

***In vitro* Propagation of orchid (*Dendrobium ovatum* (L.) Kraenzl.) through Somatic Embryogenesis**

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Key words: *Dendrobium*, somatic embryogenesis, embryogenic callus, protocorm like bodies, plant growth regulators

Abstract

An efficient *in vitro* regeneration protocol through somatic embryogenesis was established from longitudinally bisected protocorm (ITCL) of an endangered orchid *Dendrobium ovatum*. The efficiency of EC and SEs from the protocorms significantly relied on the concentration of PGRs. MS medium supplemented with TDZ (1.0 mg/l) induced optimum of EC (31.8%) and SEs (28.1/explant). Similarly, ZEA (0.5 mg/l) induced EC (27.6%) and SEs (17.1/explant). The combined treatment of TDZ (1.0 mg/l) and NAA (0.5 mg/l) resulted in the maximum induction of EC (58.6%) and SEs (39.8/explant) in an upright incubated explant. In another combined effect of ZEA (1.0 mg/l) and NAA (0.5 mg/l) induced EC (43.8%) and SEs (24.3/explant), whereas the combination of BAP (0.5 mg/l) and NAA (0.5 mg/l) produced EC (34.4%) and SEs (16.8/explants). The induced EC and SEs were healthier on medium containing TDZ + NAA than on the medium containing ZEA+NAA and BAP+NAA. The orientation of ITCL explants seemed to interact with position to affect the morphogenesis. The ITCL explants incubated in the upright orientation induced higher percentage of EC and a greater number of SEs/explants (EC-58.6% and SEs-39.8/explants) than that in the inverted orientation (EC-16.4% and SEs-9.1/explants) irrespective of the PGRs. The SEs developed from the EC and the intermediate stage of PLBs were finally differentiated into plantlets. About 89% of plantlets were successfully acclimatized in the green house conditions.

Introduction

The Orchidaceae is one of the largest and highly evolved plant family of the flowering plants contains about 35000 species belonging to 850 genera (Hossain et al. 2013). These ornamental plants are widely distributed, cultivated for their beautiful flowers and are of

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economic importance. Orchids are outstanding in many ways as they have diverse shapes, forms, colors and represent the most highly evolved family among monocotyledons. Eastern Himalaya, North-Eastern region and Western Ghats are the most prominent orchid regions in India. Orchids occupy top position among all flowering plants marketed as cut flowers and potted plants, fetching a very high price in the national and international market. Commercial importance of orchids has led to their tremendous production in recent years. Apart from their ornamental value orchids are also known for their medicinal usage especially in the traditional system of medicine (Besra et al. 2011). It is believed that the Chinese were the first to cultivate and describe orchids for medicinal purpose.

Among various orchid categories in the family the *Dendrobiums* have become increasingly popular due to its floriferous flower sprays, wide range of colors, size and shapes, year round availability and long flowering life of several weeks to months.

Among the total cut flowers orchid species of the world the *Dendrobium* contributing about 85% to the floriculture industry. This genus also exhibits a vast diversity in vegetative and floral characteristics and is considerable interest due to its broad geographic distribution and high value of hybrids as a floriculture commodity. Many species and wild populations are endangered as direct or indirect results of two human activities: habitat alteration and over collection. 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 Convention on International Trade in Endangered Species of wild Fauna and Flora (CITES), where the international trade is strictly controlled and monitored (Pant 2013).

Dendrobium ovatum is a rare and endemic species of Western ghat. It is therapeutically significant due to the presence of an anticancer bibenzyl derivative Viz., Moscatilin (Shetty et al. 2015). It is also called as green lipped *Dendrobium*. It grows at the elevations of 50 to 1520 meters above the sea level. The juice of the fresh plant is used to cure stomach ache, as carminative, antispasmodic, laxative and liver tonic. There has been concern over the habitat loss in the Western Ghats due to environmental and manmade disasters like indiscriminate collection, deforestation of host trees and illegal trade of specimens. There are also possibilities that wild endemic orchids under intense pressure due to biotic stress and might face extinction soon. *Dendrobium ovatum* capsule contains thousands of micro fusiform seeds. The zygotic embryo is poorly differentiated composed of 80-100 cells, meristem and cotyledons are usually not present at the time of seed dispersal, non-endospermic and contain almost no nutrients. In nature for germination and early developmental stages they must be symbiotic with highly specialized fungi. Consequently <5% of the seeds germinate in the wild. Hence there is an utmost need to preserve and conserve the germplasm of these medicinally valuable wild endemic orchid resources. So, it is essential to take measures such as plant tissue culture

method for propagating this rare orchid species. With the introduction of plant tissue culture technique, the need of mycorrhiza symbiotic is eliminated and hence orchids can be massively propagated in vitro. This is advantageous to pharmaceutical industry that uses orchids as raw material in bulk quantity. Different plant tissues such as shoot tips (Mamun et al. 2018; Mandal et al. 2020) protocorm like bodies (Ashok Pyati 2020, Klaocheed et al. 2021) pseudobulbs (Bowmik and Rahman 2020), nodal explants (Bhattacharyya et al. 2016, Kaur 2017), leaf and roots (Lee and Chen 2014) stem thin cell layers (Parthibhan et al. 2018) have been widely used as explants to obtain regenerative plantlets in several *Dendrobiums*. In *D. ovatum* different tissue culture systems for micropropagation have been established using mature and immature seeds as explants (Gurudeva 2019, Deeksha Raj et al. 2021, Shetty et al. 2015, Thejaswini and Narasimhan, 2017, Pujari et al. 2021, Mary and Divakar 2015). Altogether these studies made a great progress in propagating and exploiting of *D. ovatum*.

Materials and Methods

Aseptically raised protocorm like bodies (PLBs) from the matured seeds of *D. ovatum* on MS medium supplemented with 15% coconut water used as the explant source. The PLBs approximately 2.0 mm thickness were longitudinally bisected into two slices, approximately 1.0 mm thickness using a sharp surgical blade under sterile conditions, these were used as ITCL explants for regeneration. The ITCLs explants were inoculated on MS medium with 2% sucrose with different concentrations (0.5, 1.0, 2.0 and 3.0 mg/l) of NAA, BAP, Thidiazuron (TDZ) and Zeatin (ZEA) alone or in combinations of BAP + NAA, TDZ + NAA and ZEA + NAA. 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 prior to autoclaving at 121°C for 20 minutes. The explants were cultured in two orientations upright (with the basal end touching the media) and inverted (the apical end touching the media). The cultures were under the illuminations of 40 μ mol m⁻²s⁻¹ with 12/12 h of photoperiod at 25 \pm 2°C in the culture room. To check the reproducibility of the protocol the experiment was repeated twice. Subculturing was done as and when required. For each treatment at least 12 explants were used. Data on number of explants induced the embryogenic callus (EC), number of somatic embryos (SEs)/explant and PLB formation and differentiation were recorded.

The SE/PLB derived well developed plantlets from the cultures (20 Weeks) were removed and washed thoroughly in water to remove any adhering medium. The plantlets were then transferred to seedling trays and paper cups containing an autoclaved potting mixture of vermiculite, chopped coconut husk, charcoal and brick pieces in the ratio of 1:1:1:1. The plantlets were covered with transparent polythene sheets for a month in order to reduce the infection and to maintain high relative humidity (80-85%) and a day/night temperature 25/18° C in 16/8 h photoperiod and 35 μ mol m⁻²s⁻¹ light intensity. After one month the polythene bags were removed and

humidity was gradually reduced to 55-60%. The plantlets were watered daily and sprayed with ½ MS liquid medium without sucrose for alternate days. The number of surviving plants was recorded then transferred to green house.

All experiments were conducted as a completely randomized design. Each experiment included twelve replicates and experiments were repeated twice. Data on induction percentage of EC of upright and invert-oriented explants, time taken for SE/PLB formation and time taken in weeks to form complete plantlets were tested applying Tukey's multiple comparison test ($p \leq 0.5$) in one-way ANNOVA, to separate of significantly different groups. The statistical analysis was performed by using the SPSS (version 18) software package (SPSS Inc., Chicago, IL, USA). The results were expressed as mean \pm SD of twelve replicates.

Results and Discussion

The regeneration potential of *D. ovatum* ITCLs were positively tested in MS medium and with the individual and combinations of various PGRs. ITCL explants cultured on the medium without any PGRs remained green for 2 weeks and gradually turned brown and necrotic. In contrast, the explants cultured on the medium supplemented with PGRs remained green and induced EC from the cut ends after 5-6 weeks of culture. The callus was yellowish green compact and nodular in texture. Among the tested PGRs the maximum EC induction was observed on MS medium fortified with TDZ (1.0 mg/l), where 31.8% of explants induced the EC, this was followed by ZEA (0.5 mg/l) 27.6% and BAP (0.5 mg/l) 10.5% of explants induced EC in upright oriented explants after 5-6 weeks of culture. Similarly, the optimum percentage of EC formation from the inverted explants was recorded on the medium supplemented with TDZ (0.5 mg/l), ZEA (0.5 mg/l) and BAP (0.5 mg/l) where 11.2, 5.3 and 3.4% of EC was formed respectively (Table 1). But the explants cultured on medium supplemented with NAA remained green for 2 weeks and gradually turned brown and died. EC induction was enhanced as the concentration of TDZ in the medium increased from 0 to 1.0 mg/l, but was inhibited from 2.0 to 3.0 mg/l. Among the individual PGRs tested for the induction efficiency of EC, TDZ was highest while that of ZEA was moderate and BAP was lowest.

To increase EC induction, proliferation and SEs formation, combinations of TDZ, ZEA, BAP and NAA were tested to look for possible synergistic effects. As inoculated on the medium supplemented with TDZ (1.0 mg/l) with NAA (0.5 mg/l) and ZEA (1.0 mg/l) with NAA (0.5 mg/l), the explants exhibited a higher frequency of EC. In the combination of TDZ with NAA, 58.6% of explants induced EC in upright oriented explants after 5 weeks of culture (Fig.1A), whereas the invert-oriented explants produced only 14.9% of EC. Similarly, in ZEA with NAA 43.8% of explants formed EC in upright and only 16.4% of EC from the inverted explants after 5 weeks of culture. On the other hand, in the combination of BAP (0.5 mg/l) + NAA (0.5 mg/l) maximum of 34.4% of explants induced EC in upright and only 9.8% of EC from the inverted explants after 6 weeks of culture.

From these combinations EC that formed on the medium with TDZ + NAA were healthier than the medium with ZEA + NAA and BAP + NAA. When the explants cultured on the media with having higher concentrations of these PGRs inhibited the formation of EC and SEs and tissue browning was observed (Table 1). The EC induced from the above treatments developed further and continued to grow on the same medium. The optimum EC induction, proliferation, multiplication and SE/PLB formation was recorded on medium containing TDZ (1.0 mg/l) + NAA (0.5 mg/l) in upright oriented explants.

The rate of SE formation and the quality of SEs varied with the type, concentration and combination of PGRs. The highest number of SEs per ITCL (28.1) was recorded on the medium supplemented with TDZ (1.0 mg/l), followed by ZEA (0.5 mg/l), where average number of 17.1 SEs were formed after 3 weeks of subculture in upright oriented explants. The efficiency of ZEA on SE formation from the explants was no better than that of TDZ. On the other hand, BAP (0.5 mg/l) induced very low, an average of only 0.4 SE/explant after 5 weeks of subculture, but it was almost failed to induce SE (Table 1). To look for possible synergistic effect of auxin and cytokinin for the formation of SEs, various combinations were tested. On medium supplemented with TDZ (1.0 mg/l) with NAA (0.5 mg/l) the highest number of SEs (39.8/explant) formed on upright oriented ITCL explants and the EC of inverted ITCL explants produced only 9.1 SEs after 3 weeks of subculture. Similarly on the medium fortified with ZEA (1.0 mg/l) with NAA (0.5 mg/l) where 24.3 SEs were formed from the EC of upright oriented ITCL explants and only 7.0 SEs were recorded on EC of inverted ITCL explants after 3 weeks of subculture. On the other hand, medium supplemented with BAP (0.5 mg/l) with NAA (0.5 mg/l) produced 16.8 SEs from the EC of upright oriented ITCL explants and only 7.3 SEs were recorded on EC of inverted ITCL explants after 4 weeks of subculture. Generally, EC was compact yellowish green, the SE initials were observed as either protuberances or closely packed clusters on the surface of the callus mass. These clusters of SEs continued to enlarge as globular stage and at maturity subsequently formed typical elongated torpedo shaped structures after 3 weeks of subculture (Fig. 1B). These mature torpedo SEs grew into PLBs with shoot apex after 5 weeks of subculture (Fig. 1C), these PLBs differentiated into shoots (Fig.1D), further developed into leafy shoots after 8 weeks of subculture (Fig. 1E) and initiation of roots after 10 weeks of culture on the MS medium supplemented with TDZ (1.0 mg/l) + NAA (0.5 mg/l) (Table 2).

Highest frequency of EC and SE and PLB induction occurred on the medium supplemented with TDZ (1.0 mg/l) and NAA (0.5 mg/l). However, efficiency of EC induction was related to the orientation of ITCL explants. If the explants were incubated in an upright orientation on the medium, EC and SEs were induced more efficiently. Among all explants irrespective of the PGRs tested, the highest EC induction was obtained from the upright incubated explants. As the ITCL explants were cultured in an upright orientation for 5-6 weeks the optimum percentage of explants with EC was 58.6%. Nevertheless, the percentage of explants with EC per responsive explants

Table 1. Effect of PGRs and Orientation of ITCL explants on the induction of EC and SEs in *Dendrobium ovatum*.

PGRs (mg/l)	Induction of EC (%)		Time taken for EC formation (WKS)	Formation of SEs (Numbers/explant)		Time taken for SE formation after subculture (WKS)
	Upright ITCLs	Inverted ITCLs		Upright cultured EC	Invert cultured EC	
BAP						
0.5	10.5 ± 0.5ij	3.4 ± 1.2kl	6	0.4 ± 1.8op	0	5
1.0	10.5 ± 0.5ij	0	6	0.4 ± 1.8op	0	5
2.0	0	0	0	0	0	0
3.0	0	0	0	0	0	0
NAA						
0.5	0	0	0	0	0	0
1.0	0	0	0	0	0	0
2.0	0	0	0	0	0	0
3.0	0	0	0	0	0	0
TDZ						
0.5	21.3 ± 1.8e	11.2 ± 0.6ij	5	16.0 ± 1.2gh	2.1 ± 1.9mn	3
1.0	31.8 ± 2.4cd	11.2 ± 0.6ij	5	28.1 ± 2.1cd	6.3 ± 1.4kl	3
2.0	11.0 ± 0.6ij	6.0 ± 1.0kl	5	7.2 ± 1.8k	0	3
3.0	0	0	0	0	0	0
ZEA						
0.5	27.6 ± 2.0d	5.3 ± 0.9l	5	17.1 ± 2.4g	2.1 ± 1.2mn	3
1.0	27.0 ± 1.9d	5.0 ± 0.9l	5	6.6 ± 1.8kl	2.0 ± 1.2mn	3
2.0	11.2 ± 0.6ij	5.0 ± 0.9l	6	6.0 ± 1.8kl	2.0 ± 1.2mn	3
3.0	10.0 ± 1.2j	0	5	0	0	0
BAP + NAA						
0.5 + 0.5	34.4 ± 2.0c	9.8 ± 1.7jk	6	16.8 ± 1.2g	7.3 ± 2.3k	4
1.0 + 0.5	32.0 ± 1.9c	7.2 ± 1.8k	6	16.1 ± 1.2g	4.2 ± 1.8jk	4
2.0 + 0.5	0	0	0	0	0	0
3.0 + 0.5	0	0	0	0	0	0
TDZ + NAA						
0.5 + 0.5	41.4 ± 0.8b	14.6 ± 2.2gh	5	28.2 ± 2.1cd	7.6 ± 2.0k	3
1.0 + 0.5	58.6 ± 0.2a	14.9 ± 1.5gh	5	39.8 ± 1.8bc	9.1 ± 1.4jk	3
2.0 + 0.5	18.1 ± 1.3f	9.0 ± 2.4jk	5	12.4 ± 2.81hi	0	3
3.0 + 0.5	0	2.1 ± 1.9mn	6	0	0	0
ZEA + NAA						
0.5 + 0.5	33.4 ± 1.8c	11.2 ± 0.8ij	5	20.1 ± 1.6e	2.1 ± 1.2mn	3
1.0 + 0.5	43.8 ± 0.5b	16.4 ± 1.9g	5	24.3 ± 1.8de	7.0 ± 1.9k	3
2.0 + 0.5	7.0 ± 2.3k	0	6	11.3 ± 1.1ij	0	4
3.0 + 0.5	2.1 ± 1.2mn	0	6	0	0	0

Means ± Standard error the same letters within a column are not significantly different based on ANOVA at $P \leq 0.5$ as indicated by Tukey's test.

decreased significantly, if the explants were cultured in an inverted orientation, with the maximum of 16.4% of ITCL explants induced EC. Similarly, the EC obtained from the upright incubated explants yielded a greater number of SEs (0.4-39.8/explant) when compared to the EC obtained from the inverted incubated explants (2.0-9.1/explant).

Well developed plantlets (20 weeks) measuring 3.0-4.0 cm in height (Fig.1F) were transferred to seedling trays and paper cups containing an autoclaved potting mixture of vermiculite, chopped coconut husk, charcoal and brick pieces in the ratio of 1:1:1:1, covered with transparent polythene sheets. After a month sheets were removed, plants were successfully established in the green house with 89.0% survival rate (Fig 2A and 2B).

Table 2. Development and Differentiation of *Dendrobium ovatum* PLBs into plantlets

PGRs (mg/l)	Time taken for PLBs formation (Wks)	PLB formation (%)	Differentiation of (After Subculture) (Weeks)	
			Shoots	Roots
BAP 0.5	6	18.6 ± 1.0kl	10	14
TDZ 1.0	5	59.2 ± 0.9cd	8	11
ZEA 0.5	5	41.3 ± 1.3g	8	11
BAP + NAA 0.5 + 0.5	6	60.3 ± 1.1cd	9	12
TDZ + NAA 1.0 + 0.5	5	74.8 ± 0.3a	8	10
ZEA+ NAA 1.0 + 0.5	5	62.4 ± 0.8bc	8	11

Means ± Standard error the same letters within a column are not significantly different based on ANOVA at P≤0.5 as indicated by Tukey's test.

In orchid TCLs have been widely used in tissue culture for the induction of callus, somatic embryogenesis and genetic transformation of many plants including orchids (Teixeira da Silva and Dobranszki 2013). In the similar way, in this work the PLBs of *D. ovatum* were bisected longitudinally into two segments used as ITCL explants, to develop an efficient in vitro regeneration system through somatic embryogenesis will allow large scale multiplication of plants. Since the plants are medicinally useful and threatened, the use of an efficient micropropagation system as a means to multiply for controlled production of the desired plants will take the pressure off the wild populations. Orchids were once considered to be particularly difficult to propagate plants in vitro, but TCL technology has been one method that has advanced their tissue culture, making mass clonal propagation easier and more reproducible (Teixeira da Silva, 2012). The advantage of the TCL system is to produce high frequency organ regeneration and to reduce the time interval required to generate required plantlets. The TCL have been used to culture

several orchid species in vitro, namely *Coelogyne cristata* (Naing et al. 2011), *Dendrobium candidum* (Zhao et al. 2007), *Dendrobium aqueum* (Parthibhan et al. 2018), *Cymbidium* hybrid (Teixeira da Silva, 2012).

In this present system plant regeneration via indirect organogenesis has been developed from EC and SEs derived from ITCL explants. Since callus is a potential source for regeneration by virtue of having the capacity to form many meristematic regions. It can play an important role in genetic transformation studies and secondary metabolite production. The induction of EC and SEs were mainly influenced by PGRs. Among the individual PGRs tested, TDZ induced maximum EC and SEs formation followed by ZEA from upright oriented explants. TDZ involved either directly or indirectly in several morphological and physiological responses in plant tissues (Guo et al. 2011). TDZ had a stronger effect on shoot induction in *Paphiopedilum callosum* var. *sublaeve* (Wattanapan et al. 2018). The ability of TDZ resists cytokinin oxidase providing an internal suitable balance between cytokinin and auxin, enhancing the synthesis of adenine type cytokinins (Baghel and Bansal, 2015). In this study the optimal individual concentration of TDZ at a lower level (1.0 mg/l) stimulated the formation of EC (31.8%) and SEs (28.1/Explant) in an upright oriented ITCLs. The similar positive effect of TDZ was reported by Roy et al. (2007), that both of callus induction and direct PLB formation from cut surface of shoot tip explants in *Dendrobium chrysotoxum*. An observation similar to the present study the TDZ was also effective in induction of in vitro morphogenesis in several orchids, namely *Paphiopedilum Alma* Gavaert (Hong et al. 2008), *Xenikophyton smeeanum* (Mulgund et al. 2011), *Paphiopedilum gigantea* (Latip et al. 2010), *Vanilla planifolia* (Giridhar and Ravishankar, 2004) and *Anectochilus elatus* (Ahamed Sherif et al. 2016).

In this study TDZ was more beneficial in inducing both EC and SEs than other cytokinins. The induction rate varies with type and concentration of growth regulators. Of the different combinations of auxin-cytokinin meant for EC and SEs induction, TDZ (1.0 mg/l) with NAA (0.5 mg/l) induced maximum response with 58.6% upright oriented cultures were induced EC and with an average number of 39.8 SEs/explant. Thus, in this study TDZ played a central role for the induction of SEs through the formation of EC in *D. ovatum*. Similarly, TDZ + NAA are reported to promote the formation of PLBs, callus, SEs and shoots from different explants and from different species of terrestrial and epiphytic orchids. Moreover TDZ (0.5 mg/l) with NAA (1.0 mg/l) could provide a high number of microshoots/seedlings of hybrid orchid (*Aerides vandarum* Reichb.f X *Vanda strangeana* Reichb.f) (Kishor and Devi 2009). Jitsopakul et al. (2013) also reported that MVM medium containing TDZ (2.0 mg/l) with NAA (0.5 mg/l) provide a high mean number of shoots/explants of *Vanda coerulea*. The combination of TDZ (1.0 mg/l) and NAA (0.5 mg/l) was also stimulated the formation of callus and proliferation from node, internode and leaf explants of *Anoectochilus elatus* on Mitra medium (Ahamed Sherif et al. 2016). Similar positive effect of TDZ + NAA was also reported in *Malaxis acuminata* (Meena et al. 2010). All these results very clear that the TDZ alone or in combination with other PGR stimulated various morphogenic response in various orchid taxa.

However, contrast to these the use of TDZ did not induce callus formation or direct PLB formation from leaf, stem and nodal segments in *Dendrobium huoshanense* (Lee and Chen, 2014).

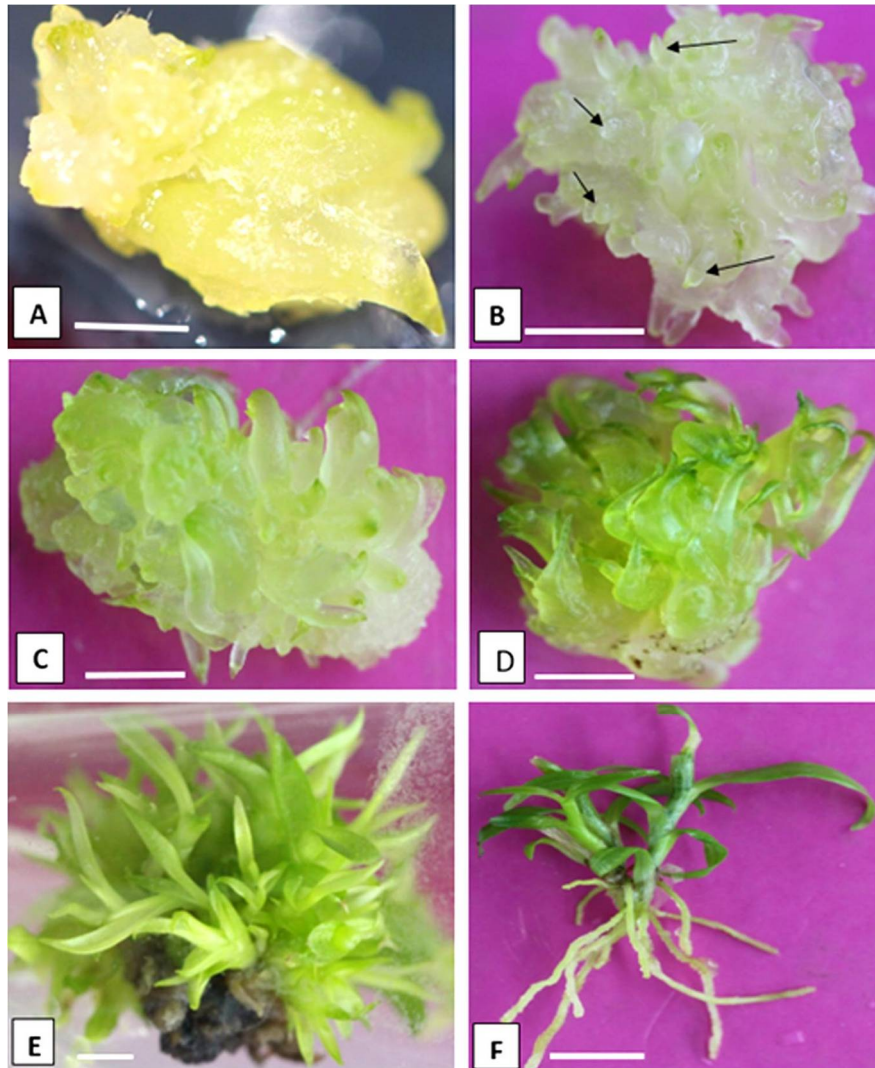


Fig. 1. Plant regeneration through ITLC sections of *Dendrobium ovatum* grown on MS supplemented with TDZ (1.0 mg/l) + NAA (0.5 mg/l). A. Initiation of EC after five weeks of culture (bar = 5 mm). B. Initiation of SEs showing globular (small arrows) and torpedo shaped embryos (large arrows) after three weeks of subculture (bar - 5 mm). C. Development of PLBs after five weeks of subculture (bar = 5 mm). D-E. Differentiation of shoots and leafy shoots from the PLBs after eight weeks of subculture (bar = 5 mm). F. Well-developed plantlets after 20 weeks of culture (bar = 1 cm).

In this work the individual concentrations of BAP (0.5-3.0 mg/l) and NAA (0.5-3.0 mg/l) were not influenced/supported the formation of either EC or SEs in *D. ovatum*. When the MS medium supplemented with BAP at lower concentrations (0.5-1.0 mg/l) showed the formation of EC (10.5%) in an upright oriented ITCL explants. The callus was whitish yellow and the formation of SEs was very few, an average of 0.4 SE/explant was recorded. But in higher concentration (2.0-3.0 mg/l) it was inhibited the formation of EC and SEs. The similar finding of inhibitory effect of BAP on the conversion of protocorms into plantlets and root formation was reported in *Dendrobium nobile* (Nayak et al. 2002), *Dendrobium huoshanense* (Luo et al. 2009) and *Doritis pulcherrima* (Mondal et al. 2013). In contrast to these results BAP also stimulated the formation of highest number of shoots in *Dendrobium chryseum* (Maharjan et al. 2020), highest percentage of callus in *Dendrobium barbatulum* (Ashok Pyati, 2020) and highest mean values of PLBs in *Phalaenopsis hybrid* (Lo et al. 2022). However, NAA was showed inhibitory effect to induce either EC or SEs in the medium. It is in conformity with the earlier work on *Dendrobium Sonia Earsakul*, where NAA could not initiate somatic embryogenesis (Juntada et al. 2015). Many orchid species require auxin and/or cytokinin for the induction of callus, SEs, neoformation of PLBs and plantlet development. It was observed that when MS medium supplemented with the combination of BAP and NAA, stimulated the formation of EC after 6 weeks of culture and further development of SEs were observed in lower concentrations. This finding could be correlated with the effect of BAP and NAA to induce maximum number of PLBs from thin cell layer culture of *Dendrobium hybrid Sonia* (Mandal et al. 2020). The similar synergistic effect of BAP and NAA were also reported in *Dendrobium palpebrae* (Bhowmik and Rahman, 2020) and *Cymbidium finlaysonianum* (Shahinul Islam et al. 2015). The effect of ZEA on the induction of EC and SEs were also examined in *D. ovatum*. ZEA at lower concentration (0.5 mg/l) had a positive effect on inducing EC (27.6%) in an upright oriented explants and also stimulated the formation of SEs (17.1/explants), when the concentration was increased EC induction and SEs formation decreased. These results however in line with the results in sweet potato cv. Brondal, where ZEA produced highest number of shoots and roots from the petiole explants (Masekesa et al. 2016). Similarly, the ZEA at lower concentrations induced EC and in higher concentrations decreased the formation of EC in *Dendrobium aqueum* (Parthibhan et al. 2018) and positive effect in inducing PLBs from TCL explants of *Cymbidium aloifolium* (Nayak et al. 2002). In contrast to this the ZEA showed inhibitory effect on callus and secondary PLBs formation in *Dendrobium barbatulum* (Ashok Pyati, 2020). The combination of ZEA with NAA induced the formation of EC (43.8%) and SEs (24.3/explants). But the EC and SEs produced in the combination of TDZ and NAA were healthier and superior than in medium containing ZEA and NAA.

The important effect of the orientation and position of the explant on the morphogenetic capacity were clearly demonstrated the formation of EC and SEs. From my results the orientation seemed to interact with position to affect the morphogenesis. For the explants incubated in the upright orientation was higher (58.6% - EC and 39.8

SEs/explant) than that in the inverted orientation (16.4% - EC and 9.1 SEs/explant), irrespective of the PGRs. These results were in accordance with the results of Zhao et al. (2007), where the efficiency of shoot regeneration was related to the orientation and position of tTLC explants. If the explants were incubated in an upright orientation on the medium, shoots were induced more efficiently in *Dendrobium candidum*. Among all explants, the highest induction frequency was obtained from the upright incubated explants. Similar results were also reported by Rosas et al. (2010) in *Lycaste aromatica*, analyzing the effect of treatments and type of explant on shoot formation. It is possible to observe that the highest averages of shoots per explants were achieved from basal sections cultured in media supplemented with TDZ. Saini and Jaiwal(2002), pointed out that the orientation appears to interact with polarity to affect shoot regeneration in black gram. But the type of basal and apical oriented tTCL explants of *Brasiliidium forbesii* showed no effect on PLB regeneration (Gomes et al. 2015).

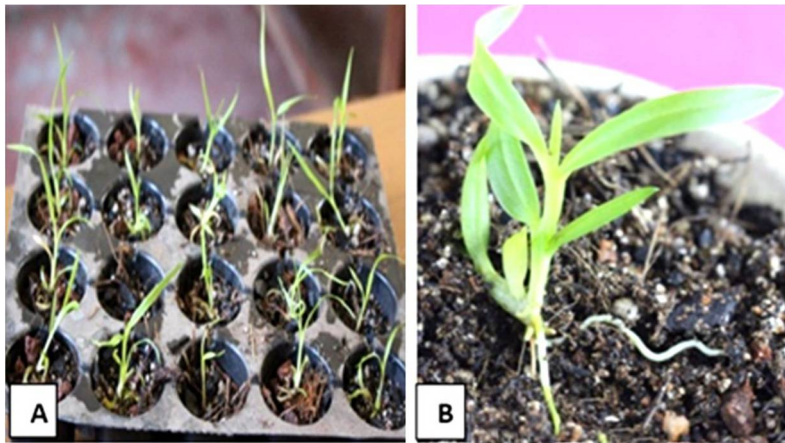


Fig. 2. A-B. Well acclimatized *Dendrobium ovatum* plantlets in greenhouse.

In the present study ITLC culture system was developed, for the induction of EC, SEs and PLBs. The MS medium containing TDZ alone or in combination with NAA resulted in the optimum percentage of EC induction and a greater number of SEs formation per explant and PLBs formation. As for the effect of explant orientation on EC, SEs and PLBs formation, placing the upright oriented ITCL on medium gave maximum results. These SEs/PLBs differentiated into healthy plantlets. This protocol should be useful for large scale multiplication and conservation of this species and for other research and commercial applications.

Acknowledgements

The author gratefully acknowledges the financial support provided by the University Grants Commission, New-Delhi, India, in the form of Major Research Project F.No. 42-927/2013(SR).

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(Manuscript received on 23 March, 2022; revised on 25 April, 2022)