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Short Communication

In Vitro Propagation and Conservation of *Dendrobium lituiflorum* Lindl Through Protocorm-Like Bodies

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Axillary buds obtained from 5-month-old *in vitro* growing plants of *Dendrobium lituiflorum* Lindl were cultured in Murashige and Skoog (MS) medium supplemented with different concentrations of 2,4-D. Fastest initiation (13.3 days) of protocorm-like bodies (PLBs) was observed in cultures containing MS medium supplemented with 0.5 mg l^{-1} 2,4-D. Maximum explant response of 83% was also observed in the same medium. PLBs obtained in MS medium containing 0.5 mg l^{-1} 2,4-D showed maximum regeneration potential of seedlings (19 explant^{-1}) when subcultured in MS medium. Well developed shoots and roots of the seedlings were obtained in the medium containing 0.5 mg l^{-1} each of NAA and BAP, in combination. Encapsulated PLBs of *D. lituiflorum* could be stored at 8°C for 90 days with 80% regeneration. However, it was observed that regeneration potential of encapsulated PLBs reduced with further storage. Seventy seven per cent hardened plants survived and bloomed after 2.5 years of hardening.

Key words: orchid axillary buds, protocorm-like bodies, *in vitro* propagation.

Dendrobium lituiflorum is one of the most popular orchids in North-East India, but it is fast depleting due to increasing deforestation and ruthless exploitation. Moreover, the available protocols have shortfall of low rate in protocorm-like body (PLB) formation, low viability of PLB and longer time for PLB formation (1). Hence, it is necessary to develop methods for *in vitro* propagation and conservation of this orchid. The present research was undertaken to develop rapid and reproducible *in vitro* method for PLB formation and subsequent growth of *D. lituiflorum*. The storability of encapsulated PLBs at low temperature, their regeneration and *ex vitro* establishment is also reported.

Five-month-old *in vitro* grown cultures of *D. lituiflorum* raised from seeds on Murashige and Skoog medium were used for setting up the experiments. Nodal sections measuring 0.5-1.0 cm were cut and cultured in MS medium supplemented with different concentrations of 2,4-D (0.0-10.0 mg l^{-1}) for PLB initiation. Regeneration of PLBs to seedlings in 2,4-D supplemented medium was slow. Therefore, 40-day-old PLBs obtained from axillary buds were subcultured in either MS medium without 2,4-D or in the medium supplemented with NAA and BAP. The pH of the medium was adjusted to 5.8 prior to autoclaving at 121°C and 1.06 kg cm^{-2} pressure for 20 min. The cultures were kept at 24 ± 2°C under 16 h photoperiod (irradiance of 150 µmol m^{-2} s^{-1}) and relative humidity maintained at 70-80%. Each treatment had 20 axillary buds as replicates, and the experiment was repeated thrice.

For storage studies, PLBs obtained from axillary buds were encapsulated in calcium alginate beads. Four per cent sodium alginate solution was prepared in basal MS liquid medium (devoid of calcium nitrate) containing 3% sucrose, and the pH was adjusted to 5.8 before autoclaving. Round and firm beads were obtained when sodium alginate solution containing single PLB was dropped into 100 mM of $Ca(NO_3)_2.4H_2O$ for 30 min on gyratory shaker (75 rpm). Five sets of 60 x 15 mm Petri plates each containing 20 encapsulated PLBs were stored at 4°C and 8°C in the dark. For control, encapsulated PLBs were kept at room temperature (24 ± 2°C) maintaining all other physical conditions constant. The experiment was repeated thrice. At an interval of every 30 days, the encapsulated PLBs were taken out and cultured on regeneration medium. The encapsulated PLBs were considered to have germinated with the emergence of PLBs and regenerated with the emergence of shoots and roots from the beads. Germination percentage of encapsulated PLBs was recorded at 40 days of culture. Time taken in germination and subsequent regeneration was also recorded for each treatment.

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Abbreviations: BAP, N^6- benzylaminopurine; 2,4-D, 2,4-dichlorophenoxy acetic acid; MS, Murashige & Skoog; NAA; α-naphthalene acetic acid; PLB, protocorm- like body.

Table 1. PLB formation on axillary buds of *D. lituiflorum* on MS medium supplemented with 2,4-D

2,4-D (mg l^{-1})	Days required for PLB initiation	Explant response (%)	Fr wt of PLBs $explant^{-1}$ (g)	No. of seedlings $explant^{-1}$*
0.0	34.3	16.6	0.04	3.2
0.5	13.3	83.3	0.62	19.0
2.5	14.3	56.6	0.59	6.0
5.0	17.7	23.3	0.35	4.3
10.0	19.0	10.0	0.06	3.9
$LSD_{(0.05)}$	5.94	4.70	0.24	5.55

*Data recorded after 70 days of culture i.e., 40 days culture in 2,4-D supplemented medium and 30 days without 2,4-D.

In vitro raised plantlets were transferred to thermocol pots of 8 cm diameter containing different mixtures: (i) brick, charcoal and decaying litter (1:1:1), (ii) brick, charcoal, decaying litter and cowdung (1:1:1:1), and (iii) brick, charcoal, decaying litter and coconut husks (1:1:1:1) with a layer of moss on top. The plantlets were fed with MS salt solution (diluted 10 times) every alternate day for about a month. Observations were recorded after 90 days of hardening. Twenty cultures were raised for each treatment, which were replicated thrice. The data were analyzed using one way ANOVA and comparisons between the mean values of treatments were made by the Least Significant Difference (LSD) test (2).

It was observed that PLB initiation was significantly fastest (13.3 days) with 83.3% explant response on MS medium supplemented with 0.5 mg l^{-1} 2,4-D (Table 1). In this treatment; the fresh weight of PLBs $explant^{-1}$ (0.62 g) was also the maximum. The efficacy of 2,4-D in the enhancement of callusing and PLB formation in other orchids has also been reported (3,4). With further increase in 2,4-D concentrations (2.5-10.0 mg l^{-1}) in the medium, subsequent delay and decrease in explant response was observed. Also, the regeneration potential of PLBs was found to be dependant on the concentrations of 2,4-D used during the initial culture period. The PLBs cultured initially in medium containing 0.5 mg l^{-1} 2,4-D showed maximum regeneration potential with high number (19) of seedlings $explant^{-1}$ (Fig. 1a) in medium without 2,4-D. The initiation culture medium with 2,4-D (>0.5 mg l^{-1}) or without it resulted in decrease of PLB regeneration. Such variations in regeneration of PLBs due to different concentrations of 2,4-D in the initiation medium, have also been reported in

Table 2. Effect of BAP and NAA on growth of plantlets derived from axillary bud PLBs of *D. lituiflorum*

Treatment (mg l^{-1})	Plantlet height $explant^{-1}$ (cm)	Shoot number $explant^{-1}$	Leaf number $explant^{-1}$	Root number $explant^{-1}$	Root length $explant^{-1}$ (cm)
0.0	1.7	2.0	2.1	2.3	0.21
NAA					
0.5	3.3	4.0	3.4	8.0	1.43
2.5	4.2	4.7	3.9	4.7	2.57
5.0	2.4	3.0	3.1	2.7	1.70
$LSD_{(0.05)}$	0.82	2.16	0.64	3.52	1.13
BAP					
0.5	2.7	2.3	3.0	2.3	0.62
2.5	3.5	3.7	4.0	3.3	0.77
5.0	2.2	2.7	2.1	0.0	0.0
$LSD_{(0.05)}$	1.07	3.08	1.37	1.22	0.09
BAP + NAA					
0.5+0.5	3.1	8.0	4.9	7.0	0.69
0.5+2.5	2.2	6.0	2.8	4.7	0.40
2.5+0.5	1.8	4.7	2.6	3.0	0.27
2.5+2.5	1.5	3.3	2.4	2.0	0.19
$LSD_{(0.05)}$	1.4	2.44	0.88	1.55	0.29

Note: Data recorded after 60 days of treatment.

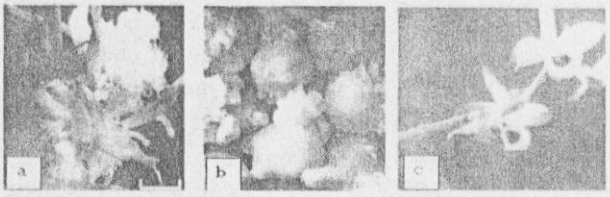

Fig.1. *In vitro* grown cultures of axillary buds of *D. lituiflorum*. (a) Eighty-day-old culture showing regeneration of PLBs in MS medium pretreated with 0.5 mg l^{-1} 2,4-D (bar = 1.2 cm), (b) 20-day-old germinating encapsulated PLBs cultured in MS medium after 90 days of storage at 8°C (bar = 0.8 cm), and (c) Flowered *in vitro* raised hardened plants of about 2.5 years in the glass house.

D. formosum (5). However, further growth of the seedlings *viz*, height, number and length of shoots and roots was poor in the medium devoid of growth regulators. Therefore, addition of NAA and BAP in the medium (0.5 mg l^{-1} each) was found promotory for overall growth of seedlings (Table 2). This is in conformity with the earlier reports (6,7). Higher concentrations of NAA and BAP, in combination, in the medium were found to have no significant effect on the growth parameters studied.

Encapsulated PLBs stored at different temperatures and time had potential to germinate and regenerate on MS medium (Fig. 1b). The results showed that only 3.3% of encapsulated PLBs germinated after 90 days of storage at room temperature (Table 3). Of the two temperature (4°C and 8°C) regimes studied, beads stored at 8°C for 90 days showed 80% of PLB germination. However, further storage significantly reduced germination percentage of encapsulated PLBs. Table 3 compares the germination percentage, time taken in germination and regeneration of encapsulated PLBs stored at different temperatures following 30, 60, 90 and 120 days of storage. Emergence of PLBs from encapsulated beads was earliest in the beads stored at room temperature for 30 days. However, the difference in time taken between PLB germination and shoot initiation, and between shoot and root initiation of cultured encapsulated beads stored at room temperature was higher and further increased with increase in storage time. On the other hand, the difference in time taken for the same parameters was less in the cultured encapsulated beads stored at 8°C. Similar results were obtained in case of encapsulation of micro-shoots of pineapple (8). Encapsulation of PLBs of several orchids with the ultimate objective of artificial seed production and storage at low temperature has been attempted in a number of systems (9,10). This shows that encapsulation of PLBs enables proper storage, transportation and regeneration, and hence serves as a useful tool for conservation of this endangered orchid.

Conservation becomes incomplete without successful transplantation of the *in vitro* grown plantlets. Higher survival percentage (77%) of hardened plantlets of *D. lituiflorum* was obtained in substratum containing brick,

Table 3. Effect of different storage temperatures and time on regeneration of encapsulated PLBs of *D. lituiflorum*

Storage temperature	Storage time (days)	Germination (%)	Time taken in (days)		
			Germination	Regeneration	
				Shoot initiation	Root initiation
Control (24 ± 2°C)	30	60.0	4.7	17.7	31.7
	60	30.0	12.0	33.3	51.0
	90	3.3	22.0	48.0	69.0
$LSD_{(0.05)}$		17.6	4.05	9.77	8.89
4°C	30	95.0	20.6	36.7	54.7
	60	85.0	24.7	40.7	58.0
	90	48.3	32.0	47.7	64.3
	120	13.3	33.7	49.0	64.0
$LSD_{(0.05)}$		14.6	9.32	11.75	6.15
8°C	30	100	10.3	19.0	28.0
	60	100	14.0	22.0	30.3
	90	80.0	17.0	24.0	31.0
	120	40.0	23.7	30.0	37.0
$LSD_{(0.05)}$		13.3	3.85	5.73	3.92

charcoal, decaying litter (1:1:1) with a layer of moss as compared to the other substrata used. The hardened *in vitro* raised plantlets flowered after 2.5 years in the glass house (Fig. 1c). The best method to ensure survival of cultured plantlets under natural conditions is to expose them gradually to relatively lower humidity, higher temperature and higher light intensity. The promotory effect of application of dilute nutrient solution for initial hardening has been reported earlier (11).

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