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Direct and Callus-mediated Protocorm-like Body Induction and High Frequency Adventitious Shoot Regeneration in an Endangered Orchid – *Dendrobium farmeri* Paxt. (Orchidaceae)

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ABSTRACT

A rapid and reliable micropropagation method was developed for *Dendrobium farmeri* Paxt. using shoot-tip culture system. Shoot-tip explants were excised from aseptically raised 3-month old seedlings and were cultured on modified Knudson's C nutrient solution supplemented with different concentrations of α-naphthaleneacetic acid (NAA) and thidiazurone (TDZ) in factorial combinations. After 3-4 weeks of culture, both direct as well as callus mediated protocorm-like body (PLB) formation from the basal cut ends of explants were observed. Optimum frequency of callusing (51.28%) was recorded in the presence of 2 μM each of TDZ and NAA. Following transfer to PGR-free medium, the callus exhibited vigorous growth and regeneration of PLBs and was maintained for more than 1 year without any loss of embryogenic potential. TDZ at higher concentrations showed an inhibitory effect on callus-mediated PLB formation and caused the extensive necrosis of callus. For direct initiation of PLBs, maximum frequency (61.90%) was found at 1 μM TDZ, yielding 13 PLBs/explant. These direct as well as callus-mediated PLBs readily germinated into well rooted plantlets. The explants that failed to respond to both callus induction and direct PLB formation media generated proliferating shoot buds which ultimately produced healthy adventitious shoots with several branches. The optimal combination for maximum shoot regeneration (69.22%) was 1 μM each of TDZ and NAA, giving rise to 1.89 shoots/explant. Rooted plantlets were readily acclimatized to greenhouse condition and a preliminary planting trial of the plantlets in the natural habitat resulted in 60% survival rate.

Keywords: adventitious shoots, micropropagation, PGR-free medium, plantlets, shoot-tip culture
Abbreviations: NAA, α-napthaleneacetic acid; **PGR**, plant growth regulator; **PLB**, protocorm-like body; **TDZ**, *N*-phenyl-N'-(1, 2.3-thiadiazol-5-yl)-urea (thidiazurone)

INTRODUCTION

The family Orchidaceae generally includes an interesting group of plants that are normally cultivated for their exquisite flowers. In several tropical countries such as Thailand, Singapore and Malaysia orchids have become a major export cut flower crop. Orchid populations are experiencing a steady decline in tropical countries due to habitat destruction (Native Orchid.org 2009). So, it is essential to take appropriate measures for the conservation and propagation of these endangered orchid species. However, as the orchids shall continue to be exploited from their natural habitat and as long as they contribute to an economic status and demands, rapid multiplication in mass scale by micropropagation is the most important strategy to ease anthropogenic pressures on the available population.

Dendrobium farmeri Paxt. is an endangered sympodial epiphytic orchid (Singh *et al*. 2001), having a geographical distribution ranging from Sikkim, Darjeeling, Nepal, Bhutan, Assam and Khasia hills at 300-1000 m to Burma and Thailand (Pradhan 1979). In these regions this species ranks among the important ornamental floricultural orchids (Pradhan 1979). It bears pendulous racemes bearing medium-sized flowers, usually with pink-mauve white sepals and petals having distinct yellow lips. Asexual propagation in this group of plants through traditional means is an extremely slow process, which generally gives rise to 2-4 plants per year (Nasiruddin *et al.* 2003; Martin and Madassery 2006; Luo *et al*. 2008). Although they may be propagated sexually by seeds, they are generally difficult to grow into complete plants in nature because seed germination needs a symbiotic fungus due to lack of endosperm and nutritive substances (Anjum *et al.* 2006). *In vitro* propagation, on the contrary, has opened up new possibilities in conservation and commercialization of plants. This method also ensures rapid multiplication of desired genotypes, shortening of time and has thus proved to be more advantageous than conventional means of propagation (Vij *et al.* 2000; Pathak *et al.* 2001; Vij *et al.* 2004). Therefore, *in vitro* propagation would be the most frequently used convenient technique where commercial demands exceed the frequency of natural regeneration in developed countries. Several species of *Dendrobium* have been reported to be propagated *in vitro* through asymbiotic immature seed germination or through direct shoot regeneration or protocorm-like bodies (PLBs) from different explants, including *D. candidum* (Shiau *et al.* 2005), *D. chrysotoxum* (Xu *et al.* 2001; Roy *et al.* 2007), *D. fimbriatum* (Roy and Banerjee 2003; Sharma *et al*. 2005), *D. nobile* (Nayak *et al.* 2002; Malabadi *et al*. 2005), *D. tosaense* (Lo *et al.* 2004), *D. densiflorum* (Luo *et al*. 2008). Depending on the type of explant and the culture conditions, sometimes an intermediary callus phase appears to be a prerequisite for PLB regeneration as observed earlier in *Dendrobium fimbriatum* (Roy and Banerjee 2001), *Dendrobium officinale* (Wei *et al*. 2010), *Dendrobium chrysotoxum* (Roy *et al*. 2007), leaf explants of *Dendrobium* (Chung *et al.* 2007), *Phalaenopsis* (Ishii *et al.* 1998; Gow *et al.* 2010) and in *Pleione formosana* (Chung *et al.* 2007). The present experiment was conducted to investigate the role of exogenous auxins and cytokinins in the induction of em-

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bryogenic callus and the subsequent formation of adventitious shoots and PLBs from the shoot-tip explants of *D. farmeri*. Shoot-tip culture is an established method for plant propagation and holds great challenge for maintaining uniformity of genotypes. In the present study, thidiazurone (TDZ) and α-napthaleneacetic acid and (NAA) were used as exogenous sources of cytokinin and auxin, respectively. TDZ is a substituted phenyl urea which has been considered to be more potent than most of the commonly used cytokinins (Malik and Saxena 1992; Massimo *et al.* 1996; Eapen *et al.* 1998). However, TDZ has also been used in some monocotyledonous species for regeneration (Singh *et al.* 2001). TDZ promotes growth directly due to its own biological activities (Sajid and Aftab 2009). In this investigation attempts have been made to establish an *in vitro* protocol by using combined treatments of TDZ and NAA for large-scale production of healthy *D. farmeri* plants, essential for commercialization of this orchid.

MATERIALS AND METHODS

Chemicals used

All the inorganic macro- and micro-salts, iron-EDTA, sucrose and potassium iodide were purchased from Sisco Research Laboratory (SRL), Mumbai, India. Solidifying agent agar and peptone were purchased from Merck Ltd., Mumbai, India. TDZ and NAA were purchased from Sigma Chemicals Co., St Louis, USA. Fungicide Thiram solution was purchased from Sree Ramcides Chemicals Pvt. Ltd., Chennai, Tamil Nadu, India.

Plant material and inoculation

The experiment was performed with shoot tips of about 4-6 mm in length, excised from aseptically raised 3-month old seedlings of *D. farmeri.* The seeds were obtained from 7-month old undehisced green capsules. For each treatment 42 shoot-tips were cultured in five replicate flasks (250 ml Erlenmeyer), each containing 100 ml culture medium. The cultures were incubated in the culture room at 25 ± 2°C under a 10-h photoperiod provided by Philips white fluorescent lights of 37.5 µmol/m^2/S intensity.

Culture medium

The basic culture medium was based on modified nutrient solution of Knudson's C (1946) and the original iron source was replaced by Fe–EDTA as described by Murashige and Skoog (1962). The medium was supplemented with 2% (w/v) sucrose and 0.1% (w/v) peptone. The PGR treatments consisted of different concentrations and combinations of TDZ at 0, 1, 2, 4, 8 and 16 µM and NAA at 0, 1 and 2 µM. The pH of the culture medium was adjusted to 5.2 prior to autoclaving. After adding 0.9% (w/v) agar the media were autoclaved at 1.02 kg/cm^2 for 20 min. The shoot-tip derived callus masses and PLBs were later maintained in PGR-free medium. The callus-derived PLBs, 2-3 mm in length with a visible shoot apex, were also later transferred to fresh PGR-free medium (pH 5.2) for further development of plantlets.

Acclimatisation of *in vitro* plantlets

For *ex vitro* establishment of plantlets, well rooted plantlets were rinsed thoroughly with distilled water to remove nutrient medium from the plant body and transplanted to the clay pots containing a mixture of dried coconut husk, small pieces of brick and charcoal (1: 1: 1 v/v/v). Immediately after transplantation, plantlets were treated with 0.1% (w/v) thiram solution (tetramethyl thiram disulphide) to control fungal infection. Plantlets were watered adequately. The transplants were kept in a well-ventilated location in the experimental garden and maintained under subdued light and 80% humidity condition for initiation of further growth.

Data collection and statistical analysis

Various morphogenetic responses of the shoot-tips were recorded periodically and the final data for analysis were gathered after 3 months of culture. Single factor ANOVA followed by Duncan's multiple range test (Little and Hill 1978) for significance of each character was performed and data is presented in **Table 1**. Since the morphogenetic responses are multi-character in nature, multivariate analysis of variance (MANOVA) was conducted to evaluate the significance of the main effects of TDZ and NAA and their interaction (TDZ × NAA), and data is presented in **Table 2**.

RESULTS

Survival of explants

Results of shoot-tip culture after 3 months are shown in **Table 1**. Browning or necrosis of shoot tips was a common phenomenon which exhibited variation in frequency from 9 to 66% depending on the concentration and TDZ + NAA combination. In the presence of a cytokinin like TDZ, higher concentrations of NAA (1-2 µM) increased necrosis to some extent while in combined TDZ+NAA treatments, necrosis also increased considerably with increasing concentrations of both TDZ (16 µM) and NAA (2 µM). In the control, maximum number of shoot tips survived indicating a possible role of endogenous PGRs. Cytokinins at higher

Table 1 Effects of TDZ and NAA on shoot-tip culture of *Dendrobium farmeri* after 3 months of culture.

Treatments (µM)		Necrosis of explants (%) ± S.E.[a]	Adventitious shoot formation (%) ± S.E.[a]	Mean No. of adventitious shoots ± S.E.[a]	PLB formation (%) ± S.E.[a]	Mean No. of PLBs ± S.E.[a]	Callus formation (%) ± S.E.[a]
TDZ	NAA						
0	0	38.45 ± 7.70 abcdefghijk	53.84 ± 8.89 ab	1.22 ± 0.27 abc	10.25 ± 6.78 abcdefghi	1.92 ± 1.32 abcd	0 ± 0
0	1	23.07 ± 4.44 abcdefghijklm	69.22 ± 4.44 a	1.89 ± 0.18 abc	23.07 ± 1.76 abcdefghi	1.53 ± 0.81 abcd	15.38 ± 1.69 abcd
0	2	9.52 ± 2.38 abcdefghijklm	45.23 ± 2.38 abc	1.16 ± 0.23 abc	35.89 ± 2.56 a	3.69± 0.74 a	0 ± 0
1	0	56.40 ± 2.56 abc	5.12± 2.55 abcde	0.12 ± 0.06 abc	61.90 ± 8.59 abcdefgh	13.61± 3.44 abcd	15.38 ± 0.89 abcd
1	1	38.45 ± 8.89 abcdefghij	12.81 ± 2.56 abcde	0.38 ± 0 a	51.27 ± 6.78 ab	5.74 ± 0.54 abcd	10.25 ± 6.78 abcd
1	2	35.89 ± 2.56 abcdefghijklm	12.81 ± 6.78 abcde	0.33 ± 0.22 abc	46.15 ± 0 abcde	6.27 ± 1.16 abc	33.33 ± 5.13 abcd
2	0	41.02 ± 2.56 abcdefghi	0 ± 0	0 ± 0	48.71 ± 6.78 abc	3.89 ± 0.90 abcd	35.89 ± 9.25 abc
2	1	56.40 ± 5.13 ab	10.25 ± 2.56 abcde	0.20 ± 0.02 abc	25.63 ± 6.79 abcdefghi	1.96 ± 0.61 abcd	7.69 ± 0 abcd
2	2	25.63 ± 7.96 abcdefghijklm	0 ± 0	0 ± 0	17.94 ± 9.25 abcdefghi	3.24 ± 0.55 abcd	51.28 ± 6.05 a
4	0	43.58 ± 2.56 abcdefg	23.07 ± 4.44 abcd	0.38 ± 0.07 abc	33.32 ± 2.56 abcdefghi	3.12 ± 0.29 abcd	0 ± 0
4	1	46.15 ± 0 abcdef	7.69 ± 4.44 abcde	0.15 ± 0.08 abc	35.89 ± 2.56 abcdefgh	3.04 ± 0.32 abcd	28.20 ± 5.13 abcd
4	2	56.40 ± 2.56 abc	10.25 ± 2.56 abcde	0.25 ± 0.06 abc	30.76± 4.44 abcdefghi	6.25 ± 1.36 abc	10.25 ± 2.56 abcd
8	0	41.02 ± 1.18 abcdefgh	7.69 ± 4.44 abcde	0.20 ± 0.16 abc	46.15 ± 4.44 abcd	7.99 ± 1.51 ab	15.38 ± 4.44 abcd
8	1	56.40 ± 5.13 ab	0 ± 0	0 ± 0	30.76 ± 4.44 abcdefghi	2.68 ± 0.90 abcd	28.19 ± 2.56 abcd
8	2	51.27 ± 3. 58 abcd	23.07 ± 4.44 abcd	0.30 ± 0.04 abc	23.07 ± 1.76 abcdefghi	1.58 ± 1.09 abcd	7.69 ± 4.44 abcd
16	0	38.45 ± 4.44 abcdefghijkl	0 ± 0	0 ± 0	43.58 ± 2.56 abcdefg	3.10 ± 0.58 abcd	46.15 ± 1.76 ab
16	1	46.15± 11.76 abcde	20.51 ± 9.25 abcde	0.56 ± 0.22 ab	43.58 ± 1.18 abcdef	6.25 ± 0.93 abc	25.63 ± 1.29 abcd
16	2	66.66 ± 2.56 a	10.25 ± 5.13 abcde	0.12 ± 0.06 abc	20.50 ± 5.13 abcdefghi	1.27 ± 0.12 abcd	7.69 ± 0 abcd

[a]S.E: Standard Error

Data shown are the mean of five replicates ± SE. In each column, mean values followed by the same letter are not significantly different at 0.05 level (DMRT)

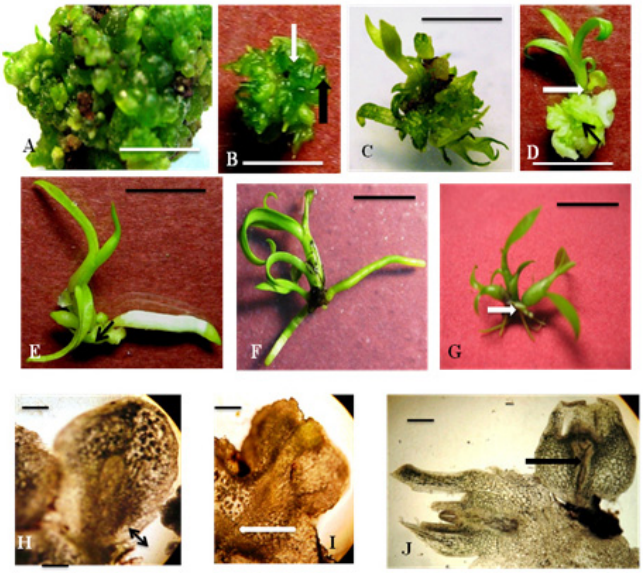

Fig. 1 Morphogenetic response of shoot-tip culture and light microscopic observations of PLBs of *Dendrobium farmeri* Paxt. (**A**) Development of callus from shoot apices (Bar = 10 mm). (**B**) Emergence of PLBs with (a) or without (b) visible shoot-buds from callus (Bar = 10 mm). (**C**) Cluster of PLBs at different stages of development regenerated directly from shoot-tip (Bar = 10 mm). (**D**) Asynchronous PLB development in a callus mass, showing both globular PLBs (a) and leafy plantlets (b) (Bar = 10 mm). (**E**) PLB derived plantlet showing development of root (a) (Bar = 10 mm). (**F**) And (**G**) Proliferation of single and multiple adventitious shoots (a) directly from shoot-tips followed by the formation of roots (Bar = 7.5 mm). (**H**) and (**I**) Histology of callus derived globular PLB showing well demarcated protoderm (a) and constricted basal attachment with vascular connection (b) (Bar = 50 µm). (**J**) Histology of directly formed somatic embryo (PLB) showing vascular connection (c) (Bar = 50 µm).

concentrations (4, 8, 16 µM) had a negative effect on necrosis (**Table 1**). The possible role of endogenous auxins in the survival of explants in the present experiment supports the findings of Bandurski and Nonhebel (1987) who reported that shoot tips bearing one to two small leaves may be considered as the major sites of auxin biosynthesis.

Induction of embryogenic callus

Initially shoot tip explants were cultured on basal medium supplemented with 18 different NAA+TDZ combinations for callus induction. This combination allowed most responsive callus induction from the basal cut-end of explants after about 3-4 weeks of inoculation of shoot tips. The callus appeared soft, translucent, friable and yellowish to deep green in color. Callus induction was not observed in PGR-free medium (**Table 1**).

The frequency of callus induction was very low in the medium containing only NAA (**Table 1**). The addition of TDZ was necessary to achieve better callus development. However, only a narrow range of NAA+TDZ combinations gave promising results, particularly at low levels of NAA and TDZ. The optimum frequency of callusing (51.28%) was recorded at 2 µM each of TDZ and NAA (**Fig. 1A**). A further increase in the concentration of TDZ resulted in no significant improvement. However TDZ at 8-16 µM exhibited an inhibitory effect.

Maintenance of callus and PLB regeneration

Calli derived from shoot tips were subcultured in basal medium without any PGR supplementation. In this condition, the subcultured calli exhibited vigorous growth and eventually turned green. On PGR-free medium the totipotent callus mass turned green and translucent which continued steady growth and the globular granules transformed into PLBs (**Fig. 1B**). The initiation of PLB formation was observed within 3 months of callus initiation. Many of these PLBs further proliferated via secondary PLB formation. Development of PLBs was most significant in the medium supplemented with 1, 2 and 4 µM TDZ and 2 µM NAA (**Table 1**). Germination of PLBs into rooted plantlets occurs readily in the same medium where PLBs were formed (**Fig. 1D**).

Necrosis of callus was a common occurrence observed to varying extents. The amount of necrotic callus was quite low in the absence of exogenous PGRs. The influence of an exogenous cytokinin on necrosis of callus varied with the type of cytokinin applied. A considerable increase in callus necrosis was recorded in the presence of TDZ, which was also not suitable for callus growth. So, from the above findings, only endogenous PGRs might be playing a key role in the maintenance and proliferation of embryogenic calli without a loss of regeneration potential for more than 12 months.

Direct PLB formation

In vitro regeneration of *D. farmeri* first appeared in the form of PLB formation. Such structures developed without an intermediary callus phase. The TDZ-NAA combination was found to be most effective in PLB regeneration (**Table 1**). The direct formation of PLBs and the subsequent development of plantlets from shoot tips were significantly influenced by the application of cytokinin at certain concentrations. Among the different concentrations applied, TDZ at a very low concentration (1 µM) induced maximum PLBs (61.90%) directly from the explants. Generally, all 18 combinations of NAA and TDZ promoted PLB formation.

In the TDZ+NAA combination, the frequency of direct PLB regeneration ranged from 10.25 to 61.90% (**Table 1**) and the maximum number of PLBs per explants ranged from 1.53 to 13.61/explant (**Table 1**). The highest number of PLBs (13.61 PLBs/explant) was recorded with 1 µM TDZ (**Fig. 1C**), while a very low percentage (10.25%) was observed in the control (**Table 1**). Morover, the frequency of PLB formation was augmented from 2-16 µM TDZ. The results of the present study confirm the fact that combined treatments of TDZ and NAA were very effective for PLB regeneration directly from shoot-tip explants.

Histological studies on direct and callus-mediated PLBs

Thin free-hand sections of the callus showed that regeneration of PLBs occurred from the surface of callus (**Fig. 1H, 1I**). Histological observation revealed that at an early stage of development, PLBs retained a globular shape, characterized by a well-demarcated protoderm and a constricted basal attachment (**Fig. 1H**), but at a later stage a vascular connection was established between each PLB and the mother tissue (**Fig. 1I, 1J**). The PLB structure is considered to be unique to the orchidaceous members, including *D. farmeri*, and is the earliest structure formed during embryo development process. This finding suggests that PLBs derived from calli could be considered as somatic embryos and the callus induced in this study was embryogenic in nature.

Plantlet regeneration from PLBs

PLBs further developed into plantlets at all combinations of TDZ (1-16 µM) and NAA (1, 2 µM). However, this process was relatively slow in the medium where PLB production was high. The medium supplemented with 1, 2 and 4 µM TDZ initiated plantlets (**Fig. 1E**) directly from PLBs. The frequency of PLB-derived plant regeneration in these media was markedly high compared to control. Thus the best result (76.66%) was achieved with the lowest level of exogenous cytokinin. However, continued proliferation of embryogenic callus as well as the regeneration of PLB-derived

plantlets exhibited an efficient protocol for mass-scale production of plantlets.

Adventitious shoot formation

The morphogenetic response through the formation of adventitious shoots is a natural ontogenic route for axial growth. Multiple shoots with several branches were initiated from shoot tip explants after 21-28 days of culture. Generally those explants that failed to respond to callus induction as well as PLB formation media followed the direct ontogenetic pathway by producing adventitious shoots with several branches. Among the different treatments tested, consistent shoot formation occurred with the application of TDZ. However, the addition of NAA in the same medium proved to be more effective in this regard. In the TDZ+NAA combination, the frequency of initiation of adventitious shoots varied from 5.12 to 69.22% (**Fig. 1F, 1G**). High frequency shoot regeneration (69.22%) coupled with maximum shoot formation (1.89 shoots/explant) was recorded in the combined treatment of 1 µM each of TDZ and NAA (**Table 1**). Therefore, 1 µM TDZ was considered as the optimum concentration for shoot regeneration, depending upon the level of accompanying NAA. A decrease in the number of shoots that regenerated was noticed when the concentration of TDZ was increased from 1 to 16 µM. Also, at a supra-optimal (8-16 µM) concentrations, TDZ exhibited inhibitory effects in most treatments. However, when NAA was applied alone, the frequency of adventitious shoot proliferation was very low but the application of NAA (1-2 µM), together with TDZ, might prove effective in enhancing shoot regeneration (**Table 1**).

In vitro rooting and acclimatization of plantlets

In vitro generated shoots of about 1-2 cm were harvested for rooting experiment and cultured only on Knudson's C medium supplemented with peptone as well as on the medium containing either IBA or NAA alone. Of the two auxins tested for inducing roots, NAA was more effective than IBA. KCP with 2 µM NAA supported the best rooting response (80%), in which an average of 3.5 roots/explant of 2 cm in length were produced within 4 weeks. PGR-free basal medium also enhanced root induction within 3 weeks.

The potted plantlets were successfully acclimatized in a moist and shady place and eventually transferred to an orchid house. About 60% of the plantlets survived finally and initiated new growth after 4-5 weeks (**Fig. 1F**).

DISCUSSION

Induction of callus and subsequent PLB regeneration

The results of this study on *D. farmeri* indicate that the application of auxin and cytokinin in combination was most suitable for induction of callus from shoot tip explants. A similar result was observed in *D. officinale* (Wei *et al.* 2010). This fact corroborates previous reports where it was clearly evident that in the majority of angiosperms, callus formation was induced by combined treatments of exogenous cytokinin and auxin. Callus culture of most orchids also exhibits an absolute requirement of both auxin and cytokinin for their induction which was previously observed in different species like *Paphiopedilum* hybrid (Lin et al. 2000), *Cypripedium formosum* (Lee and Lee 2003) and in *Cymbidium* (Huan *et al*. 2004; Huan and Tanaka 2004; Teixeira da Silva and Tanaka 2006). The TDZ+NAA combination was found to be most effective for callus induction while BAP failed to exhibit any promising result. This finding, however, does not tally with our previous reports on *D. fimbriatum* and *D. chrysotoxum* (Roy and Banerjee 2003; Roy *et al.* 2007) where a purine type cytokinin 6-benzylamino-purine (BAP) also showed promising effect for callus induction from shoot tip explants. TDZ combined with 2,4-dichlorophenoxyacetic acid (2,4-D) also induced embryonic calli from rhizomes of *Cymbidium ensifolium* var. *misericors* (Chang and Chang 1998) and longitudinally bisected segments of PLBs of *Cymbidium* hybrid orchid (Huan *et al*. 2004). Generally cytokinin shows a very low callus-inducing effect but it is generally required for overcoming callus necrosis and thus promotes growth particularly in members of Orchidaceae (Lin *et al.* 2000; Roy and Banerjee 2003; Lu 2004). Although the presence of exogenous PGRs was essential for callus induction, it could be possible to maintain this callus growth in PGR-free medium for more than 12 months, without any loss of embryogenic potential. Such an observation was also recorded in other *Dendrobium* species, namely *D. fimbriatum* (Roy and Banerjee 2003), *D. chrysotoxum* (Roy *et al.* 2007) and *Dendrobium formosum* (Nasiruddin *et al.* 2003). Therefore, it was evident that an adequate endogenous hormonal system was responsible for the maintenance of callus in the PGR-free medium The growth characteristic of the callus of *D. farmeri*, which has been initiated in presence of plant growth regulators exhibits a similarity with that of the habituated callus (Horgan 1987; Meins 1989). But unlike the habituated callus that exhibits loss of morphogenetic potential (Choi *et al.* 2003), the callus of *D. farmeri* sustains its regenerative potential through continued production of PLBs for more than 12 months. This hormone-autonomous callus culture through PLB regeneration was earlier noted in *Panex ginseng* and *Mammillaria gracillis* (Choi *et al.* 2003; Poljuha *et al*. 2003). Histological studies of PLBs show that those are actual somatic embryos which strongly support a previous report on *Cymbidium* (Teixeira da Silva and Tanaka 2006).

Necrosis of callus was a common occurrence during maintenance of callus in orchids. Similar observation was previously reported in several orchids including *Paphiopedilum* hybrid (Lin *et al.* 2000), *Pleione formosana* Hayata (Lu 2004) and *Oncidium* 'Gower Ramsey' (Wu *et al*. 2004). In the present study we confirm that callus necrosis was quite low in PGR-free control medium compared to TDZ treatments.

Regeneration of direct PLBs

Auxins and/or cytokinins were most effective for neoformation of PLBs and subsequent plantlet development. However, the ratio of auxin and cytokinin for PLB formation depends upon the specific species (Le *et al.* 1999; Roy *et al.* 2003, 2007). Some species do not require exogenous PGRs, which supports the findings of Chen and Chang (2000) and Roy *et al.* (2003, 2007). The rate of PLB regeneration either directly or mediated via callus was much lower in the absence of exogenous PGRs. NAA in combination with a cytokinin such as BAP has been used in many monopodial orchid species for enhancing PLB formation (Kim and Kim 2003; Puchooa 2004). TDZ was quite an effective cytokinin in the production of PLBs. TDZ was first reported to mimic cytokinin activity by Mok *et al.* (1982) and since then it has been used as a more potent cytokinin for induction of both organogenesis as well as embryogenesis in several plants belonging to diverse groups or families such as *Hordeum vulgare* (Ganeshan *et al*. 2003), *Oryza sativa* (Gairi and Rashid 2004), *Hyoscyamus niger* (Uranbey 2005) and *Solanum tuberosum* (Sajid and Aftab 2009). It has also been observed that the TDZ+NAA combination enhanced PLB formation significantly, also observed earlier in leaf explants of *Dendrobium* (Chung *et al*. 2007), *Oncidium* (Chen and Chang 2001), *Doritaenopsis* (Park *et al.* 2003) and *Phalaenopsis amabilis* var. Formosa Shimadzu (Chen and Chang 2004). However the optimum concentration required for direct adventitious PLB regeneration was quite efficient at a low level of cytokinin which was previously noted in *D. chrysotoxum* (Roy *et al.* 2007) and *Phalaenopsis* (Gow *et al.* 2009). Our result also depicts that intermediary concentrations of TDZ and NAA have an inhibitory effect which leads to enhanced callus formation.

Table 2 Multivariate Analysis of Variance (MANOVA) of the combined treatments of NAA and TDZ.

Treatment	Df	Wilks Lambda	Approx F	Num df	Den df	P-value
TDZ	5	0.0252	6.3381	30	126	0
NAA	2	0.4912	2.205	12	62	0.0221
TDZ × NAA	10	0.0082	4.1884	60	167.4742	0

P-values < 0.05 indicate that interaction of TDZ x NAA and single effect of TDZ and NAA are significant.

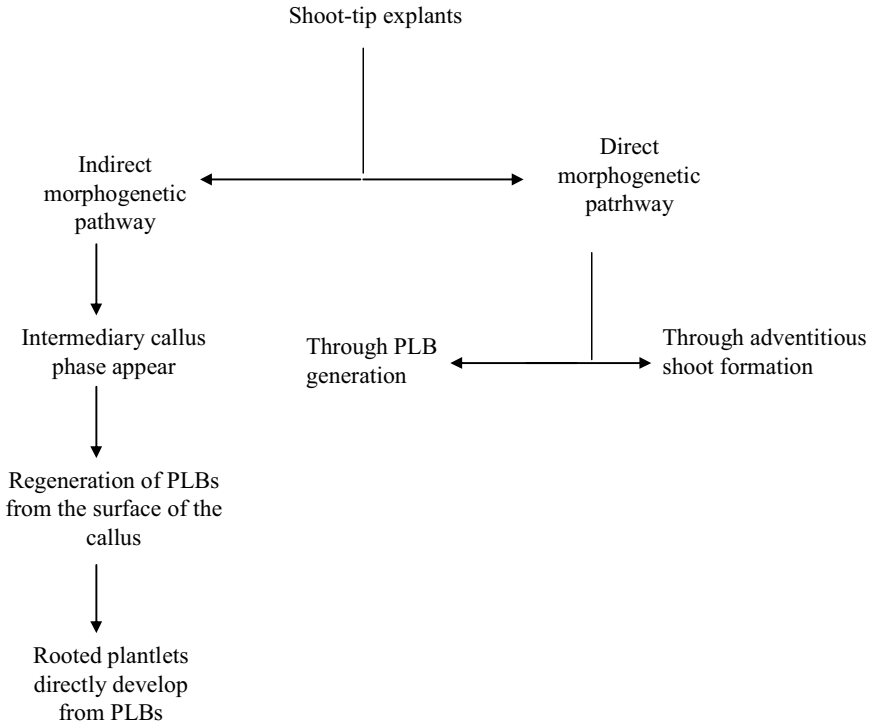

Fig. 2 In the presence of TDZ and NAA, shoot tip explants of *Dendrobium farmeri* exhibit two morphogenetic pathways for plant regeneration: (1) indirect pathway through an intermediary callus phase and (2) direct PLB formation and adventitious shoot initiation.

Formation of adventitious shoots

A number of reports are available on the multiplication of orchid species through shoot tip culture (Seeni and Latha 2000; Malabadi *et al*. 2005). The results observed herein have shown the ability and potential of shoot tip explants to produce phenotypically uniform, stable plants in an intermediary callus-free state for conservation purposes. The synergistic action of an auxin (NAA) and a cytokinin (TDZ) in the formation of multiple shoots supports earlier reports on orchids. Ket *et al*. (2004) showed that multiple shoot proliferation was induced by the application of exogenous cytokinin. Similar finding was also observed by Seeni and Latha (2000) in Blue *Vanda*.

In the case of shoot tip culture of orchids, it appeared that the resident axillary meristems of the condensed nodes of the basal part of shoot apices relieved apical dominance by the defunct apical meristems and proliferated to produce callus-free shoot buds (Seeni and Latha 2000). In the present study on *D. farmeri* it was noted that exogenous auxin along with cytokinin also suppressed apical dominance, which led to proliferation of adventitious shoots. The combined TDZ+NAA treatments increased the number of shoots compared to TDZ alone. So the results confirmed the fact that exogenous cytokinin is a crucial PGR for adventitious shoot formation. On top of that, it was also evident that for better responses exogenous auxin could be effectively applied along with cytokinin.

The success of rapid and direct shoot regeneration without a callus phase or PLB formation from the shoot tip explants of *D. farmeri* also showed a successful pathway for rapid regeneration and multiplication of shoots. Similar results have been reported earlier on *Dendrobium* hybrids (Martin and Madassery 2006). From the perspective of propagation, it could be noted that the multiplication rate of shoots with a greater number of branches might prove effective in increasing the yield of *D. farmeri*. The branching frequency of adventitious shoots was higher in the TDZ +NAA combination of (69.66%) than the control (28.86%).

Statistical analysis on the experimental data, assessed through DMRT (**Table 1**), clearly reveals the significant effects of TDZ and NAA on the responses. As the responses are correlated, a multivariate analysis of variance (**Table 2**) performed with same experimental data also justifies that which was proved through DMRT.

CONCLUDING REMARKS

This study indicates that in the presence of TDZ and NAA, shoot tip explants of *Dendrobium farmeri* exhibit two morphogenetic pathways leading to plant regeneration (**Fig. 2**). Firstly, it depicts an indirect pathway through an intermediary callus phase which could best be achieved in a combination of 2 µM each of TDZ and NAA. Although the presence of PGR was essential for callus induction, it was possible to maintain this callus through subculturing in PGR-free medium for 12 months without any loss of regeneration potential. Such habituated callus in PGR-free medium is capable of producing PLBs efficiently. Since this process does not require any exogenous PGR, it has immense practical implication for mass-scale production of commercial orchids with less possibility of the occurrence of chromosomal variation. Secondly, direct PLB formation and adventitious shoot initiation could also be achieved from shoot tips for which 1 µM TDZ was most effective. Therefore it could be concluded that cyokinin-like compounds such as TDZ, a phenyl urea derivative, bypass the normal cytokinin metabolism pathway which was catabolized by cytokinin oxidases. Instead, TDZ showed a different novel biological activity (Horgan 1987; Hare and van Staden 1994; De Pauw *et al.* 1995).

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