

Plant Regeneration from Protocorm Like Body (PLB) derived Callus of *Dendrobium barbatulum* Lindl.

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Abstract

In vitro regenerative potential of protocorm like bodies (PLBs) of *Dendrobium barbatulum* were assessed. The formation of secondary PLBs from the primary PLBs that formed from the callus, it is derived from a few larger injured protocorm like bodies. The injured larger PLBs were cultured on half strength MS supplemented with different concentrations of BAP (0.32, 1.62, 3.23 and 16.15 μ M), Zn (0.46, 2.28, 4.56 and 22.80 μ M) and Kn (0.47, 2.33, 4.65 and 23.25 μ M). The highest percentage of secondary PLBs (66.33%) were obtained on half strength MS medium supplemented with BAP 3.23 μ M, after 5 weeks of culture. Then these clumps of PLBs were subcultured on half strength MS fortified with coconut water (CW), cane juice (CJ), peptone (P) and casein hydrolysate (CH) for regeneration. Among the complex organic additives 20% CW and 2.0 g/l P to half strength MS resulted in the development of plantlet 78.31 and 62.60%, respectively. Well developed plantlets were successfully acclimatized in the community pots having brick pieces, charcoal, decaying litter and coconut husk (1 : 1 : 1 : 1) gave maximum survival rate of 87.03%.

Introduction

Orchidaceae is the most widely distributed family having beautiful vast and varied, offer lot of variations in their flower size, form, colour, delicacy and design. The *Dendrobium* is one of the most popular orchid genera which have so far contributed to the trade of floriculture. Due to ruthless collection, over exploitation, destruction of habitats, deforestation and unauthorized trade have led to reduction in natural population of this species, including many other orchids. At present most of the wild orchids are listed in the International Union of Conservation of Nature and Natural Resources (IUCN) Red Data Book. This family is also included in the Appendix-II of the Convention of Interna-

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tional Trade in Endangered Species of Wild Flora and Fauna (CITES) (Senthilkumar 2001). The species *D. barbatulum* is an ornamental and having floriculture importance, flowers are white with pink lilac make an attractive unique addition to one's orchid collection. When it produces beautiful flowers with long stems and long vase life, thus giving this species great potential as a novel alternative for the floriculture industry. *D. barbatulum* is now becoming rare because of the destruction of its habitat, the heavy exploitation of its wild resources, its low propagation rate and its slow growth. Therefore, to meet the growing demand of the horticulture and floriculture industries artificial cultivation is beginning to be investigated and attempted. *D. barbatulum* is usually propagated sexually by seeds. However, the conventional method of propagation is very inefficient and time consuming as seeds lack endosperm and require mycorrhizal association for germination. Now an effective strategy becomes essential to conserve and multiply this orchid species. An efficient propagation system followed by callus induction, PLBs formation, shoot proliferation and acclimatization has been demonstrated. Such a protocol would allow for large scale propagation to meet commercial demand and conserve this rare orchid species by reducing wild collection. Culture work on South Indian orchids with the objective of mass clonal multiplication of indigenous species and to preserve endangered/rare species has been initiated.

Materials and Methods

A six month old light yellowish green capsule of *Dendrobium barbatulum* that formed after self-pollination were collected from greenhouse plants at Plant Tissue Culture Laboratory, Department of PG Botany, Maharani's Science College for Women, Mysore, Karnataka, India. The capsules were washed under running tap water, then dipped in 90% (v/v) ethanol, flamed for a few seconds, under sterile conditions and split open longitudinally with sterilized scalpel. The seeds were inoculated on the surface of the semi-solid half strength MS with 0.8% agar. The pH of the medium was adjusted to 5.8. Cultures were maintained under 60 $\mu\text{mol m}^{-2}/\text{s}$ light intensity at 16/08 (light/dark) photoperiod at $25 \pm 2^\circ\text{C}$, these were subcultured on to similar half strength MS every 6 weeks for further proliferation and development.

The seeds imbibed the nutrients and became swollen after 3 weeks of culture. Then these swollen seeds forming sphere shaped protocorm like bodies (PLBs) after a week in a half strength MS. The seeds inoculated on half strength MS with CW (20%) grew larger (3.0 - 3.5 mm diam.) than the normal PLBs (1.5 - 2.0 mm diameter). These larger PLBs were selected from the cultures and injured by piercing the sharp needle at the base for inducing callus. When these injured larger PLBs cultured on half strength MS with BAP (3.23 μM) initiated the development of callus after 7 weeks. On the same medium this callus on subculture developed primary PLBs after 6 weeks and later developed secondary PLBs in another 5 weeks of subculture. The complex organic additives such as coconut water (5, 10 and 20%), cane juice (5,10 and 20%), casein hydrolysate (0.5, 1.0 and

2.0 g/l) and peptone (0.5, 1.0 and 2.0 g/l) were added individually into the medium prior to autoclaving for the regeneration of PLBs. The clumps of PLBs were cultured on each of the media containing the additives. Complete regenerated plantlets (3.0 - 4.0 cm) of *D. barbatulum* were transferred to community pots containing compost mixture of brick pieces, charcoal, decaying litter and coconut husk in the proportion of 1 : 1 : 1 : 1. The temperature of the greenhouse for acclimatization was maintained at $26 \pm 2^\circ\text{C}$, the relative humidity initially around 80 - 90% for a week then it is reduced to 60 - 70%, plants were watered daily. Readings were recorded after 10 weeks of transfer.

The experiment was repeated thrice with 10 replicates per treatment. Statistical analysis was done by ANOVA at $p < 0.05$ and means compared using Tukey's test (PC version Origin 8.0).

Results and Discussion

Germination of seeds on half strength MS started within 4 weeks. The undifferentiated embryo gradually developed a round/ellipsoidal protocorm (Batygina et al. 2003). For callus induction some of the larger protocorms were selected and injured with a sharp needle at the base and cultured on half strength MS. Half strength MS supplemented with different PGRs viz., BAP (0.32, 1.62, 3.23 and 16.15 μM), Zn (0.46, 2.28, 4.56 and 22.80 μM) and Kn (0.47, 2.33, 4.65 and 23.25 μM). In half strength MS, Kn and ZT, the PLB explants failed to form callus and gradually became necrotic after 10 weeks of culture. On the other hand, the callus induction was observed only on half strength MS with BAP. The lower surface of the explants prolific callus induction was achieved from the seed derived injured protocorms. The frequency of callus induction increased with the increasing BAP concentration from 0 - 3.23 μM , it decreased as BAP concentration raised to 16.15 μM (Table 1). The highest percentage of PLB explants producing calluses was 56.71 in BAP 3.23 μM after 7 weeks of culture (Fig. 1A). The initiated callus was subcultured on to the same medium, the proliferation of whitish callus became more obvious and gradually developed into compact texture after 11 weeks (Fig. 1B). The callus was induced only in half strength MS with BAP hence the same PGR was used to initiate primary PLBs. The callus was cultured on half MS with various concentrations of BAP. The whitish compact callus developed irregular granules which subsequently turned green and developed into primary protocorm like bodies. In this experiment the optimum development of primary PLBs were on medium supplemented with BAP 3.23 μM (Fig. 2A), where about 60.24 % of callus developed into primary PLBs after 6 weeks of culture. BAP at low and relatively high dosages showed different results on PLB induction. At low dosages of BAP (0.32 μM) there was no response on PLB induction. On the other hand, the high frequency of callus browning was observed on the medium containing BAP (16.15 μM) and 18.90% of primary PLBs have been developed (Table 2).

Table 1. Initiation of callus from PLBs of *D. barbatulum* in half MS and with different PGRs.

	BAP (μM)	Kn (μM)	Zn (μM)	Time taken (Weeks)	% of callus formation
Half MS	-	-	-	-	-
	0.32	-	-	7	$2.3 \pm 2.0^{\text{hi}}$
	1.62	-	-	7	$11.06 \pm 1.2^{\text{de}}$
	3.23	-	-	7	$56.71 \pm 0.6^{\text{ab}}$
	16.15	-	-	8	$8.23 \pm 1.9^{\text{e}}$
	-	0.47	-	-	-
	-	2.33	-	-	-
	-	4.65	-	-	-
	-	23.25	-	-	-
	-	-	0.46	-	-
	-	-	2.28	-	-
	-	-	4.56	-	-
	-	-	22.80	-	-

Values represent mean \pm SE; the same letters within a column are not significantly different based on ANOVA followed by Tukey's test at $p < 0.05$.

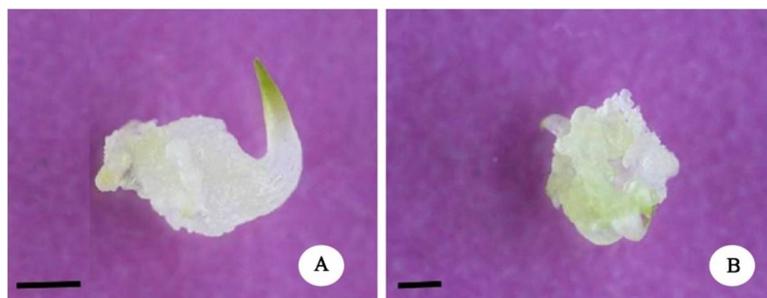


Fig. 1. Callus induction and proliferation in *Dendrobium barbatulum*. A. Injured PLB initiated the development of callus on half strength of MS + BAP $3.23 \mu\text{M}$ after 7 weeks. Scale bar = 1 mm. B. Proliferation of callus on the same medium after 11 weeks. Scale bar = 1 mm.

Table 2. Development of Primary PLBs from the protocorm derived callus of *D. barbatulum* in half strength MS with BAP.

BAP (μM)	Time taken for the formation of primary PLBs (Weeks)	Percentage of callus developed primary PLBs
0.32	-	-
1.62	7	$18.31 \pm 1.6^{\text{ef}}$
3.23	6	$60.24 \pm 0.7^{\text{ab}}$
16.15	6	$18.90 \pm 1.8^{\text{e}}$

Values represent mean \pm standard error; the same letters within a column are not significantly different based on ANOVA followed by Tukey's test at $p < 0.05$.

The effect of different kinds of cytokinins BAP (0.32, 1.62, 3.23 and 16.15 μM), Kn (0.47, 2.33, 4.65 and 23.25 μM) and Zn (0.46, 2.28, 4.56 and 22.80 μM) were studied for the induction of secondary PLBs from primary PLBs. The development of secondary PLBs on the primary PLBs was not recorded on some of the treatments including PGR free medium. Of the three cytokinins tested BAP was found to be most effective once again in inducing secondary PLBs. The percentage of secondary PLBs increased with increasing concentration of BAP. The primary PLBs started to imbibe nutrients and swelled considerably after 2 weeks and globular structures were developed on the swollen PLBs after 3 weeks of culture. The optimum percentage of secondary PLBs (66.33%) developed from primary PLBs cultured on half MS with BAP 3.23 μM (Fig. 2B), the addition of cytokinins specially BAP, significantly increased the formation of secondary PLBs. The primary PLBs cultured on PGR free medium did not show any response. However, the addition of Zn was found to inhibit the development of secondary PLB. The medium containing Zn where the primary PLBs imbibed the nutrients but did not show any secondary PLB after 10 weeks, then the swollen PLBs became necrotic. Hence the addition of Zn was found to inhibit the induction of secondary PLB formation. On the other hand Kn (0.47 μM) was slightly stimulated the formation of secondary PLBs at lower concentration (Table 3), in this medium the primary PLBs swelled considerably and developed secondary PLBs (36.83%) after 6 weeks, but the secondary PLBs were not healthy. The higher concentration of Kn (23.25 μM) also inhibited the formation of secondary PLBs. The primary PLBs did not show any response and they became brown after 10 weeks.

The propagation of orchids through the formation of secondary PLBs from protocorms/PLBs has been reported in several orchids. Induction of secondary PLBs from primary PLBs in orchids is greatly influenced by the specific PGRs (Hossain et al. 2010). The secondary protocorms can be regenerated directly from the outer tissues of the protocorms (Huan et al. 2004), protocorm segments or thin cell layers (Teixeira da Silva and Tanaka 2006). The propagation of orchids through the formation of secondary PLBs from primary protocorms/PLBs has been reported for several orchids including *Cymbidium* (Teixeira da Silva and Tanaka 2006), *Phalaenopsis* (Murdad et al. 2006), *Phaphiopedilum* (Chen and Chang 2004, Chyuam and Saleh 2011) and *Dendrobium* (Saiprasad et al. 2004). Various results have shown that the presence of BA proceeded the formation and further proliferation of PLBs in some orchids like *Cymbidium aloifolium*, *Dendrobium densiflorum*, and *Aerides maculosum* (Nayak et al. 2002, Luo et al. 2008 and Sheelavanthmath et al. 2005). However Teixeira da Silva and Tanaka (2006) reported that Kinetin was more effective in the formation of PLBs in *Cymbidium* hybrids when added with NAA to the culture medium. In contrast Chen et al. (2002) reported that both BA and Kn were equally effective for PLB formation in *Epidendrum radicans*. Here it was found that optimum secondary PLB formation is possible in *D. barbatulum* cultured on half strength of MS with BAP alone. It indicated that the requirement of exogenous PGRs

for PLB formation are species specific as has been observed in other orchids (Lee and Lee 2003, Yan et al. 2006).

Table 3. Formation of secondary PLBs from the primary PLBs of *D. barbatulum* in half MS medium and supplemented with different PGRs.

	BAP (μ M)	Kn (μ M)	Zn (μ M)	Time taken for secondary PLB formation (weeks)	% of secondary PLB formation
Half MS	-	-	-	-	-
	0.32	-	-	5	30.03 \pm 2.4 ^{de}
	1.62	-	-	5	29.06 \pm 3.3 ^{de}
	3.23	-	-	5	66.33 \pm 0.9 ^a
	16.15	-	-	7	18.92 \pm 1.8 ^e
	-	0.47	-	6	36.83 \pm 0.4 ^{cd}
	-	2.33	-	6	24.6 \pm 1.9 ^c
	-	4.65	-	6	24.8 \pm 2.7 ^c
	-	23.25	-	-	-
			0.46	-	-
			2.28	-	-
			4.56	8	3.2 \pm 2.9 ^{hi}
			22.80	-	-

Values represent mean \pm standard error; the same letters within a column are not significantly different based on ANOVA followed by Tukey's test at $p < 0.05$.

The potentiality of secondary PLBs to regenerate into plantlets was also investigated by using different types and concentrations of PGRs and complex organic additives. The clumps of PLBs were subcultured on PGR enriched (BAP, Kn and Zn) medium did not show further proliferation even after 14 weeks of culture. This indicated that the exogenous cytokinins in the proliferation of PLBs did not support the regeneration of plantlets. However, very poor proliferation was recorded from these PLBs in PGR free medium also (data not shown). Further work was carried out on the proliferation of these PLBs with of complex organic additives *viz.*, CW (5, 10, 20%), CJ (5, 10, 20%), P (0.5, 1.0, 2.0 g/l) and CH (0.5, 1.0 and 2.0 g/l) to the culture media. The clumps of PLBs were cultured on these additives for regeneration of plantlets. It was observed that the addition of CW (20%) and P (2.0 g/l) to half strength of MS promoted optimum proliferation of PLBs in *D. barbatulum*. The apical part of the protocorm consists of small number of cells formed a tubercle which turns into shoot apex. A green shoot elongated further and small leaves with stout roots were initiated from it after 16 weeks of subculture (Fig. 2C).

Similar observations on plantlet development was reported in *Paphiopedilum*, banana- or potato homogenate to the culture medium promoted shoot proliferation two to three-folds (Chyuam and Saleh 2011) and in *Dendrobium* (Aktar et al. 2008). The addition of complex organic additives to the culture medium to promote *in vitro* growth and proliferation of orchid is a common practice (Arditti and Ernst 1993, Arditti 2008 and George et al. 2008). The addition of complex organic additives may enhance the development and proliferation of PLBs and minimize the use of exogenous PGRs. Here further proliferation and regeneration of PLBs were devoid of supply of exogenous PGRs

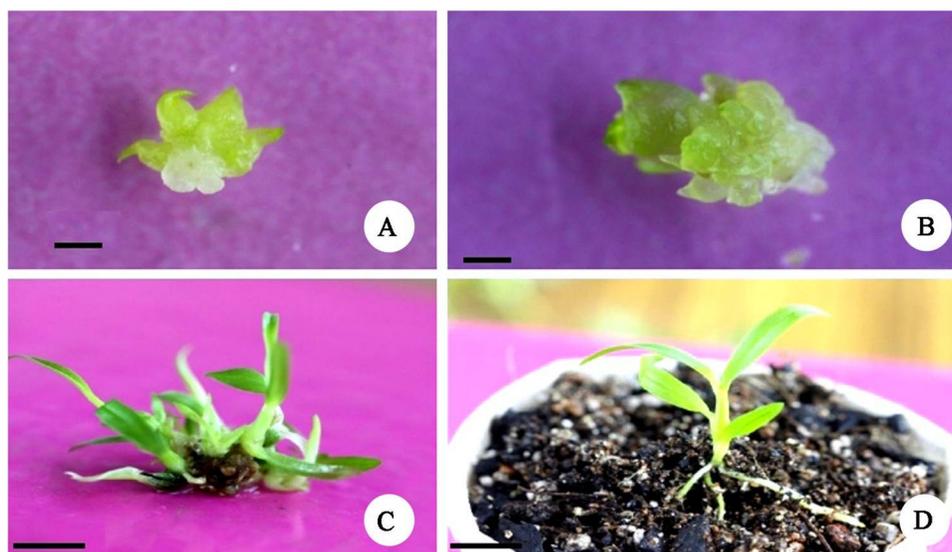


Fig. 2. Initiation of primary, secondary PLBs and plantlet development in *Dendrobium barbatulum*. A. Induction of primary PLBs on callus on half strength MS + BAP 3.23 μ M after 6 weeks. Scale Bar = 1 mm. B. Development of secondary PLBs on the surface of primary PLB explants on half strength MS + BAP 3.23 μ M after 5 weeks. Scale bar = 1 mm. C. Regeneration of PLBs on half strength MS + CW 20% after 16 weeks. Scale bar = 1 cm. D. A complete plantlet acclimatized in greenhouse. Scale bar = 1 cm.

as suggested by Roy et al. (2007) and Chyuam et al. (2010). The highest number of PLBs converted into plantlets (78.31%) was achieved using half strength MS with 20% CW. But addition of CJ and CH was not beneficial in converting the PLBs into plantlets (Table 4). On the other hand, the addition of P 2.0 g/l stimulated the conversion of PLBs into plantlets (62.60%). These plantlets developed 3 to 4 leaves and roots after another 6 weeks of subculture. The addition of CW proliferated the PLBs of *Phalaenopsis gigantea* (Murdad et al. 2006), *Paphiopedilum rothschildianum* (Chyuam et al. 2010) and *Vanilla planifolia* (Kalimuttu et al. 2006). However in *Aerides crispum* the addition of CW did not show regeneration any PLB (Sheelavanthmath et al. 2005). Similarly the addition of P significantly enhanced germination and shoot formation in *Dendrobium lasianthera* (Utami et al. 2017). In contrast to the above results, it was found that the addition of CW and P into the basal medium stimulated PLB regeneration in *D. barbatulum*.

Well developed plantlets measuring about 3.0 - 4.0 cm in height were transferred to community pots containing the potting mixture of brick pieces, charcoal, decaying litter and coconut husk in the ratio of 1 : 1 : 1 : 1 in greenhouse for acclimatization (Fig. 2D). The high humidity prevented the wilting of healthy plants. Charcoal and brick pieces provided maximum water holding capacity, porosity and drainage is essential for proper growth and development of *in vitro* grown plantlets. Also the required moisture content was maintained by the husk. About 87.03% of the *in vitro* grown plantlets survived and continued to grow.

Table 4. Effect of complex organic additives on proliferation and regeneration of PLBs of *D. barbatulum*.

Complex organic additives	Concentration	Time taken for regeneration (weeks)	Percentage of regeneration
CW	5%	5	51.40 ± 1.3 ^{bc}
	10%	5	51.34 ± 2.6 ^{bc}
	20%	4	78.31 ± 0.7 ^a
CJ	5%	5	21.0 ± 1.8 ^{ef}
	10%	6	21.0 ± 1.4 ^{ef}
	20%	6	18.30 ± 1.2 ^f
P	0.5 g	6	34.46 ± 2.8 ^{cd}
	1.0 g	6	32.30 ± 1.9 ^d
	2.0 g	5	62.60 ± 3.0 ^{ab}
CH	0.5 g	8	11.21 ± 1.8 ^f
	1.0 g	8	9.04 ± 3.6 ^{fg}
	2.0 g	8	9.01 ± 3.0 ^{fg}

Values represent mean ± SE; the same letters within a column are not significantly different based on ANOVA followed by Tukey's test at $p < 0.05$.

The procedure described here is relatively simple and reliable where the formation of callus, primary PLBs, secondary PLBs and the clumps of PLBs developed into normal and healthy plants. From this study half MS with BAP was found to be best for the induction of callus, primary PLBs and secondary PLBs. The complex organic additives, namely CW and P played a crucial role in the regeneration of PLBs into plantlets. The propagation method reported here is an ideal tool for rapid mass propagation and conservation purpose.

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