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IN VITRO REGENERATION OF AN EPIPHYTIC ORCHID, *DENDROBIUM APHYLLUM* USING LEAF EXPLANTS

M Rahamtulla[1] and S M Khasim

Department of Botany and Microbiology, Acharya Nagarjuna University, Nagarjuna Nagar- 522 510, Guntur, Andhra Pradesh, India
[1]Department of Botany, K B N College (Autonomous), Vijayawada- 520 001, Andhra Pradesh, India

Abstract

The present investigation deals with *in vitro* regeneration of an epiphytic orchid, *Dendrobium aphyllum* (Roxb.) C.E.C Fischer using leaf explants through Protocorm-like bodies (PLBs) proliferation. The species collected from the Darjeeling Himalaya (Latpanchar forest, Kurseong) has been classified as *Least Concern* by the IUCN Red List (2020). Ethnomedicinally, this plant has been used for curing skin disorders, scorpion bite, and wounds. Additional presence of 2,4-D/BAP (0.5, 1.0, 1.5, 2.0 mgl^{-1}) in MS medium proved obligatory for inducing callusing in the leaf explants; callusing was however, highly promoted in the former combination. Further, the combination of both these additives showed synergistic effect in inducing better response. The growth and differentiation of callus tissues derived from leaf explants was further investigated on MS medium supplemented with different concentrations of 2,4-D and BAP. Optimal results were obtained on 2, 4-D (1.5 mgl^{-1}) and BAP (1 mgl^{-1}) combination; the callus differentiated Protocorm-like bodies (PLBs). After subculturing, calli initially exhibited 3 different appearances: light green, yellow, and light brown. The light green calli grew rapidly, consisting compact mass of isodiametric granules, and produced 1.2 PLBs on an average per 0.5 cm diameter of callus on MS medium without growth regulators. Subsequently, the PLBs were inoculated on 4 different nutrient media such as MS, Gamborg B5, Vacin and Went, and M for the differentiation of shoots and roots. After 6 wks, about 4.2 plantlets were formed from each PLB on MS basal medium; the highest shoot length (7.4 cm) and a greater number of leaves (3.8) on average were observed in the plantlets in this combination. The regenerated plantlets displayed maximum survival frequency (100%) in the potting medium with the combination of charcoal and brick pieces. The method can be successfully used for mass propagation of the species.

Introduction

DENDROBIUM APHYLLUM C.E.C. Fischer (Orchidaceae) is therapeutically potent and highly preferred as a medicinal plant by traditional healers in the Darjeeling Himalaya and other parts of the NorthEastern region of India (Hossain, 2009; Rahamtulla *et al.,* 2020a,b,c; Singh *et al.,* 2019). Further *D. aphyllum* has also floricultural potential for its magnificent flowers of great delicacy and beautiness. It is well known for its leaflessness during its blooming stage and the whole stem gets covered by pale, pinkish, and fragrant flowers. Indiscriminate collections, unprecedented destruction of its natural habitat, over-exploitation, and unauthorized trade has caused this species to register its name in the IUCN red list of threatened species (Romand-Monnier, 2013). The present status of *D. aphyllum* is the least concern (LC) and its population trend is decreasing at an alarming rate. In China, it is also reported as an endangered species according to the Chinese Red List (Fu and Chin, 1992; Gao *et al.,* 2014).

Propagation through orchid seeds especially *in vivo* is a slow process. Alternatively, in most cases, seeds, leaf, nodal explants, shoot tips, and pseudobulbs have been employed *in vitro* for the regeneration of the plantlets (Anuprabha and Pathak, 2020; Bhowmik and Rahman, 2020; Du *et al.,* 2012; Kumari and Pathak, 2021; Lalduhsanga *et al*., 2021; Roy and Banerjee, 2003; Sembi *et al*., 2020; Sunita *et al*., 2021; Thakur and Pathak, 2021; Vasundhra *et al*., 2021). However, leaf explants prove better than the other explants because they can be procured without sacrificing the mother plant and are available throughout the year. The genus *Dendrobium* is sympodial and propagated through methods like cutting, separation of off-shoots, and keikis, produced from the stem, which are very slow (Hossain *et al.,* 2013). Hence, the present study is aimed to develop an effective regeneration protocol using leaf explants employing various combinations of 2,4-Dichlorophenoxy acetic acid (2,4-D) and 6-Benzylaminopurine (BAP).

Material and Methods

Plant Material

Plant samples of *D. aphyllum* were collected from Latpanechar forest, Kurseong, Darjeeling Himalaya (West Bengal), and grown in the Herbal garden of Botany and Microbiology Department, Acharya Nagarjuna University, Guntur. Leaves procured from well-developed plants were surface-sterilized with an aqueous solution of $HgCl_2$ (0.5%) for 5-7 min in a laminar airflow cabinet. Later, they were cleaned with

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ethanol (50%) for 2-3 min and rinsed thrice with autoclaved distilled water.

Callus Induction

Leaf segments (0.5 cm each in length and width) were placed on MS basal medium (Murashige and Skoog, 1962). Plant growth regulators (PGRs) such as 2,4-D and BAP in different combinations (0.5, 1.0, 1.5, 2.0 mgl^{-1}) were also used alone and in different combinations in the medium. In each treatment, three 55 ml glass tubes with 15 ml of the medium were taken. One leaf explant was placed in each glass tube and it was closed with a plastic cap. Parameters such as callus growth (diameter) and morphology of callus were recorded.

Callus Subculturing and Proliferation of PLBs

The growth and differentiation of callus tissues derived from leaf explants was further investigated on MS medium supplemented with different concentrations of 2,4-D and BAP. Optimal results were obtained on 2, 4-D (1.5 mgl^{-1}) and BAP (1 mgl^{-1}) combination; the callus differentiated Protocorm-like bodies (PLBs). Later, the 6-wk-old callus was segmented into approximately 0.3-0.5 cm diameter segments. About 10 callus segments were placed on 12 ml of culture medium poured into Petri plates with the same combinations of 2,4-D and BAP. Five replicates were maintained per treatment. After 6 wks, the percentage of Protocorm-like bodies (PLBs) formation was calculated.

The percentage of PLB formation was calculated by using the following formula:

$$\text{PLB formation (\%)} = \frac{\text{Number of segments giving PLBs}}{\text{Number of segments cultured}} \times 100$$

Regeneration of Plantlets from PLB

The PLBs obtained were subsequently inoculated on 4 different nutrient media like MS, M, Vacin and Went, and Gamborg B5 for the differentiation of shoots and roots. Later, the time taken for initiation of shoots and the average number of shoots per PLB were recorded. Further, subculturing was done on the same medium for the development of the complete plantlets. Plant growth was estimated by measuring the stem length, counting the number of leaves and roots formed, root length, and fresh weight. The final values were calculated by taking the average of five independent replicates of each treatment.

Acclimatization of In Vitro Derived Plants

Three-months-old *in vitro* derived plants were placed under natural conditions for acclimatization. Firstly, the plantlets were cleaned with tap water to remove the stuck agar, treated with Diathane M-45 (0.2%) and transplanted into small pots. About three different types of Diathane-treated materials such as coco peat, charcoal, and red brick pieces were used as potting media separately and also in combinations. The potted plantlets were arranged in groups and placed on a wooden table, where the temperature ranged between 22°C to 25°C, illumination between 1200-1400 lux, and the relative humidity of 80-90% had been maintained. Potted plantlets were sprayed with water daily, and foliar spray of Nitrogen, Phosphorus, and Potassium (1:1:1) was given once in a week. Observations on the vegetative growth parameters such as the height of the stem, number of leaves and roots, root length, and fresh weight were recorded at regular intervals, in each of the treatments.

Statistical Analysis

The survival percentage of plants was calculated after 2 months of potting. The final values were calculated by taking the average of ten independent replicates of a similar treatment. Statistical analysis of the data was performed using Statistics Kingdom Software (http://www.statskingdom.com).

Results

In Vitro Regeneration using Leaf Explants

Additional presence of 2,4-D/BAP (0.5, 1.0, 1.5, 2.0 mgl^{-1}) in MS medium proved obligatory for inducing callusing in the leaf explants; callusing was however, highly promoted in the former combination. Further, the combination of both these additives showed synergistic effect in inducing better response. Amongst the treatments, 2,4-D (1.5 mgl^{-1})+BAP (1.0 mgl^{-1}) combination showed green compact callus with a diameter of about 1.90 cm whereas, other treatments exhibited moderate effects on callus growth. Callus was not observed in the case of the control treatment (Fig. 1A).

2,4-D Treatment

Among the different concentrations used, 2,4-D (0.5 mgl^{-1}) induced low callus growth (0.28 cm) with yellow and nodular morphology. At 1.0 mgl^{-1} of 2,4-D, the callus was yellow, nodular and its diameter was found to be higher up to 0.64 cm. However, callus initiation was observed after 18-20 days when this auxin was used at 0.5 mgl^{-1}. The nodular structures which develop at this concentration failed to form PLBs. Callus size was found to be high (1.49 cm) in 2,4-D (1.5 mgl^{-1}) (Fig. 1B). Though the number of days (13-15 days) for callus initiation remained the same as compared with 2,4-D (1.0 mgl^{-1}), green compact callus was observed at this

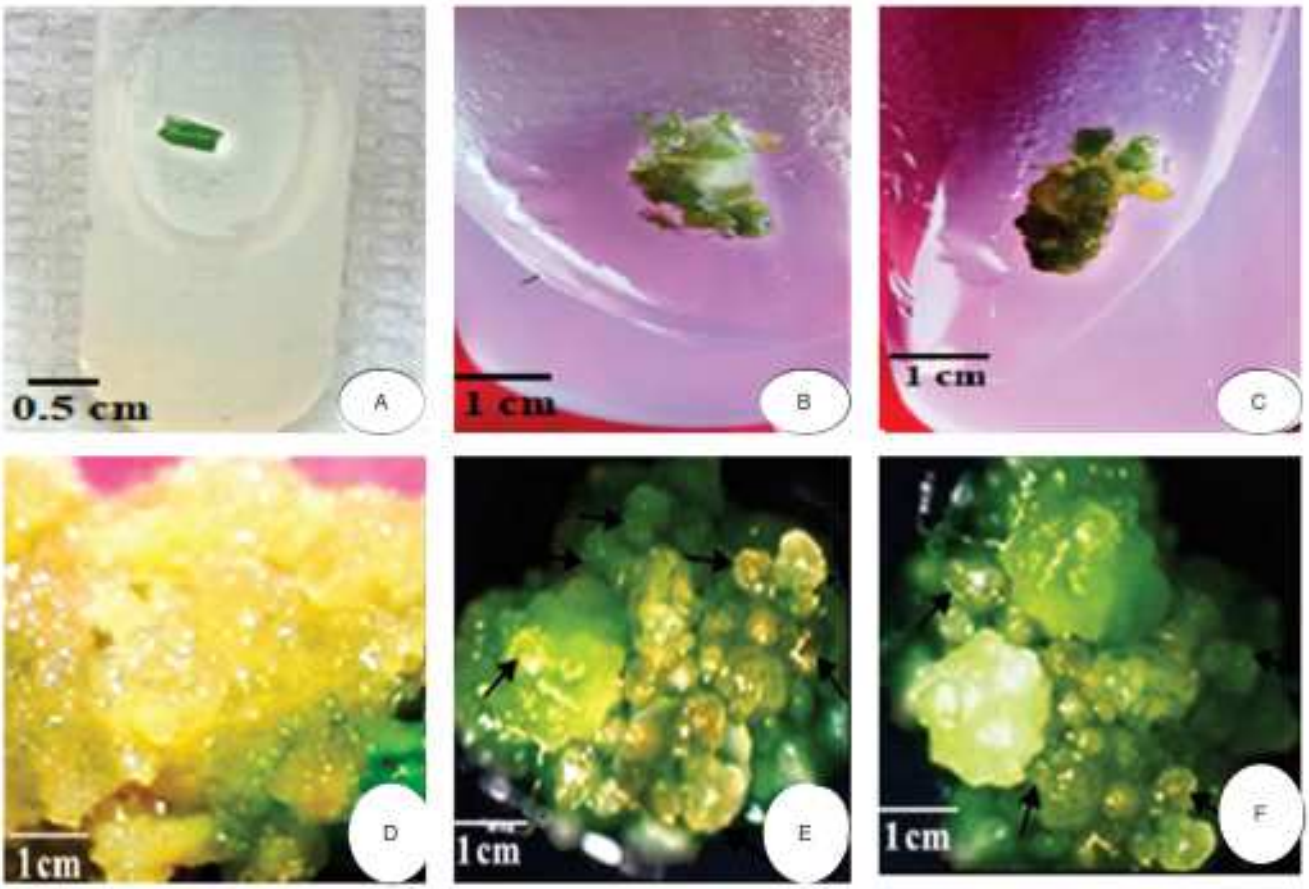

Fig. 1. A-F. Regeneration of leaf explants of *Dendrobium aphyllum* and the proliferation of *PLBs* from the callus segments: A, Leaf explant on MS medium without PGRs; B, Green compact callus [MS+2,4-D (1.5 mgl^{-1})]; C, Green compact callus [MS+2,4-D (1.5 mgl^{-1})+BAP (1.0 mgl^{-1})]; D, Less number of PLBs from yellow coloured callus on MS medium without PGRs; E, Appearance of 7-8 PLBs from callus segments [MS+2,4-D (0.5 mgl^{-1})+BAP (1.5 mgl^{-1})]; F, Appearance of 6-7 PLBs from callus segments [MS +2,4-D (0.5 mgl^{-1})+BAP (1.0 mgl^{-1})].

concentration. The above auxin when used at 1.5 mgl^{-1} concentration in the medium, was found to be the best amongst other treatments. Further, at high concentrations of this auxin, the callus growth was found to be moderate to poor callus induction.

BAP Treatment

Amongst the different concentrations of BAP (0.5, 1.0, 1.5, and 2.0 mgl^{-1}) used, low levels of BAP like 0.5 mgl^{-1} and 1.0 mgl^{-1} were suitable concentrations for the callus induction.

2,4-D and BAP Treatment

Combined treatments of 2,4-D and BAP showed an additive effect on the increasing callus growth. MS medium supplemented with 2,4-D (1.5 mgl^{-1}) and BAP (1.0 mgl^{-1}), produced a green compact callus with a diameter of 1.92 cm (Fig. 1C). However, a green compact callus (1.71 cm) also observed in MS+2,4-D (1.5 mgl^{-1})+BAP (0.5 mgl^{-1}) medium. Incidentally, it was observed that when the concentration of BAP exceeded the concentration of 2,4-D, the additive effect drastically reduced and became nullified. In contrast, when the concentration of 2,4-D exceeded the concentration of BAP, the additive effect was operative and there was an increment in the callus growth. Lower concentrations of 2,4-D (0.5 mgl^{-1}) and BAP (0.5 mgl^{-1}) induced green compact callus on leaf explants. However, the combination of 2,4-D (1.5 mgl^{-1}) and BAP (1.0 mgl^{-1}) proved as the optimal for early callus induction.

Callus Subculturing and PLBs Proliferation

After subculturing on the optimal medium for 3 wks, the calli initially exhibited three different appearances such as light green, yellow, and light brown. Further, the callus mass differentiated protocorm-like bodies (PLBs). The light green calli grew rapidly, consisting of a compact mass of granules, and produced 1.2 PLBs on an average per 0.5 cm diameter of callus on basal MS medium. The yellow-coloured callus developed granules slowly and produced less number of PLBs (Fig. 1D); the brown callus showed initial multiplication

but turned dark brown. However, the number of PLBs increased when selective growth regulators (2,4-D and BAP) were used in different combinations. Therefore, both 2, 4-D and BAP promoted the good proliferation of PLBs. When the callus segments from the optimal medium were transferred to the MS medium supplemented with 2,4-D, the percentage of PLBs formation slightly increased. 2,4-D (1.5 mgl^{-1}) favoured two PLBs on average from the 0.5 callus segments. The light green calli have rapidly produced 2-3 PLBs. Further 2,4-D (2.0 mgl^{-1}) showed a decline in PLB formation. However, it did not show any effect on the yellow and brown coloured calli.

BAP showed a good effect on PLB formation as compared to 2,4-D. BAP (1.5 mgl^{-1}) produced 3.6 PLBs on an average from the callus segments. A high concentration of BAP generated a smaller number of PLBs. Interestingly, in the medium supplemented with BAP, yellow-coloured calli produced light green colour granules which formed PLBs. However, the combined treatment of 2,4-D and BAP generated a greater number of PLBs. Medium supplemented with 2,4-D (0.5 mgl^{-1}) and BAP (1.5 mgl^{-1}) produced 7.6 PLBs on an average from the callus segments and the highest per cent (76%) of PLB formation was observed (Table 1; Fig. 1E). Similarly, 2,4-D (0.5 mgl^{-1})+BAP (1.0 mgl^{-1}) generated 6.4 PLBs on an average and showed 64% PLB formation (Fig. 1F). A low concentration of 2,4-D (0.5 mgl^{-1}) and a high concentration of BAP (1.5 mgl^{-1}) promoted PLBs formation.

Subculturing of PLBs

The PLBs were transferred to four different nutrient media. MS medium showed early shoot formation (27.6 days). Vacin and Went, M, and Gamborg B5 media showed regeneration after 31.4, 35, and 40.2 days respectively (Table 2). PLBs generated multicellular epidermal bulges, however, the exact origin of shoots could not be ascertained. However, a bright green area with high meristematic activity was observed at the place of origin of the shoots. In the present study, PLBs have regenerated additional shoots and about 4.2 shoots per PLB had formed on an average on MS medium (Table 2; Fig. 2A). PLBs on Vacin and Went medium produced 3.4 shoots on an average per PLB. On M and Gamborg B5 media, 2.6 and 2.2 shoots per PLB were observed respectively. PLBs subsequently developed into plantlets.

Fig. 2. A-D. Effect of MS medium and potting mixture on *Dendrobium aphyllum* plantlets*:* A, Differentiation of PLBs; B-C, Plantlets; D, Plantlets transferred to a pot with potting mixture comprising brickbats and charcoal pieces.

Complete plantlets were obtained after subculturing of these PLBs in the same nutrient medium. After 6 wks, about 4.2 plantlets were formed from each PLB on MS medium. The height of the stem and number of leaves per plantlet greatly varied amongst the different nutrient media tested. The highest shoot length (7.4 cm) and a greater number of leaves (3.8) on an average were observed in the plantlets (Fig. 2B, C); plantlets exhibited a greater number of roots, high root length, and a high amount of fresh weight in MS medium. Plantlets cultured on M medium and Vacin and Went medium, had showed similar number of leaves. The plantlets grown on the M medium remained dwarf (2.6 cm) as compared to the plantlets (5 cm) on the Vacin and Went medium. The number of roots, root length, and fresh weight of plantlets were almost similar in M medium and Vacin and Went media. Plantlets cultured on Gamborg B5 medium showed poor plant growth.

Effect of Various Potting Mixture on In Vitro Derived Plantlets and their Acclimatization to the Natural Conditions

The effect of different potting mixture such as coco peat, charcoal, and brick pieces was tested with respect to the growth-promoting ability. Further, appropriate quantities of water and enough air circulation for the roots were provided to young plantlets. The plantlets with well-formed leaves and

Table 1. Effect of combination of 2,4-D and BAP on callus differentiation and per cent PLBs formation.

2,4-D (mgl^{-1})	BAP (mgl^{-1})	Total number of callus segments cultured	Number of cultured segments differentiating PLBs						Total number of callus segments differentiating PLBs	PLBs formation
		($R_1+R_2+R_3+R_4+R_5$)	R_1	R_2	R_3	R_4	R_5	Mean±S.E	($R_1+R_2+R_3+R_4+R_5$)	(%)
0 (Control)	0	50	2	1	1	2	0	1.2±0.374	6	12
0.5	0.5	50	3	5	3	4	3	3.6±0.400	18	26
0.5	1.0	50	7	7	6	6	6	6.4±0.244	32	64
0.5	1.5	50	8	7	8	8	7	7.6±0.244	38	76
0.5	2.0	50	6	4	4	5	3	4.4±0.500	22	44
1.0	0.5	50	5	4	5	4	5	4.6±0.244	23	46
1.0	1.0	50	5	5	4	5	4	4.6±0.244	23	46
1.0	1.5	50	6	6	4	6	5	5.4±0.400	27	54
1.0	2.0	50	5	4	5	4	4	4.4±0.244	22	44
1.5	0.5	50	4	3	5	3	2	3.4±1.14	17	34
1.5	1.0	50	3	5	3	2	2	3.0±0.547	15	30
1.5	1.5	50	4	3	2	1	2	2.4±0.509	12	24
1.5	2.0	50	2	1	2	3	2	2.0±0.316	10	24
2.0	0.5	50	2	1	3	2	1	1.8±0.374	9	18
2.0	1.0	50	2	1	2	2	2	1.8±0.20	9	18
2.0	1.5	50	1	2	2	2	1	1.6±0.244	8	16
2.0	2.0	50	2	2	2	2	1	1.8±0.20	9	18

roots got established within 20 days of transplantation in the pots. Plantlets grown in the coco peat displayed poor results and showed retarded growth. Amongst the various potting mixture used, the combination of charcoal and brick pieces supported the maximum survival frequency (100%) and growth of roots and leaves (Fig. 2D). The combination of charcoal and brick pieces proved as an ideal potting mixture for the growth of *in vitro* derived *D. aphyllum* plantlets.

Discussion

Callus-mediated PLB Induction from Leaf Explants

Callus Induction

In the present study, the callusing response of leaf explants was encouraging as callus production was observed to be high in medium supplemented with 2,4-D (1.5 mgl^{-1}); the callus initiation appeared after 14±0.57 days and showed a growth diameter of 1.496 cm. Zhao *et al.* (2008) also reported that amongst the 2,4-D treatments, 2,4-D at 1.5 mgl^{-1} showed the highest percentage of callus induction in *Dendrobium candidum*. However, the studies by Budisantoso *et al.* (2017) reported that 2,4-D at 2 ppm was the best concentration for quickening the callus growth time (14.3 days) in *Vanda* spp. Van Minh *et al.* (2017) investigated callus formation in *Oncidium* (Vu Nu Orchid) on MS medium containing various concentrations of 2,4-D and concluded that callus induction varied depending on the concentration of 2,4-D in the culture media. Jheng *et al.* (2006) reported that low concentrations of 2,4-D influenced cell proliferations in callus cultures of *Oncidium* 'Gower Ramsey,' as reported in the present investigation. However, higher concentrations of 2,4-D showed poor callus induction. Additional presence of BAP showed slight effect during callus formation as compared to 2,4-D containing medium. Nonetheless, a small amount of BAP (0.5 mgl^{-1}) improved callus formation. Similar observations were made by Binh and Tai (2018). Different concentrations of auxins along with cytokinins could produce exuberant callus growth. Several studies reported that auxin and cytokinin are required for the maintenance of most of the orchid callus cultures (Borah *et al.,* 2015; Chen and Chang 2000; Huan *et al.,* 2004; Lee and Lee 2003; Lin *et al.,* 2000; Zeng *et al.,* 2013). In the present study, it was observed that 2,4-D in combination with BAP was more efficient than 2,4-D alone in inducing callus generation in leaf explants of *D. aphyllum*. The combination of auxin and cytokinin [2,4-D (1.5 mgl^{-1})+BAP (1.0 mgl^{-1})] in MS medium generated the best callus induction, forming a green compact callus with a growth diameter of 1.92 cm within 8-10 days. Janarthanam and Seshadri (2008) also reported that MS medium fortified with BAP and 2,4-D was more successful in producing callus from *Vanilla planifolia* leaf explants.

Present studies indicated that when the BAP concentrations used was similar to the 2,4-D, the additive impact was greatly reduced and eventually zeroed. However, when the concentration of 2,4-D was higher than that of BAP in the medium, the synergistic effect was evident, and hence the callus growth was increased. Auxins and cytokinins act synergistically to promote either cell division or cell expansion depending upon the factors within the cell which react with these hormones (Gamborg *et al.,* 1977; Setterfield, 1963; Steinhart *et al.,* 1961).

Proliferation of Protocorm-Like Bodies (PLBs)

After three wks of subculturing on the optimal medium [2,4-D (1.5 mgl^{-1})+BAP (1.0 mgl^{-1})], the light green compact calli initially had three different appearances: light green, yellow, and light brown. In the control treatment, the light glistening green callus remained compact, with some growing points developing into pear-shaped PLBs whereas yellow and brown coloured calli showed no response. Plant regeneration from callus culture is typically accomplished via an intermediate PLB phase. This type of morphogenetic development was noticed by several workers (Chen and Chang, 2000; Lee and Lee, 2003; Roy and Banerjee, 2003; Zhao *et al.,* 2008). With respect to the PLB differentiation, two kinds of orchid calli are generally noticed (Roy *et al.,* 2007). Some orchid species do not need an exogenous supply

Table 2. Regeneration response of Protocorm like bodies (PLBs) on different nutrient media after 2 months of inoculation.

Nutrient media	Number of days taken for the initiation of shoots from PLBs					Average number of days taken for the initiation of shoots from PLBs	Number of shoots formed per PLB					Average number of shoots formed for PLB
	R_1	R_2	R_3	R_4	R_5	(Mean±S.E)	R_1	R_2	R_3	R_4	R_5	(Mean±S.E)
MS	28	27	28	27	28	27.6±0.244	4	4	4	5	4	4.2±0.20
M	36	34	36	35	34	35.0±0.447	4	2	3	2	2	2.6±0.40
Vacin and Went	31	32	32	30	32	31.4±0.400	3	4	4	2	4	3.4±0.40
Gamborg B5	39	38	40	41	43	40.2±0.860	3	2	2	2	2	2.2±0.20

of PGRs to produce PLBs (Chen and Chang, 2000; Huan *et al.,* 2004; Roy and Banerjee 2003) whereas others need PGRs (Lee and Lee 2003; Lin *et al.,* 2000; Lu, 2004; Wu *et al.,* 2004). In the present investigation, even though PLB formation was quite effective in the control treatment, the rate was increased further with the addition of BAP and 2,4-D. This observation is in accordance with the studies of Roy *et al.* (2007).

Only a slight change in the rate of PLB proliferation from light green calli was observed in the MS medium amended with 2,4-D. However, like the control treatment, it had no effect on yellow and brown calli. BAP, unlike 2,4-D, had a positive effect on PLB proliferation from light green and yellow coloured calli. Studies by Chen *et al.* (2002) on the efficient production of PLBs from *Epidendrum radicans* concluded that BAP (1 mgl^{-1}) showed the best response for enhancing the number of PLBs. In the present study, BAP (1.5 mgl^{-1}) produced a good response in the percentage of PLB formation (Table 1). Cytokinins, alone or in combination with auxins, have been shown to induce PLBs in the majority of orchids (Bhattacharyya *et al.,* 2014; Dohling *et al.,* 2012; Zhao *et al.,* 2008). MS medium fortified with 2,4-D (0.5 mgl^{-1}) and BAP (1.5 mgl^{-1}) proved the best combination as it produced 7.6 PLBs on an average (76%), on the callus explants.

Response of PLBs and Plantlets on Different Nutrient Media

Well-developed PLBs were transferred to four different nutrient media. PLBs are capable of forming the shoot and root meristem. As a result, these structures can easily transform into plantlets (Kalyan and Sil, 2015; Ng and Saleh, 2011). In the present study, the prolonged culture of PLBs on the optimal medium for 3 months showed little signs of shoot formation. However, PLBs responded well after being transferred to different basal media. At the point of origin of the shoots, a bright green area with high meristematic activity was observed. These bright green areas (protuberances) of the PLBs developed into shoots. Here, PLBs produced more shoots, with an average of 4.2 shoots per PLB (Table 2) in the MS medium and it was the best as compared to other basal media. Further, subculturing on the same basal medium resulted in shoot enlargement and root formation. After 6 wks, approximately 4.2 plantlets were formed from each PLB on MS basal medium. However, studies by Hong *et al.* (2010) reported that PLB aggregates of *Zygopetalum mackayi* gradually regenerated shoots after being transferred onto half-strength MS basal medium in light, for 1-2 months. Their studies further concluded that PLBs proliferated more in the presence of Thidiazuron (TDZ), but shoot development from PLBs was retarded.

In all the basal media (without PGRs) used in the present study, PLBs regenerated shoots and roots, and finally the complete plantlets. However, Plantlets grown on MS medium showed greater fresh weight, after one month as compared to other basal media. Chen and Chang (2000) reported that embryo-derived PLBs in *Oncidium* 'Sweet Sugar' had regenerated plantlets on half-strength PGR-free MS basal medium for 3-4 wks. A better response of *D. aphyllum* plantlets on the MS medium suggested the importance of an interaction (compatibility) between the orchid genotype and its nutrient regime in the culture medium (Reddy *et al.,*1992). The superiority of MS medium for plantlet growth may be due to its high nitrate content such as ammonium nitrate and potassium nitrate (Dutta *et al.,* 2011).

In vitro-derived plantlets complete with roots and leaves, got established within 20 days of transplantation in the pots; their subsequent performance, however, varied with the type of the potting mixture used. Amongst the various potting mixtures tried, the one comprising charcoal and brick pieces supported the maximum survival frequency of *in vitro*-derived plantlets of *D. aphyllum* due probably to its support to the plantlets and providing enough air and moisture to the roots. Similar observations were made earlier by Bhattacharyya *et al.* (2014) and Paul and Rajeevan (1992).

Conclusion

The presently developed *in vitro* protocol was proved very effective during regeneration of an epiphytic orchid, *Dendrobium aphyllum* using leaf explants. Each PLB can yield approximately four plantlets after six wks and if the callus is subcultured, a large number of plantlets can be obtained. The protocol would be highly beneficial for the mass production of the presently investigated species, *D. aphyllum* and other similar orchids to counteract population decline in their natural habitats.

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