Chaturvedi *et al*., 2004). Moreover, as for most *in vitro* techniques, the successful implementation of minimal-growth technology required the establishment of specific protocols for each type of explant and species under consideration (Watt *et al*., 2000).

MATERIALS AND METHODS

Well-developed PLBs (90 d-old; 3 - 4 mm in diameter) of *C. devonianum* were obtained from the apical meristems or leaf bases of *in vitro*-grown seedlings. These PLBs were used for the experiments described here after

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three sub-culturings in four different strengths of hormone-free Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing all vitamins, but without agar. All MS media contained 0.3% (w/v) sucrose and were solidified with 4% (w/v) sodium alginate. The pH values of all media were adjusted to 5.8 using 0.1 M NaOH before autoclaving. Encapsulation was carried out by mixing the PLBs in 4% (w/v) sodium alginate in 1.0×, 0.5×, 0.25×, or 0.125× MS medium without calcium nitrate [Ca $(NO_3)_2$ $4H_2O$]. Approx. 1.0 ml of sodium alginate containing a single PLB was dropped, using a small spatula, into 100 ml of 100 mM Ca $(NO_3)_2$ $4H_2O$ dissolved in MS medium (as described above). Firm spherical beads of calcium alginate were obtained after 30 min of gel complexation.

The experimental design included a set of 40 Petri dishes (60 mm × 15 mm) for storage studies with (i) reduced nutrient levels, or (ii) one reduced nutrient-level (0.25× MS) at a reduced temperature. Each study included four different nutrient treatments, each containing ten replicates with 20 encapsulated PLBs per Petri dish. For storage studies at the four different nutrient strengths, all encapsulated PLBs were stored in the dark at room temperature (25º ± 2ºC).

For the study on storage at reduced temperatures, Petri dishes (n = 10) each containing 20 encapsulated PLBs were kept in the dark at 0ºC, 4ºC, 8ºC or at room temperature (RT). One set of control (non-encapsulated PLBs) was maintained for each treatment.

In both studies, Petri dishes from each treatment were sampled at 30 d intervals and the alginate beads were transferred to MS regeneration medium (1.0× MS + vitamins + 3.0% (w/v) sucrose + 0.8% (w/v) agar) and incubated at 25º ± 2ºC with a 16-h photoperiod at a light intensity of 15 µmol m^{-2} s^{-1} provided by cool-white fluorescent tubes. The time taken for initial germination (i.e., emergence of the protocorms from each bead) and the percentage germination were recorded over 10 weeks of culture. At this stage, germinated beads were sub-cultured on fresh MS regeneration medium and the times taken for subsequent regeneration of shoots and roots were recorded.

Each experiment was repeated three-times and comparisons of mean values within each treatment were statistically analysed by Least Significant Difference (LSD) using Duncan's Multiple Range Test (Harter, 1960)

In vitro-grown plantlets, 2.5 – 3.0 cm in height, were removed, together with a small amount of adhering agar, using a long-handled spoon. The agar medium that stuck to the roots was carefully removed using a brush to avoid damage. The plantlets were then transferred to clean "Thermocol" pots (120 mm × 80 mm) containing one of four compost mixures (PM1 – PM4) containing equal (w/w/w) amounts of: (a) charcoal pieces + brick pieces + decaying litter (PM1); (b) charcoal pieces + brick pieces + decaying litter + sawdust (PM2); (c) charcoal pieces + brick pieces + decaying litter + coconut husk (PM3); and (d) charcoal pieces + brick pieces + decaying litter + cow dung (PM4). Each compost mixure was further divided into two types, with or without peat moss on its surface. All pots were covered with pierced polythene bags for 2 – 3 weeks, to maintain a high humidity, and transferred to a glasshouse (80 ± 2.0% RH; 27º ± 2ºC). The plantlets were watered every second day, in the evening, and supplied with 0.1× MS nutrient salt solution every fourth day for approx. 1 month, after which time they were transferred to soil in earthen pots for further hardening and acclimatisation.

RESULTS AND DISCUSSION

Germination of stored, encapsulated PLBs and their subsequent conversion into shoots and roots occurred sequentially on MS regeneration medium. Initially, small green globular protrusions of PLBs were observed on the surface of the beads in all treatments. These protrusions multiplied further to form clusters, followed by leaf initiation and the development of roots (Figure 1 A–D).

The concentration of MS nutrient in the alginate matrix, and the time in storage, affected the survival and regeneration of encapsulated PLBs. A high germination percentage (85%) was recorded for PLBs in 0.25× MS up to 90 d in storage, after which time, the survival and germination percentages declined to 40% at 120 d of storage. The germination of PLBs encapsulated in 1.0× or 0.5× MS was impaired after 60 d of storage, and after 30 d for PLBs encapsulated in 0.125× MS (Table I). This could be due to optimal levels of nutrients for germination being present in the 1.0× and 0.5× MS media. Although studies on slow-growth caused by modifying the culture medium have been reported (Withers, 1987; Negri and Standardi, 2000; Hao and Deng, 2003), studies on reduced nutrient levels in the encapsulating matrix are limited (Maruyama *et al*., 1997; Nassar, 2003).

PLBs were considered to have survived upon emergence of the PLB from the encapsulation matrix on MS regeneration medium. Multiplication of a single PLB to form a cluster of approx. 15 – 20 PLBs per explant was recorded in all those PLBs that survived. Development of a pointed vegetative apex (Figure 1 C) was observed in a cluster of PLBs that subsequently expanded to form green leaves and initiate roots.

The time required for encapsulated PLBs to germinate and produce plantlets at each MS nutrient level differed for the different storage periods (Table I). For each nutrient treatment, the percentage of PLBs that survived declined, and the time taken to produce both leaves and roots increased with increasing storage time. Nassar (2003) reported similar results with encapsulated shoot buds of *Coffea arabica*.

PLBs encapsulated in 0.25× MS and stored at RT, 0ºC, 4ºC, or 8ºC showed prolonged survival at 4ºC or 8ºC without loss of viability (Table II). The maximum storage time for encapsulated PLBs (180 d) was recorded at 8ºC without loss of viability on MS regeneration medium (Table II). However, a significant decrease in the percentage survival of encapsulated PLBs was recorded after 180 d of storage, and only 16.6% survival was recorded after 330 d of storage. The successful regeneration of encapsulated PLBs has been reported in a number of orchids stored at 4ºC (Sharma *et al*., 1992; Kumaria *et al*., 2005; Sarmah *et al*., 2010), but not at 8ºC. The results of the present study indicate that it may be worth investigating 8ºC for the storage of PLBs of other orchid species. Encapsulated microshoots of pineapple stored at 8ºC showed a

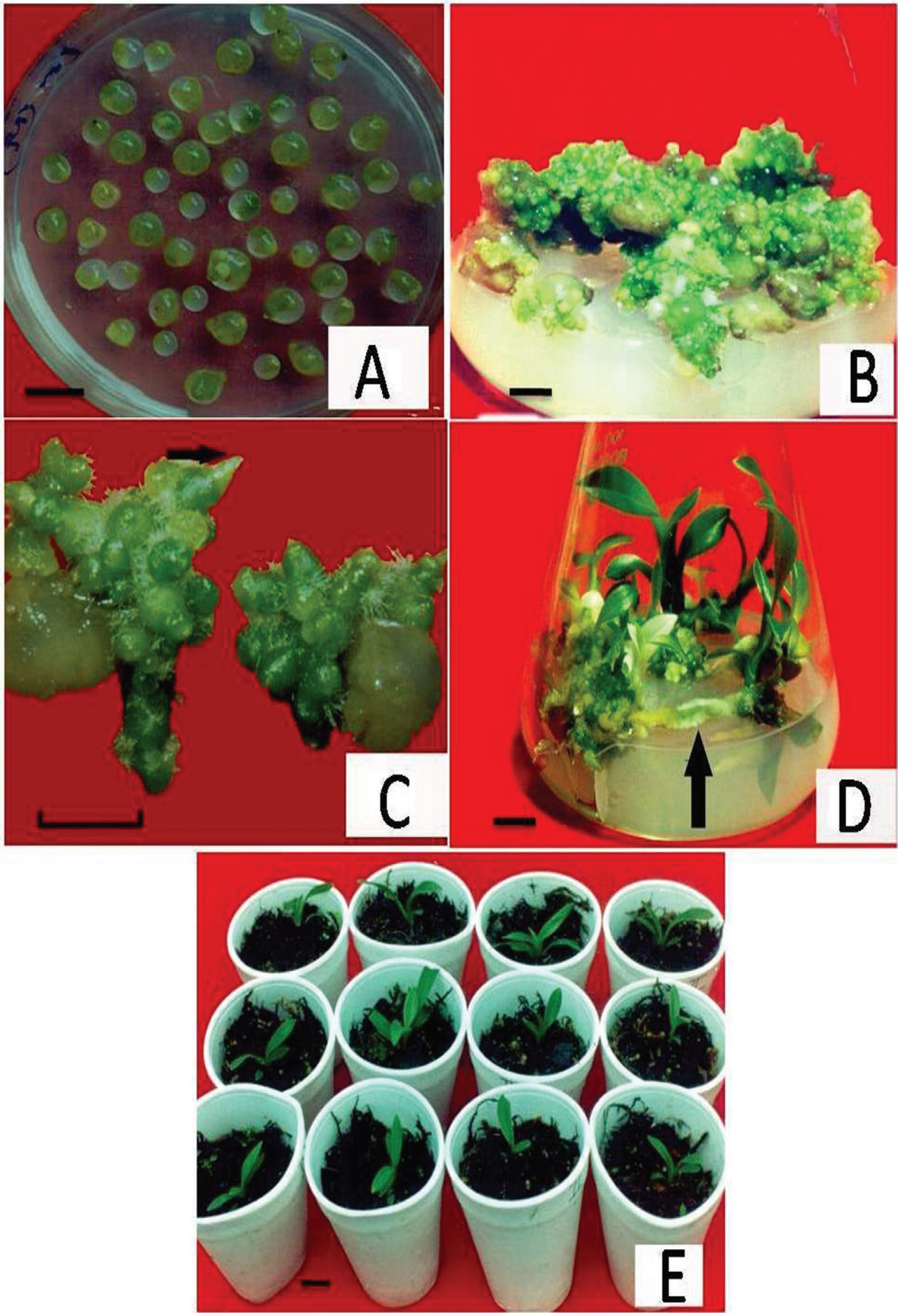

Fig. 1
Stages of development of encapsulated protocorm-like bodies (PLBs) of *C. devonianum* cultured on regeneration medium [1.0× MS nutrients + 3.0% (w/v) sucrose + 0.8% (w/v) agar] after 180 d storage at 8ºC. Panel A, encapsulated PLBs on day-0 of culture. Panel B, protruberances emerging from encapsulated beads after 4 weeks in culture. Panel C, enlarged view of protruding PLBs with a vegetative apex (arrowed). Panel D, developing roots (arrowed) after 8 weeks in culture. Panel E, hardened plantlets after 12 weeks in potting medium (PM1) containing brick + charcoal + decaying litter with a layer of peat moss. Scale bars in Panels A, B, D, E = 10.0 mm and in Panel C = 1.0 mm.

TABLE I
Room temperature storage and regeneration of Ca-alginate-encapsulated protocorm-like bodies (PLBs) of C. devonianum *supplemented with four different levels of MS nutrients in the matrix*

MS basal nutrient medium	Storage period (d)	PLB survival (%)	Time to germination of PLBs (d)	Time to emergence of 1-2 leaves (d)	Time to emergence of 1-2 roots (d)
Full-strength (1.0×)	30	100 ± 0a†	3.6 ± 0.6a	10.6 ± 0.6a	33.3 ± 3.5a
	60	70 ± 7.2a	2.6 ± 0.6a	11.3 ± 0.6a	37.3 ± 4.1a
	90	35 ± 4.7b	2.6 ± 0.3a	12.0 ± 1.1a	42.3 ± 4.3a
	120	20 ± 5.4b	3.0 ± 0.5a	12.3 ± 1.7a	47.6 ± 2.2a
½-Strength (0.5×)	30	100 ± 0a	6.3 ± 0.3d	14.6 ± 0.3d	33.3 ± 2.8a
	60	80 ± 4.7a	9.0 ± 0.5c	20.6 ± 0.6c	37.3 ± 4.7a
	90	40 ± 5.4b	12.6 ± 0.6b	24.6 ± 0.3b	43.6 ± 2.7a
	120	30 ± 4.7b	15.6 ± 0.3a	28.6 ± 0.6a	49.3 ± 1.9a
¼-Strength (0.25×)	30	100 ± 0a	7.6 ± 1.2c	18.0 ± 0.5d	32.6 ± 1.4a
	60	93 ± 2.7a	10.6 ± 0.6bc	22.6 ± 0.6c	36.0 ± 1.8ab
	90	85 ± 2.3a	13.3 ± 0.8b	25.0 ± 0.5b	41.3 ± 1.4b
	120	50 ± 2.7b	18.0 ± 0.5a	30.6 ± 0.6a	46.3 ± 1.2b
⅛-Strength (0.125×)	30	80 ± 9.4a	15.3 ± 0.3c	26.3 ± 0.9c	50.0 ± 0.9a
	60	40 ± 4.7b	24.0 ± 1.2b	32.7 ± 1.8b	57.0 ± 2.2ab
	90	26 ± 2.7c	27.3 ± 1.2b	41.0 ± 1.5a	61.0 ± 1.2ab
	120	6.7 ± 2.7d	34.3 ± 2.3a	45.3 ± 1.7a	70.6 ± 3.1b

All percentage survival data were recorded after 10 weeks in culture. Each treatment was replicated three-times and each replicate consisted of 20 explants. Values represent means ± SE.
†Mean values followed by different lower-case letters in each column for each treatment are significantly different at $P \leq 0.05$ according to Duncan's multiple range test.

maximum percentage of shoot proliferation when placed in regeneration medium (Gangopadhyay *et al*., 2005). Encapsulated PLBs did not survive at 0ºC (data not shown), probably due to ice crystal formation and subsequent damage. Encapsulated PLBs of *Dendrobium* 'Sonia' stored at 0ºC could not germinate after 45 d of storage (Saiprasad and Polisetty, 2003). In the present study, delays in the germination, and shoot and root emergence of encapsulated PLBs were observed at 4ºC and 8ºC compared to PLBs stored at room temperature. Under these storage temperatures, the time for PLB germination, and for shooting and rooting, increased significantly with an increase in the time of storage. This study agrees with earlier reports on storage studies in members of the genera *Dendrobium*, *Oncidium*, and *Cattleya* (Saiprasad and Polisetty, 2003), and other plant species (Matsumoto *et al*., 1995; Ballester *et al*., 1997). Non-encapsulated PLBs did not survive in any treatment, but turned brown and subsequently died. This result was consistent with earlier reports in orchid (Datta *et al*., 1999; Mohanraj *et al*., 2009)

A high survival percentage (90%) of acclimatised *C. devonianum* plantlets was recorded in the potting mixture containing brick + charcoal + decaying litter (PM1) with a layer of peat moss (Figure 1E). The improved survival of plantlets in PM1 with a layer of moss could be attributed to the improved circulation of air and excellent drainage required for most epiphytic orchids. The addition of moss to the potting mixture

TABLE II
Effect of storage temperature on Ca-alginate-encapsulated protocorm-like bodies (PLBs) of C. devonianum *supplemented with 0.25× MS basal medium in the matrix*

Storage temperature (ºC)	Storage period (d)	Survival (%)	Time to germination of PLBs (d)	Time to emergence of 1-2 leaves (d)	Time to emergence of 1-2 roots (d)
RT (25ºC)	30	100 ± 0a†	7.6 ± 1.2c	18.0 ± 0.5d	32.6 ± 1.4a
	60	93 ± 2.7a	10.6 ± 0.6bc	22.6 ± 0.6c	36.0 ± 1.8ab
	90	85 ± 2.3a	13.3 ± 0.8b	25.0 ± 0.5b	41.3 ± 1.4b
	120	50 ± 2.7b	18.0 ± 0.5a	30.6 ± 0.6a	46.3 ± 1.2b
8ºC	30	100 ± 0a	8.3 ± 0.7a	21.6 ± 0.5a	47.3 ± 1.2a
	60	100 ± 0a	11.3 ± 1.4a	25.0 ± 0.5a	53.3 ± 0.5b
	90	100 ± 0a	17.7 ± 1.2b	28.0 ± 0.5ab	56.3 ± 0.7b
	120	100 ± 0a	21.3 ± 0.7b	31.6 ± 0.7ab	61.0 ± 1.2c
	150	100 ± 0a	24.0 ± 0.9c	35.3 ± 0.5abc	66.6 ± 1.1d
	180	100 ± 0a	29.0 ± 2.8cd	37.3 ± 0.3abc	72.0 ± 0.9e
	210	70.0 ± 3.6b	33.3 ± 0.7de	40.0 ± 0.5abc	76.6 ± 0.7e
	240	62.0 ± 5.4bc	36.3 ± 1.2ef	44.7 ± 1.2abc	80.7 ± 1.1f
	270	50.0 ± 2.7c	38.6 ± 1.7f	50.6 ± 1.4bcd	86.0 ± 1.2g
	300	30.0 ± 2.7d	41.0 ± 1.6f	56.3 ± 0.7cd	91.3 ± 0.7h
	330	16.6 ± 3.4e	47.3 ± 1.0g	59.3 ± 0.3d	96.3 ± 1.5i
4ºC	30	100 ± 0a	18.7 ± 1.4a	24.0 ± 1.8a	63.3 ± 0.7a
	60	100 ± 0a	23.7 ± 2.5ab	29.6 ± 1.2ab	68.0 ± 0.9ab
	90	100 ± 0a	28.0 ± 1.6abc	34.0 ± 0.5b	72.6 ± 1.2bc
	120	100 ± 0a	33.7 ± 2.4bcd	36.6 ± 0.5b	77.6 ± 1.5c
	150	70.0 ± 2.7b	36.0 ± 2.5bcd	43.3 ± 1.6cd	85.0 ± 1.4de
	180	50.0 ± 3.6c	39.7 ± 2.6cd	45.6 ± 1.1d	90.6 ± 1.1ef
	210	20.0 ± 2.5d	48.0 ± 1.2de	50.3 ± 0.7de	96.6 ± 1.2f
	240	5.00 ± 1.2e	54.0 ± 1.8e	55.0 ± 0.8e	105.0 ± 2.4g
*0ºC	30	–	–	–	–

*Encapsulated PLBs stored for different time periods at 0ºC did not germinate, but turned brown and died on MS regeneration medium.
All survival data were recorded after 10 weeks in culture. Each treatment was replicated three-times and each replicate consisted of 20 explants. Values represent means ± SE.
†Mean values followed by different lower-case letters in each column for each treatment are significantly different at $P \leq 0.05$ according to Duncan's multiple range test.

improved the percentage survival of *C. devonianum* plantlets by 10%. Various combinations of charcoal pieces, decaying litter, and moss were found to be superior for acclimatising epiphytic orchids (Das *et al*., 2007; Deb and Imchen, 2010)

The results presented here clearly demonstrate that encapsulated PLBs of *C. devonianum* can be stored for 90 d at room temperature without loss of viability following the incorporation of 0.25× MS in the encapsulation matrix. The storage time could also be extended to 180 d by reducing the temperature to 8ºC. For *in vitro* conservation, the ideal protocol should lead to maximum survival with minimum sub-culturing and without the costly requirements for the use of agar, supplementary lighting, and the maintenance of a specific temperature. The long-distance exchange of germplasm without damage in transit could also be achieved using encapsulated PLBs. Although cryopreservation offers a useful tool for conservation, it is not cost-effective and poor retrieval of cryopreserved tissue is a major drawback (Chaturvedi *et al*., 2004). An approach involving the storage of encapsulated PLBs at 8ºC might help in ensuring less damage to PLBs during temperature fluctuations in transportation, leading to better survival and regeneration.

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