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In vitro Regeneration of an Endangered Medicinal Orchid *Dendrobium crepidatum* Lindl. & Paxton through the Development of Protocorm like Bodies

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Abstract

A protocol for *in vitro* regeneration of an endangered medicinal orchid *Dendrobium crepidatum* was established mainly through the formation of indirect protocorm like bodies (PLBs). The PLBs procured from *in vitro* growth cultures was assessed in ½ MS medium alone and in combinations with 6-Benzyl amino purine (BAP), Kinetin (Kn) and α-Naphthalene acetic acid (NAA). The callus tissue was induced from longitudinally bisected segments of PLBs. The optimum percentage of callus formation was obtained on ½ MS medium supplemented with BAP (1.0 mg/l) and a combination of BAP (1.0 mg/l) with NAA (0.5 mg/l), where 64.3% and 83.3% of callus was formed respectively. Formation of PLBs were achieved when callus mass was transferred onto the combinations of BAP + NAA and Kn + NAA in ½ MS medium. The optimum percentage (91.6%) of PLBs were regenerated in the combination of BAP with NAA. These PLBs differentiated into intact plantlets. Plantlets of about 3 cm in length with 4-5 roots were transplanted into potting mixture where the highest rate of survival was 93.3% in a green house.

Introduction

Orchidaceae represents one of the largest plant family among the monocotyledons, included 899 genera and 27800 species (The plant List 2019) and distributed almost worldwide. *Dendrobium* is one of the largest genera in the orchid family, with great diversity in floral characteristics and plant metabolic features, which is of considerable value to researchers, breeders and biotechnologists. *Dendrobium* species have high values in ornamental cultivation and medicinal utilization. There are several species that have been used in traditional medicine. *Dendrobium crepidatum* Lindl. & Paxton is commonly called as Shoe-lipped *Dendrobium*, a sympodial epiphytic endangered medicinal orchid is one of the most famous orchids distributed in a few countries including India, China,

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Australia, Thailand and Philippines. It is a distinctive evergreen, popular decorative perennial and extremely beautiful flowering plant. It is also used in traditional Chinese medicine. However, its number is steadily declining because of a lower rate of propagation in nature, due to ruthless collection by increasing orchid lovers, and over exploitation for medicinal and ornamental purposes, deforestation, killing of pollinators and unauthorized trade. Meanwhile many orchid species have become extinct, and many others are on the verge of becoming rare and endangered. At present many orchids are listed in the Red data book prepared by International Union for Conservation of Nature and Natural resources (IUCN). In fact, the entire family is now included in Appendix-II of Conventional on International Trade in Endangered Species of wild fauna and flora (CITES), where the international trade is strictly controlled and monitored (Pant 2013).

Hence an *in vitro* propagation technique could be a useful approach for the mass scale propagation of this endangered orchid for ornamental, medicinal and commercial purposes. Tissue culture techniques have been widely used for *in vitro* mass propagation of several commercially important orchids over a past few decades (Chen and Chang 2004). *In vitro* culture provides an alternative way for recovery of the endangered species and it could also provide sources of herbal drug (Luo et al. 2009). Present study was carried out to establish a complete protocol for callus induction, PLB formation and plantlet conversion in this economically important and endangered medicinal orchid.

Materials and Methods

The capsules of *D. crepidatum* were collected from Madikeri Forest, Coorg district, Western Ghats of Karnataka, India. These capsules were surface sterilized with 0.75% (v/v) sodium hypochlorite plus 0.1% Tween-20 for 5 minutes followed by three rinses with distilled, autoclaved water. The capsules were then cut opened aseptically and seeds were cultured on ½ MS medium. The microscopic seeds imbibe the nutrients then developed into small oval bodies called as protocorm like bodies (PLBs) after 4-5 weeks of culture. These PLBs of about 3 mm in diameter were selected, longitudinally bisected and used as explants for callus induction. The explants inoculated in culture tubes (18 X 150 mm^2) containing 15 ml ½ MS basal medium, which has macro nutrients at half strength, 2% sucrose, supplemented with NAA (0, 0.5, 1.0, 3.0 and 5.0 mg/l), BAP (0, 0.5, 1.0, 3.0 and 5.0 mg/l) and Kn (0, 0.5, 1.0, 3.0 and 5.0 mg/l) alone or in combinations. The media were solidified with 0.8% (w/v) agar (Hi media, Mumbai, India). The pH was adjusted to 5.8 with 1N NaOH/HCl before autoclaving at 121°C for 20 minutes. Cultures were maintained at temperature 25 ± 2°C, with light intensity of 40 µmol m^2 s^{-1} and photo period of 16/8 h light/dark cycle.

The callus induced from explants were subcultured on the optimum medium for callus induction for 9 weeks. Then the callus was transferred onto the same medium for PLB regeneration. The regenerated PLBs about 2-3 mm in diameter with a cotyledon were transferred onto ½ MS medium with various concentrations of PGRs for

differentiation of shoot and roots. The plantlets with 4-5 roots were then transferred to community pots containing an autoclaved potting mixture of vermiculite, charcoal, brick pieces and finely chopped coconut husk in the ratio of 1 : 1 : 1 : 1 in a green house at 25 ± 2°C with 14 h (50 µmol m^2 s^{-1}) photoperiod and 75 ± 5% relative humidity.

Each experiment was repeated three times with ten replicates per treatment, the means and standard errors were calculated. The statistical significance between the mean values was assessed Duncan's multiple range test, where a probability of P = 0.05 was considered significant. The statistical data analysis was performed using SPSS software program (SPSS Inc., Chicago, USA).

Results and Discussion

The PLBs about 3 mm in diameter were longitudinally bisected (Fig. 1A) and cultured on ½ MS medium and the ½ MS medium supplemented with various PGRs. In ½ MS medium there was no response of the explants recorded. On the other hand, the explants imbibed the nutrients and swollen considerably after 2-3 weeks of culture and initiated the development of whitish and fragile callus mass from the cut end of the explants, inoculated on ½ MS medium supplemented with different PGRs and continued to proliferate and gradually turned green and granular calluses after 6-9 weeks of culture. Calluses could be induced from explants in most treatments with different frequencies. Of the three PGRs tested BAP was found to be most effective in inducing the calluses from explants. Callus induction was enhanced as the concentration of BAP ranged from 0 to 1.0 mg/l and reduced in the concentrations of 3.0 and 5.0 mg/l. The frequency of callus induction from explants was 64.3% on ½ MS medium with BAP (1.0 mg/l), in this PGR 61.7% explants showed the response. The effectiveness of NAA on callus induction from the explants was comparatively less to that of BAP. The frequency of callus induction was optimum on NAA concentration 0.5 mg/l (46.8%), it drastically decreased on the concentration of 1.0 mg/l, where only 20.1% of callus was formed from the explants. The optimum of 29.1% explants were responded in NAA. The effect of Kn on callus induction followed a similar pattern but to a lesser degree than BAP and NAA. The optimum percentage of explants producing calluses was 36.1% at 0.5 mg/l of Kn. But only 12.2% of explants were responded in this PGR. To further promote callus induction and proliferation, combinations of BAP, NAA and Kn were tested to look for possible synergistic effects. The explants inoculated on ½ MS medium supplemented with BAP (1.0 mg/l) with NAA (0.5 mg/l) exhibited a higher frequency of callus induction. In this combination 80.1% of explants responded and developed 83.3% of callus after 6 weeks of culture (Fig. 1B). Similarly, in Kn (0.5 mg/l) with NAA (0.5 mg/l) combination 51.1% of explants developed callus from 31.3% of explants responded (Table 1).

Table 1. Effect of PGRs in ½ MS medium on formation of Callus and PLBs in ***D. crepidatum***

Concentration (mg/l)			Explant response (%)	Time taken for callus formation (Wks)	Percentage of callus formation	Time taken for PLB formation (Wks)	Percentage of PLB formation
Control (½ MS)			-	-	-	-	-
NAA	BAP	Kn					
0.5	-	-	29.1 ± 0.6^{c}	8	46.8 ± 0.8^{d}	7	61.6 ± 1.0^{f}
1.0	-	-	19.0 ± 0.7^{b}	8	20.1 ± 1.8^{b}	7	40.8 ± 0.9^{d}
3.0	-	-	18.2 ± 0.4^{b}	8	20.1 ± 1.8^{b}	7	40.0 ± 0.9^{d}
5.0	-	-	5.8 ± 1.0^{a}	8	4.6 ± 1.2^{a}	-	-
-	0.5	-	52.0 ± 0.2^{f}	8	48.9 ± 0.9^{d}	7	68.3 ± 0.8^{f}
-	1.0	-	61.7 ± 1.1^{g}	7	64.3 ± 0.9^{f}	6	81.8 ± 1.0^{h}
-	3.0	-	42.3 ± 1.3^{e}	7	42.8 ± 1.1^{d}	6	60.3 ± 0.3^{f}
-	5.0	-	40.6 ± 0.9^{e}	8	31.3 ± 1.0^{c}	6	60.0 ± 0.3^{f}
-	-	0.5	12.2 ± 0.6^{a}	8	36.1 ± 1.0^{c}	7	51.2 ± 1.2^{e}
-	-	1.0	12.0 ± 0.5^{a}	8	36.0 ± 1.0^{c}	7	51.0 ± 1.4^{e}
-	-	3.0	10.9 ± 1.9^{a}	9	21.6 ± 1.8^{b}	8	28.3 ± 0.1^{b}
-	-	5.0	-	-	-	-	-
0.5	0.5	-	66.2 ± 0.4^{g}	6	63.5 ± 1.2^{f}	6	72.3 ± 0.7^{g}
0.5	1.0	-	80.1 ± 0.3^{i}	6	83.3 ± 0.2^{i}	6	91.6 ± 0.9^{i}
0.5	3.0	-	60.3 ± 1.1^{g}	7	61.9 ± 0.4^{f}	6	72.0 ± 1.3^{g}
0.5	-	0.5	31.3 ± 0.3^{d}	8	51.1 ± 1.4^{e}	7	21.3 ± 1.1^{b}
0.5	-	1.0	16.4 ± 1.0^{b}	8	19.8 ± 1.5^{b}	7	13.5 ± 1.0^{a}
0.5	-	3.0	16.7 ± 0.9^{b}	8	-	-	-
0.5	-	5.0	-	-	-	-	-

The results are based on ten replicates per treatment in three repeated experiments. ± indicates the SE values. Means followed by the same letters are not significantly different at P = 0.05.

The callus mass emerged from the disorganized PLB explants continued to proliferate after being transferred to the fresh medium. The calli continued to proliferate and gradually turned shiny whitish green elongated protocorms were obtained in the combination of BAP (1.0 mg/l) with NAA (0.5 mg/l), where 91.6% of PLBs were developed after 6 weeks of subculture. A large number of differentiating PLBs appeared as thick elongated clumps (Fig. 1C). The intensity of PLB development was found to be enhanced in this combination of PGR. But the combination of NAA with Kn found to be,

not effective in developing the PLBs from the callus mass, optimum of 21.3% of PLBs were formed in this combination after 7 weeks of subculture. The differentiation of PLBs into shoots readily occurred on the same medium or after transferred to a fresh medium with the same combination and concentrations of PGRs (Fig. 1D and 1E). The PLBs first differentiated with a protrusion and then the protrusion developed into a cotyledon. Subsequently the first leaf of the foliage emerged from the cotyledon. Succeeding leaves and roots were differentiated in an alternating sequence. The BAP alone and with the combination of NAA influenced the response of plant regeneration. The maximum PLB differentiation and formation of healthy plantlets (96.4%) was recorded in BAP (1.0 mg/l) with the combination of NAA (0.5 mg/l), in this combination the shoot and root differentiation were observed after 17 and 19 weeks of subculture respectively. The healthy plantlets were significantly developed due to the supplement of combination with BAP and NAA (Fig. 1F) (Table 2). The well-developed rooted plantlets were planted out in community pots containing an autoclaved potting mixture of vermiculite, charcoal, brick pieces and finely chopped coconut husk in the ratio of 1:1:1:1 in a greenhouse with a highest survival rate of 93.3% (data not shown).

Table 2. Effect of PGRs in ½ MS medium on differentiation of shoot and roots from PLBs in *D. crepidatum*

Concentration (mg/l)		Time taken for Shoot Development (Wks)	Time taken for root Development (Wks)	Percentage of Healthy Plantlets
BAP	0.5	20	24	73.8 ± 1.6[a]
	1.0	18	21	86.6 ± 1.0[b]
	3.0	20	24	70.0 ± 0.9[a]
NAA + BAP				
	0.5 + 0.5	17	20	90.9 ± 0.8[c]
	0.5 + 1.0	17	19	96.4 ± 1.3[c]
	0.5 + 3.0	18	22	90.2 ± 1.6[c]

The results are based on ten replicates per treatment in three repeated experiments. ± indicates the SE values. Means followed by the same letters are not significantly different at P = 0.05.

The propagation of orchids through indirect PLB formation may be more effective than through the direct formation in orchids. Because of the high frequency of somatic embryos formation and efficiency of embryo conversion into plants, somatic embryogenesis could be best accomplished through indirect PLB formation from callus (Zhao et al. 2008). Although callus exhibited great importance for mass propagation in many species and have been included in a number of orchids, tissue culture in orchids is not focused on callus because of the lower growth rate and necrosis in culture. In recent years callus lines can be successfully established in some orchid species and these calluses gives rise to plantlets through indirect PLB formation for mass propagation (Pyati 2020). It is reported that calluses could be formed from stem thin cell layer cultures

on ½ MS medium supplemented with Zeatin (0.5 mg/l) and BA (0.5 mg/l), where 41.42 and 16.83% of explants developed callus respectively in *Dendrobium aqueum* (Parthibhan et al. 2018). Similarly, the combination of TDZ (1.0 mg/l) with NAA (0.5 mg/l) of thin

Fig. 1. Plant regeneration through callus from bisected PLBs of *Dendrobium crepidatum*. A. Longitudinally bisected PLB segment (bar= 3 mm). B. Induction of callus on half MS with BAP (1.0 mg/l) + NAA (0.5 mg/l) after six weeks of culture (bar= 5 mm). C. Initiation of PLBs in the same combinations of PGR after six weeks of subculture (bar = 1 cm). D and E. Differentiation of PLBs into shoots (bar=1 cm). E. Well-development of plantlet after 19 weeks of subculture (bar=1.5 cm).

cell layer explants exhibited a higher frequency of embryogenic callus (58.6%) in *Dendrobium ovatum* (Pyati 2022). The shoots of *Dendrobium aurantiacum* cultured on MS medium supplemented with 2, 4-D (10 mg/l) induced callus (Ma et al. 2020). When the root tip explants of *Dendrobium huoshanense* cultured on ½ MS medium fortified with TDZ (1.0 mg/l) with 2, 4-D (1.0 mg/l) developed into yellow callus (Lee and Chen 2014). The internodal explants of *Anoectochilus elatus* cultured on Mitra et al medium (Mitra GC et. al) added with TDZ (1.0 mg/l) and NAA (0.5 mg/l) induced callus frequency of 77.8% (Ahmed Sherif et al. 2016). The thin cell layer culture in *Dendrobium* Hybrid Sonia induced maximum frequency of callus formation (40%) in MS medium supplemented with NAA (0.5 mg/l) with BAP (0.5 mg/l) (Mandal et al. 2020). Until there are many reports about plantlet regeneration from callus cultures via PLB formation in many orchids. In the present study a novel regeneration system of *D. crepidatum* was established. Calluses were induced from protocorm segments under various conditions. Of three PGRs tested for callus induction, the explants exhibited a higher frequency of callus formation in the medium supplemented with BAP (1.0 mg/l). The superiority of BAP in promoting callus induction was also observed in *D. fimbriatum* (Roy and Banerjee 2003).

Although the presence of PGRs are essential factors for callus induction and differentiation. In some of the orchid species the process of PLB regeneration from the callus and their subsequent development is independent of exogenous PGRs in *D. frimbriatum* (Roy and Banerjee 2003) and *Cymbidium* (Takamura et al. 2004). In contrast the calluses of many other orchid species require continuous exposure to specific PGRs for further differentiation and development of PLBs in *Cymbidium formosanum* (Lee and Lee 2003), *Pleione formosana* (Lu 2004) and Oncidium "Grower Ramsey" (Wu et al. 2004).

In vitro multiplication of orchids makes an effective contribution to saving rare species from extinction. The present aspect of investigation was mainly focus on, successful and efficient protocol for in vitro propagation of the threatened medicinally useful epiphytic orchid *D. crepidatum*. The method uses bisected PLBs cultured in vitro on ½ MS semi solid medium supplemented with cytokinin BAP and the auxin NAA. Regeneration through indirect formation of PLBs and further differentiation of plantlets. The technique is likely to be widely applicable, but the growth regulator component may need adjustment depending on the species, the physiological status and nutrient environment of the source material.

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