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Efficient plant regeneration of the Threatened Medicinal Orchid *Dendrobium macrostachyum* Lindl. using Protocorm like bodies

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Abstract

A process of organogenesis via callus with successful plantlet formation was developed for *Dendrobium macrostachyum*. Indirect organogenesis was achieved from in vitro derived protocorm explants. The explants were cultured on half MS (½ MS) medium fortified with different concentrations and combinations of plant growth regulators such as α-Naphthalene acetic acid (NAA), Benzyl adenine (BA) and Kinetin (Kin). The explants cultured on ½ MS medium supplemented with NAA and Kin alone or in combinations, directly differentiated into the seedlings even after 6 weeks of dark incubation. On the other hand, these explants cultured on ½ MS medium supplemented with BA alone and combination with NAA developed callus after 6 weeks of dark incubation. The optimum callus was developed in BA (4.44µM) and Combination of BA (4.44µM) with NAA (1.08 µM) induced 36.4% and 59.9% of callus respectively. This callus on subculture to the same media developed protocorm like bodies (PLBs). These PLBs later differentiated into healthy plantlets. A survival rate of 90% under greenhouse conditions was achieved.

Orchids which belong to the orchidaceae family comprising 30000-35000 species in 850 genera and is one of the largest families of flowering plants[2], diverse in morphology and fragrance are highly valued for cut flower production and decorative purposes because of their attractive colors. The genus *Dendrobium* is a highly evolved and diversified sympodial group of orchids comprising more than 1400 plant species and widely distributed from Southeast Asia to New Guinea and Australia. Several important orchid species are listed as endangered and under threat of extinction in the Red data book of the International Union for Conservation of Nature and Natural Resources (IUCN)[9]. But in the recent years the wild orchids as a whole facing threat of extinction owing to over collection, illegal trade, human population pressure and habitat destruction[15]. Orchids

requires a combination of different factors for reproduction in nature. The population of orchids through sexual means is a very slow process as its seeds lack endosperm and need fungal association for germination in nature. In nature 2-5% of seeds germinate, even if they do so, the seeds take a long time for their germination and any disturbance in the habitat or physical environment destroys the whole population. Orchids are heterozygous and their vegetative propagation through division of clumps of rhizomes, bulbs or by the rooting of off-shoots also takes long time and difficult to obtained desired number of plants. This difficulty in natural population drives the orchids to extinction. Though orchids are grown primarily as ornamental, many are used as herbal medicine, food and cosmetics in the different parts of the world[1]. The conservation of orchid is a matter of global concern for a verity of reasons including their low generation rate, slow growth and low germination of seeds.

Dendrobium macrostachyum Lindl. is an endemic threatened species distributed through South and South-West India and possess ornamental and pharmaceutical value[12]. It is commonly called as fringed tree orchid, is a species of epiphytic orchid with long narrow pseudobulbs, that lose their leaves as they mature and up to three whitish to lime green flowers with a hairy labellum. This orchid is high in flavonoid content and is of pharmaceutical importance and is used as pain killer by tying plant materials overnight on the parts of body to relieve from pain. The tendered shoot tips are used as an ear drops for earache and also to treat boils, pimples and other skin eruptions[10]. The stem extract is having high concentration of phenolics, high scavenger activity and high anti-inflammatory activity. This indicates that the *D. macrostachyum* can be a potential source of bioactive compounds[11]. Due to its ornamental and medicinal properties the natural populations of this species are declining due to unregulated collections and lower rate of propagation in nature. Therefore an in vitro propagation technique could be a useful approach for the mass scale propagation of *D. macrostachyum* through protocorm culture, which provides a useful way to reestablish plants in the wild for germplasm preservation as well for commercial propagation.

The mature seeds from the capsules of *D. macrostachyum* were cultured for the protocorm induction in half MS medium. The 4 weeks old protocorms of 2-3 mm in diameter were used as an explant for callus induction. These protocorms were inoculated in 100 ml Erlenmeyer flasks containing 30 ml half MS basal medium, which has macronutrients at half strength, 2% sucrose with α-Naphthalene acetic acid (NAA, 1.08, 2.7, 5.4, 10.8 µM), N^6- Benzyl adenine (BA, 0.27, 2.22, 4.44, 8.88 µM) and Kinetin (Kin, 2.32, 4.65, 9.29, 13.8 µM) alone or in combinations. The media were solidified with 0.8% (w/v) agar (Hi media, India). The pH was adjusted to 5.8 with 1N NaOH/HCl before autoclaving at 121°C for 15 min. cultures were maintained at 25±2°C, 50-60% relative humidity, initially cultures were maintained in the dark for about 6 weeks later provided 12/12 hrs. photoperiod of 40 µmol m^{-2} s^{-1} irradiance level provided by cool white fluorescent tubes (Philips, India). For the induction of callus and protocorm like bodies (PLBs) formation the explants were cultured on half strength MS medium supplemented with various concentrations and combinations

of PGRs such as NAA, BA and Kin. The regenerated PLBs were subcultured on to the same medium for further differentiation into plantlets.

Hardening :

The well developed plantlets with roots were obtained in the same induction medium on being left undisturbed. After 28 weeks of culture these plantlets were washed thoroughly and transferred to community pots containing the sterilized compost mixture of crushed bricks, charcoal, decaying litter and vermiculite in the ratio of 1:1:1:1, a top layer of finely chopped coconut husk. The plantlets were grown in the greenhouse covered with a polythene sheet for 3 weeks and sprayed with water to inhibit dehydration. The temperature was maintained 25±2°C and relative humidity at 80-85%. Plantlets were sprayed with 1/10 strength of MS medium solution without sucrose every 3 days for 2 weeks, plants were assessed after 3 months.

Statistical analysis :

Protocorm explants were cultured in 100 ml Erlenmeyer flasks with 10 replicates and the experiment was repeated twice. Regular observations of cultures were made once a week. Total number of protocorms responding with callus was recorded after 8 weeks and initiation of PLBs were recorded after 19 weeks and differentiation of PLBs into plantlets were recorded after 26 weeks of culture. The frequency of callus formation, PLB induction and differentiation of PLBs into plantlets were analyzed statistically using one way analysis of variance (ANOVA) and the mean ± standard error was represented and compared using Duncan's multiple range test at 5% level of significance using SPSS-PASW statistical program software version 18.0.0.

The seeds of *D. macrostachyum* were collected from the wild used to develop the protocorms. The seeds imbibed the nutrients swollen considerably after 2 weeks of culture. However, development of protocorms could be observed only after 4 weeks of culture in half strength MS (½ MS) medium. When these protocorms subcultured onto the same medium and ½ MS medium supplemented with NAA and Kin incubated in the dark did not show the development of callus. Instead, they remained as PLBs even after 6weeks of culture (Fig. 1A) when they are exposed to light, normally differentiated into plantlets (Fig. 1B; Table 1). On the other hand, these well developed protocorms were sub cultured in the darkness for 6 weeks on ½ MS medium fortified with BA alone and in combination with NAA developed callus. The ½ MS medium fortified with BA (4.44 µM) induced white callus at the basal end of the protocorm explants after 6 weeks of culture in darkness (Fig. 2A). Then this callus was exposed to light it was started proliferating for another 4 weeks of subculture (Fig. 2B), then it gradually turned green nodular and compact in texture after another 3 weeks of subculture (Fig. 2C). The efficacy of callus induction varied with the concentrations of BA in the medium. The maximum callus induction percentage was recorded on ½ MS medium containing BA (4.44 µM), where optimum of 36.4% of protocorm explants initiated callus after 6 weeks of culture. But the higher concentration of BA (8.88µM) was negative effect on inducing callus from the explants, in this concentration the protocorm explants became necrotic and died (Table-2). To increase callus

formation and proliferation combinations of BA and NAA were tested to look for possible synergistic effects. After 6 weeks of culture ½ MS medium fortified with BA (4.44 µM) and NAA (1.08 µM) showed the highest response of callus induction, where 59.9% of explants formed the callus. All the calli formed were originated from the basal region of the explants. The percentage of callus formation was generally low in media containing BA alone. In this study the combination of BA with NAA found to be more effective than other combinations for callus induction and proliferation.

Protocorm explant derived calli showed a high potential to induce PLBs formation from the proliferating calli. Numerous appearances of small nodular structures from the surface of the calli was observed after 16-19 weeks of subculture on medium containing BA alone and combination of BA with NAA (Table 2). The ½ MS medium containing BA (4.44 µM) induced the optimum percentage of PLB formation from the callus with 69.1% in comparison to other concentrations studied. But the combination BA (4.44 µM) and NAA (1.08 µM) was generally more effective than other PGRs in inducing PLBs from the callus. In this combination the green callus developed clumps of small globular structures after 16 weeks of subculture, totally 90.2% of callus developed into PLBs (Fig. 2D). On the other hand, the addition of NAA and Kin and combinations of these PGRs has no effect on the induction

Table-1. Effect of basal medium and PGRs on the direct differentiation of PLBs into plantlets in *D. macrostachyum*

	PGRs (µM)		Callus formation	Direct differentiation of PLBs into (Wks)		% of PLBs differentiated into Plantlets
	NAA	Kin		Shoot	Roots	
½ MS			-	8	12	71.8±0.8^{d}
	1.08		-	7	11	82.8±0.9^{cd}
	2.7		-	7	11	93.8±0.8^{b}
	5.4		-	6	9	100^{a}
	10.8		-	8	12	70.3±0.3^{d}
		0.47	-	7	12	80.1±1.1^{cd}
		2.33	-	7	12	92.8±0.9^{b}
		4.65	-	6	10	100^{a}
		8.80	-	8	13	70.0±0.3^{d}
	1.08	0.47	-	7	12	91.3±1.0^{b}
	1.08	2.33	-	6	9	100^{a}
	1.08	4.65	-	6	10	80.1±0.8^{cd}
	1.08	8.80	-	8	13	70.3±0.9^{d}

The values followed by different letters within column are significantly different from each other at 5% level.
Data represents means ± SE.

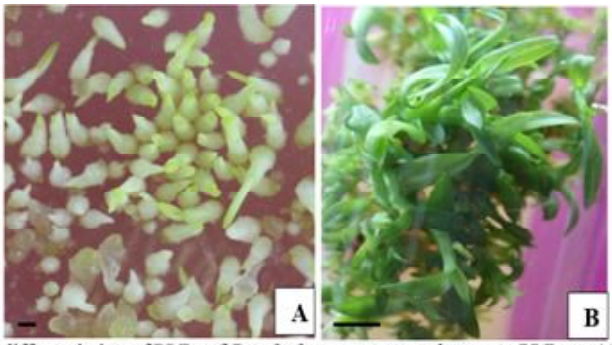

Fig.1. Development and direct differentiation of PLBs of *Dendrobium macrostachyum*. A. PLBs on ½ MS medium with Kin (4.65 µM) after 6 weeks of culture in dark (Scale bar=2mm). B. Direct differentiation of PLBs into shoots on ½ MS medium with Kin (2.33 µM) + NAA (1.08 µM) after 9 weeks of culture (Scale bar= 1cm).

of callus, but the protocorm explants cultured on to medium containing Kin, NAA and combinations of these PGRs directly differentiated in to plantlets. The ½ MS medium supplemented with Kin and NAA alone and various combinations failed to induce callus after 6 weeks of culture in the darkness. When these protocorms were illuminated after the dark period they gradually differentiated into shoot and roots after 6-8 weeks and 10-13 weeks of cultures respectively. In NAA (5.4 µM), Kin (4.65 µM) and combinations of Kin (2.33 µM) with NAA (1.08 µM) all the PLBs (100%) differentiated into plantlets (Table-1).

Table-2. Effect of BA and NAA on induction of callus, development of PLBs and differentiation of plantlets in *D. macrostachyum.*

PGRs (µM)		Initiation of Callus (Wks)	% of callus formation	Dev. of PLBs (Wks)	% of PLBs formation	Differentiation of PLBs (Wks)		% of differentiated PLBs into plantlets
NAA	BA					shoot	roots	
0.27	-	8	24.0±1.1^{d}	19	36.3±0.9^{k}	23	26	67.8±1.3^{hi}
2.22	-	8	24.6±1.8^{d}	18	51.6±1.3^{g}	21	24	80.6±1.0^{e}
4.44	-	6	36.4±0.9^{bc}	16	69.1±0.7^{de}	19	21	90.1±0.9^{b}
8.88	-	-	-	-	-	-	-	-
0.27	1.08	-	-	-	-	-	-	-
2.22	1.08	6	38.4±0.7^{b}	16	71.3±1.6^{cd}	19	22	84.9±0.9^{cd}
4.44	1.08	6	59.9±0.8^{a}	16	90.2±0.3^{a}	19	21	100^{a}
8.88	1.08	7	21.2±1.3d^{e}	17	46.3±1.2^{hi}	21	24	70.1±1.3^{gh}

The Values followed by different letters within column are significantly different from each other at 5% level. Data represents means ± SE.

The clumps of PLBs formed from the callus were subcultured for plantlet regeneration. The PLBs on subculturing on to the ½ MS medium supplemented with BA (4.44 µM) and combination of BA (4.44 µM) with NAA (1.08 µM) differentiated well grown shoots after 19 weeks of subculture (Fig. 2E). These well developed shoots differentiated roots after 21 weeks of subculture. In BA alone totally 90.1% of PLBs were differentiated into plantlets. Similarly, the ½ MS medium containing BA (4.44 µM) with NAA (1.08 µM) increased the frequency of plantlet regeneration relative to the tested PGRs, where 100% of the PLBs differentiated into plantlets after 21 weeks of subculture. The well grown shoots developed 3-4 roots in the same medium were removed from the cultures and washed thoroughly under tap water to remove all the medium attached to the roots. The plantlets were then transferred to small paper cups containing the potting mixture of charcoal, brick pieces, vermiculite and decaying litter in the ratio of 1:1:1:1 and placed under the greenhouse conditions (average temperature of 25±3°C and RH 80-90%) (Fig. 2F). Initially these cups containing plantlets were covered with translucent plastic sheet for first 30 days, the plantlets were watered daily and sprayed ½ MS salts without sucrose for alternate days. The plastic cover was then gradually removed to allow the relative humidity to decrease to 50-60%. The survival rate of 90% was recorded after 8 weeks of hardening.

In vitro regeneration of wild orchids using different explants has proven challenging regardless type of the explant used. In the present study efficient plant regeneration through direct protocorm differentiation and callus mediated organogenesis have been developed from the protocorm explants. Among the cytokinin concentrations tested the highest percentage of callus formation stood at 36.4%, which was observed on ½ MS medium fortified with BA (4.44 µM) in 6 weeks of dark treatment. However, the callus formation was suppressed by the higher concentrations of BA in the medium. This finding could be corelated with the positive effect of BA to induce callus in bisected protocorms of *Dendrobium candidum*. Callus induction was enhanced in the lower concentrations (0 to 8.8 µM) and reduced as the concentration of BA was increased (8.8 to 22.0 µM)[18]. The similar finding of 4 weeks dark treatment to in vitro grown root explants of *Dendrobium huoshanense* developed callus from the tip of roots on ½ MS supplemented with 2, 4- D and TDZ[4]. The stimulative effect of BA was also reported, it induced axillary shoots from the nodal explants of *Dendrobium macrostachyum*[12]. However, the direct induction of shoots from foliar explants was reported previously from the ½ MS medium supplemented with BA in *Dendrobium* hybrids[7]. On the other hand, incorporation of BA singly in MS medium for pseudobulb section culture showed higher PLB formation in *Lycaste aromatica*[13]. Although BA has proved to be effective cytokinin in orchid micropropagation, it was found to be very effective in inducing various morphogenic responses including callus formation, direct induction of PLBs, PLB differentiation and multiple shoot induction. In this report it was found to be very effective in inducing callus from the PLB explants and differentiation of PLBs into plantlets.

Fig. 2. Callus mediated PLB regeneration of *Dendrobium macrostachyum*. A. Callus initiation from protocorm in MS medium supplemented with BA (4.44 µM) after 6 weeks in dark (Scale bar= 0.5 mm). B. Development of callus on same medium after 4 weeks of subculture (Scale bar= 0.5 mm). C. Development of green callus on same medium after 3 weeks of subculture (Scale bar= 0.5 mm). D. Development of PLBs in MS medium fortified with BA (4.44 µM) + NAA (1.08 µM) after 16 weeks of subculture (Scale bar= 1cm). E. Differentiation of Shoots on same medium after 19 weeks of subculture (scale Bar=1cm). F. Well acclimatized plantlet in green house.

In this research work BA and NAA in combination were beneficial in inducing the callus from the explants than the other PGRs studied. The induction rate was varied with the type and concentrations of PGRs. The protocorm explants showed the maximum callus formation and proliferation in ½ MS medium supplemented with NAA + BA. These PGRs involved either directly or indirectly in several morphological and physical responses in plant tissues and these are reported to promote the formation of callus, PLBs, shoots

and development of plantlets from different explants from the various species of orchids. The previous report of synergistic effect of NAA + BA stimulated the formation and proliferation of callus in ½ MS medium supplemented with NAA + BA was the highest among all combinations of PGRs tested[18]. The similar synergistic effect of NAA + BA produced most viable PLBs and these grew well and developed the sheath leaves more quickly than other PGRs in *Dendrobium huoshanense*[4]. The combination effect of NAA + BA was also reported in *Dendrobium wangliangii*, where they exhibited with the highest shoot differentiation frequency from the protocorms[19], favored PLB mediated regeneration in *Cymbidium eburneum* from the leaf segments[14], quick differentiation of PLBs in *Dendrobium cariniferum*[5], neo-formation and regeneration response of stem node segments in *Dendrobium chrysotoxum*[3] and facilitated the initiation and elongation of shoot buds from the pseudobulb cultures in *Changnienia amoena*[17]. However, the findings in this research illustrated that the combination of BA and NAA strongly stimulated PLBs formation from the protocorm derived callus. These formed PLBs continued to differentiated into plantlets in the same medium. This confirmed the positive role of NAA and BA in inducing callus-PLB-plant regeneration in *D. macrostachyum* and the protocol developed during present investigation may provide a suitable system for conservation of this valuable and threatened orchid species.

In the present experiment NAA and Kin individually and in combinations did not show pronounced results of callus formation in *D. macrostachyum*. But in these PGRs the protocorm explants directly differentiated into the plantlets. But the results obtained in this study were contradictory with the results of NAA + Kin, this combination in MS medium was effective in initiating callus on *Dendrobium nanum* rhizome buds[6]. Similarly, this combination of PGRs also reported to induce organogenic shoots from the internodal stem segments of *Malaxis acuminata*[8]. In the present study the Kin alone did not show the formation of callus. These obtained results were conformity with the earlier report of the Kin was ineffective to induce shoots from leaf explants of *Dendrobium* cultivars Sonia 17 and 28 on ½ MS medium[7]. The NAA enriched ½ MS medium favored the proliferation of PLBs. Similarly, ½ MS medium containing Kin was superior for conversion of PLBs into plantlets in *Dendrobium* cultivars Sonia 17 and 28[7]. The Kin alone in the Mitra medium was more effective in inducing multiple shooting[16]. These previous results were in conformity with the results in *D. macrostachyum*, where NAA and Kin were stimulated the conversion of PLBs into plantlets.

This study is to establish a reliable protocol for regenerating *D. macrostachyum* via callus mediated PLB formation and plantlet regeneration. Various treatments of individual and combinations of PGRs exhibited effective growth promoting factors like callus induction, PLB formation and differentiation. Individual levels of BA, NAA and Kin alone and in combinations successfully produced the healthy plantlets. Among the tested PGRs BA (4.44 µM) and combination of NAA (1.08 µM) + BA (4.44 µM) induced highest callus formation of 36.4% and 59.9% respectively. The PLBs were developed from the callus and later differentiated into plantlets. However, the ½ MS medium with Kin (4.65 µM) and NAA

(5.4 µM) individually and in combination of Kin (2.33 µM) + NAA (1.08 µM) the protocorm explants directly differentiated into plantlets (100%) without the intervention of callus. The plants obtained in the present investigation could be used either as material for further research or to satisfy horticultural demand, thus reducing pressure on wild populations. Therefore, this research represents a direct contribution to the conservation and sustainable use of this valuable natural resource.

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