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RESEARCH ARTICLE

ASYMBIOTIC HYPERGENERATION OF PROTOCORM LIKE BODIES – AN EFFICIENT AND SIMPLE MICROPROPAGATION STRATEGY FOR CONSERVING THE THERAPEUTIC ORNAMENTAL *DENDROBIUM OVATUM*

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ABSTRACT

Dendrobium, the third largest genus of the family Orchidaceae, is a slow growing epiphyte. The capsules have thousands of micro seeds, but fail to convert into plants in nature due to the low titer of mycorrhizal associations. *Dendrobium ovatum* is a rare and endemic species of Western Ghats, India. It is therapeutically significant due to the presence of an anticancer bibenzyl derivative viz., Moscatilin. Our aim was to develop a protocol for asymbiotically convert the entire micro seeds of *Dendrobium ovatum* to plantlets. We cultured micro seeds in modified half strength MS media (Murashige and Skoog, 1962) with and without growth regulators with varied sucrose concentrations. We have achieved non-symbiotic micro seed conversion where, 348 PLBs developed from 10mg FW of spherules in half MS medium supplemented with 0.1mg/l ZN. We also compared here the use of undefined additive viz., 10% Coconut water, where we could generate 342 PLB per 10 mg fresh weight of tissue. Addition of 10% coconut water further accelerated the production of PLBs, within 15 days of culture initiation. We observed here that micro seed culture was the most rapid way of propagating this species. The culture established, generated similar sized micro shoots in one-step process. This optimized protocol has rapid and efficient spherule to plantlet conversion percentage (91%) within 21-25 days of culture initiation. This inexpensive protocol can be used serves as a platform for deriving therapeutically important natural products without destroying the *Dendrobium* populations growing in the wild.

INTRODUCTION

Dendrobium, is ranked third among the genus in Orchidaceae and is estimated to comprise 1189 species globally (Leitch, 2009). Among these, 103 species are endemic to North East part of the Indian subcontinent (Singh *et al*., 2001). This genus includes epiphytes with grandiose colourful flowers, especially in sturdy hybrid varieties that are expensive. Very few, wild species are medicinally well exploited by traditional Chinese herbal medicine. They are celebrated as 'Shihu' which is administered as a tonic to clear and nourish digestive system, for fever alleviation, as an antidepressant and for general health improvement (Anonymous, 1986, 2005). Among the therapeutic *Dendrobium* species as reported by Chinese pharmacopoeia; species like *D.aphyllum*, *D.densiflorum*, *D.fimbriatum*, *D.nobile* are native species of India (Singh *et al*., 2001). There have been many attempts to study the chemical constituents of this genus in China. This led to the isolation of medicinally potent bioactives like alkaloids, flourenones, sesquiterpenoids, phenanthrenes and bibenzyl derivatives of which many of them in isolation exhibited antitumor activity (Ye, 2002). Nevertheless, the medicinal aspects remain unexploited in endemic Indian species to date. Crude *Dendrobium* extracts are seen to exhibit many effects such as antidepressant, disease resistance and is used as a health tonic as opined by many studies (Khasin and Rao, 1999). Many *Dendrobium* species are endemic to humid and temperate regions of the tropics. Towards the beginning of the 20th century, there has been a high demand for commercially valuable *Dendrobium* hybrid species due to its floral diversity and long standing freshness (Jones *et al*., 1998). This had led to the uncontrolled outbreeding of many wild species with little concern over their curative aspects. *Dendrobium ovatum* is one such Indian species where, its medicinal property is not studied. There has been little attempt to study germination of seeds. Our observations on the morphometric characters indicated that the species possessed mauve brown pseudobulbs that are 4-55 cm long, with internodes of length 2-5 cm.

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Leaves displayed alternate phyllotaxy (alternatively arranged leaves) which are membraneously elliptical of length 4-8 cm. Inflorescence is racemose in nature, which were usually 5-15 cm long. Individual flowers of inflorescence are cream coloured, with a green lip at its centre. Sepal like tepals and petal like tepals are 7-10 mm long. Lip is 7-8 mm long and is trilobed. Lateral lobes are small, erect, while the mid lobe is longer, covered with green soft trichomes. Flowering pattern was annual, from November-April. Our survey on occurrences of *Dendrobium ovatum* helped us to understand, that the species exhibited host specificity and was found distributed in small pockets in spill over regions of the Western Ghats (unpublished data). There has been a concern over the habitat loss in the Western Ghats and its buffer zones due to environmental and man-made disasters like indiscriminate collection, deforestation of host trees and illegal trade of specimens. There are also possibilities that wild endemic orchids are under intense pressure due to biotic stress and might face extinction soon (Kishor *et al*., 2006).

Hence, there is an utmost need to preserve and conserve the germplasm of these medicinally valuable wild endemic orchid resources. *D. ovatum* fruits (capsules) produce thousands of powdery fusiform micro seeds, but very few (<1%) germinate in nature. Low germination rates of seeds in orchids is due to its minuscule, powdery non-endosperm seeds and their prerequisite for mycorrhizal association (Rao, 1977, Richardson *et al*., 1992). However, this 'facultative symbiosis' is rare in natural conditions. Mycorrhizal alliance helps the seeds to draw nutrients for their growth, especially simple sugars, which need to be metabolized facilitating seed germination (Arditti, 1967). *In vitro* cultures have successfully played a vital role in propagating many recalcitrant plants; which were unsuccessful when conventional methods of propagation were adopted (Fay, 1994). This will be advantageous to epiphytic orchids that can grow *in vitro* non-symbiotically (without any aid of fungal partners). Keeping the large-scale production in mind, there have been many attempts to micropropagate *Dendrobium* species like *D. candidum* (Zhao *et al*., 2007), *D. draconis* (Rangsayatorn, 2009), *D. gratiosissimum* (Jaiphet and Rangsayatorn, 2010) and *D. nobile* (Nayak *et al*., 2002). Some of these methods were tedious and slow.

Few studies have opined that, rhizoid like structures developing from any tissue or explants other than seeds leading to direct or indirect plantlet formation in orchids are termed as Protocorm Like Bodies or PLBs (Arditti and Ernst, 1993). If the seeds generate an interphase structure from which the protocorms develop, they must be addressed not as protocorms, but as Protocorm Like Bodies.

The PLBs once formed were able to accelerate the gradual organogenesis in a few orchid genus. However, raising PLBs is not an easy task. The first scheme would be to identify the suitable media recipe fortified with appropriate plant growth regulator. The second approach would be to identify the efficient explant which could generate PLBs. Studies elsewhere have indicated that PLBs once formed, their conversions to plantlets demand either change in media supplements in the form of PGRs (Rangsayatorn, 2009) or carbohydrate concentrations or additives which are either defined or undefined. Despite these lengthy approaches, the growth continued to remain retarded due to low PLB induction, its developmental failures and low acclimatization success rates. Thus, there is an utmost need to amplify both the induction and the conversion efficiency of PLBs in a simple one-step protocol. The present study was done primarily to identify explants capable of PLB induction and optimize an efficient protocol for PLB based micropropagation in *D.ovatum*. We believe that this study constitutes the first report on the rapid and large-scale propagation of *D. ovatum*. Here we were able to demonstrate amplified non-symbiotic micro seed germination *in vitro*, leading to PLB hypergeneration followed by plantlet development using a single step approach.

MATERIALS AND METHODS

Plant material

D. ovatum plants were collected from the wild and were established in the greenhouse of School of Life Sciences, Manipal University, Manipal, Karnataka, India. The pseudostems of the plants was inserted in pots filled with equal proportions of soil, coconut husks, tile pieces, charcoal. The species with epiphytic habitat exhibited root initiation within 2 weeks and tightly were attached to the surfaces of pots, tile pieces and coconut husks. All plants acclimatized to the greenhouse within six weeks. Flowering pattern and anthesis cycles of the plants in the greenhouse was recorded which was in consonance with plants found in the site of collection. The study design was categorized into IV phases.

Phase 1 experiment was to determine the appropriate sucrose concentration that could initiate PLBs in full strength basal MS medium (Murashige and Skoog 1962). Phase II analysis was to ascertain the impact of medium strength (Full strength, half strength and quarter strength) in the rapid PLB initiation from explants at a selected sucrose concentration. Only those explants that responded to Phase II trials and I were selected for Phase III analyses. Phase III trial was to identify the ideal PGR at three wider concentrations to identify the potential PGR primarily cytokinins and its effective concentration in PLB induction and amplification.

The cytokinins used in the present experiment were 6–benzylaminopurine (BAP), 6 furfuryl amino purine called Kinetin (KIN) and Zeatin (ZN) purchased from Sigma, USA. The three concentrations used are 0.1, 1 and 10 mg/l. Phase IV was to compare the efficiency of the media by replacing the PGR with undefined additive – coconut water (CW), in selected sucrose concentration and replacing one of the macronutrients, ammonium nitrate with ammonium sulphate in the media composition viz., (Table.1). Tender CW was collected, filtered using a Whatmans filter paper and was then added in 1, 5, 10 and 15 % in media without PGRs. 50 ml of MS medium of pH 5.8 was poured into culture bottles of 250 ml capacity each and was gelled with 0.8% agar (Hi-Media, India) prior sterilization and was autoclaved at 121ºC at 15 p.s.i for 20 minutes.

Table 1 Morphometric Characters, occurrence and life cycle of *Dendrobium ovatum* as observed in the present study

General appearance	Epiphytic orchid with small cream coloured long standing flowers. Green lipped cream coloured flowers was in racemose inflorescence which was 5-15 cms long	Exhibits tufty growth	Found in small pockets in the bark of tree trunks aged ≥ 50 year old trees infrequently found in the buffer zones of Western Ghats
Pseudobulb	Brown in colour with mauve stripes bearing parallel veined membranous elliptical leaves (4-8 cms in length) which exhibited alternate phyllotaxy	Length 4-55 cms long Internodes were 2-5 cms long	Pseudobulbs gave rise to fresh branches with tuft of aerial roots at the base
Aerial roots	Light green slender when young with dark green root tips	Turns brown on maturity which facilitate attachment of drooping tufts	Fresh aerial roots appear from the base of the new branch sprouts that in turn arise from older pseudobulb
Flowering	Flowering is annually observed from November and stands till February	Sepal like tepals and petal like tepals are 7-10 mm long. Lip is 7-8 mm long and is trilobed. Lateral lobes are small, erect, while the mid lobe is longer, hanging out, covered with green soft trichomes.	During the late flowering phase the plant defoliates followed by shrinkage of pseudobulbs.
Capsule formation	From February to May the capsule are visible in the plants	The capsule is ellipsoid and green when they are young and turns silvery cream with dark brown longitudinal running ridges on the surface	The capsule dehisce in the longitudinal axis liberating thousands of non-endospermous fusiform micro seeds
Vegetative phase	June to November	Small propagules (2-3 Numbers) emerge that cling to bark of trees with aerial roots which substantiates low seed germination rates in nature	This orchid exhibited host specificity and are found commonly attached to *Mangifera indica, Casuarina, Hopea,, Albizzia, Pithecelobium* tree sp.

Table 2 Response from various *D. ovatum* explants in Basal MS media of varied strengths and concentrations of sucrose in 10 replicates.

MS medium	Explant Type	2 weeks after culture	4weeks after culture	8 weeks after culture	12 weeks after culture
Full Strength with 1,3,4 & 5% sucrose	Nodes, Internodes, Leaves	Tissue blackening	Tissue blackening & necrosis	Tissue blackening & necrosis	Necrosis
Full Strength with 1,2,3,4,5% sucrose	Tepals	No Tissue blackening	No Tissue blackening	No Tissue blackening	No response
Full Strength with 1,2,3,4&5% sucrose	Green & Dry Capsules	No response	No response	No response	Micro seeds exhibited size enlargement
Half Strength with 1,2,3,4& 5% sucrose	Nodes, Internodes, Tepals and Leaves	No tissue blackening	No tissue blackening	No tissue blackening	No tissue blackening
Half Strength with 1, 3,4& 5% sucrose	Green &Dry Capsules	No tissue blackening	Micro seeds exhibited size enlargement	Micro seeds exhibited size enlargement	Micro seeds exhibited size enlargement
Half strength with 2% sucrose	Green &Dry Capsules	Micro seeds exhibited size enlargement	Micro seeds exhibited size enlargement	Micro seeds exhibited size enlargement	Signs of spherule formation indicating requirement of PGR
Quarter strength media with 1,2,3,4&5% sucrose	Nodes, Internodes, tepals & leaves	No tissue blackening	No tissue blackening	No tissue blackening	No response
Quarter strength media with 1,2,3,4 & 5% sucrose	Green and Dry Capsules	No response	No response	No response	Micro seeds exhibited size enlargement

The media used for culture experiments was supplemented with five concentrations of sucrose ranging from 1,2,3,4 and 5%. Plant parts, which were used for inoculation, where the leaves, pseudostem (nodes & internodes), tepals and capsules (green indehiscent and dry dehiscent).

The green capsules were harvested after 4 weeks of anthesis and dry capsules were harvested after 8 weeks of anthesis. Explants like leaves, single flowers, pseudostems and capsules were segregated into separate conical flasks and were subjected to surface sterilization procedures. The explants were first soaked in 10% Tween 20 detergent for 5 minutes and washed in running tap water for 30 minutes. All the subsequent work was performed in the laminar air-flow cabinet.

The explants were subjected to 0.1% mercuric chloride treatment. Mercuric chloride treatment duration for tender explants viz., leaves and flowers were 3 minutes and for robust explants like pseudostem and capsules, the treatment duration was 10 minutes.

After mercuric chloride treatment, the explants were rinsed thrice with sterile double distilled water.

Leaves and tepals were cut into square pieces of approximately 5mm × 5mm in a sterile Petri plate and were inoculated in culture bottles containing basal MS media (MS media free of any PGR) with varied sucrose concentrations.

Pseudostem, node and internodes of 1 cm length were placed vertically in the medium. Every culture bottle had 5 explants and 10 such culture bottles (10 replicates) were prepared for every explant. The capsules (Fig.1.a) after sterilization procedures were longitudinally slit open using a sterile surgical blade and all the fusiform micro seeds (Fig.1.b&c.) from a single capsule were layered as a thin film into a 250 ml culture bottle containing 50 ml solidified medium. Each treatment had 10 replicates. The conversion of micro seeds and its transition to spherules (the PLB initials) was calculated in percentage. Number of PLBs developed from spherules was recorded per 10 mg FW of tissue in triplicates using a hand lens and stereo zoom microscope. Sub culturing of explants was performed only when micro shoots developed.

Statistical analysis

Data was analyzed using the Statistical Package for Social Sciences (SPSS 16.0; Chicago, IL, USA). Duncan's multiple-

range test was used to determine significant differences between treatment means.

Culture maintenance

The cultures were maintained at 25±2^{0}C and 75 % relative humidity under cool fluorescent light at 50 µmol m^{-2} s^{-1} (Philips, India) with a 16-h photoperiod.

and their conversion to PLB development from the seeds at the 12th week of culture (Table.2). The protracted PLB formation was indicative for the requirement of a suitable plant growth regulator.

The PLBs when sub cultured in the same media did not have any impact on protocorm development.

Table 3 Response from micro seeds to treatments from 10 replicates. Mean values with different letters are significantly different using Duncan's multiple-range test (P<0.05)

PGR used in half strength MS fortified with 2% sucrose	Spherule formation from microseeds in % (Mean ±SE)	Time required Formation of PLB (in Weeks)	Development of protocorm like bodies per 10 mg fresh weight (Mean ±SE)	PLB Conversion Efficiency to Plantlets in % (Mean ±SE)	FW in mg of tissue at 4 weeks of culture	Time required for plantlet acclimatization (in Weeks)
Basal (control)	13.0±2.18^a	12-15	2.25±0.31^a	3.7±0.91^a	2.5±0.86	9-10
0.1mg/l 6-BAP	58.7±1.77^e	8-9	46.6±5.05^c	25.1±1.75^c	208.8±14.88^a	6-8
0.1mg/l ZN	92.8 ±1.60^h	1-3	273.2±5.48^g	81.6±3.20^e	2522.9±34.97^e	3-4
0.1mg/l KIN	15.0±1.63^a	7-9	2.55±0.32^a	5.0±0.97^a	132.5±7.12 ^{a}	8-9
1 mg/l 6-BAP	41.6±4.12^c	5-8	33.8±2.06^{bc}	18.8±1.93^b	321.6±39.01^a	4-5
1mg/l ZN	39.1±1.90^c	2-3	48.1±3.14^c	70.1±2.53^d	578.8±13.33^{bc}	3-4
1mg/l KIN	50.0±2.23^d	4-6	41.1±2.03^c	26.2±1.76^c	520.0±40.01^b	4-6
10 mg/l 6-BAP	26.6±1.88^b	4-6	33.3±2.52^{bc}	31.0±2.13^c	766.6±18.67^c	3-5
10mg/l ZN	41.2±1.90^c	3-4	35.0±2.94^{bc}	70.9±4.33^d	1640.3±55.89^d	5-6
10mg/l KIN	36.1±2.52^c	4-6	21.4±1.95^b	15.3±1.83^b	545.6±41.71^b	8-9
1% CW	80.4±2.32^g	3-4	138.7±5.58^f	83.0±1.56^e	2811.5±51.12^f	3-6
5% CW	83.5±2.01^g	3-4	121.4±7.34^e	84.3±1.77^e	3862.2±76.60^h	3-4
10% CW	86.3±2.26^g	2-4	292±11.24^h	93.0±1.65^f	5777.3±49.95^i	4-6
15% CW	68.7±3.39^f	4-6	78.9±2.66^d	69.6±1.25^d	3253.8±63.42^g	3-4
Modified ½ MS+0.1mg/l ZN	94.7±1.01^h	1-2	348.6±6.90^i	91.0±1.20^f	8049.5±83.87^j	3-4
Modified ½ MS +10%CW	95.5±0.97^h	1-2	342.5±12.63^i	91.0±1.20^f	7870.9±195.29^j	3-4

RESULTS

Phase I trial showed that, the only explant that responded to basal media was fusiform micro seeds from green and dry capsules (Table.2). The fusiform microseeds (Fig.1.c) exhibited size enlargement irrespective of the strength of media or sucrose concentrations. Enlargement of *D. ovatum* fusiform micro seeds altered into small miniature globular bodies called spherules (Fig.1.d) which later transformed into rhizoid like structures termed PLBs (Fig.1.e).

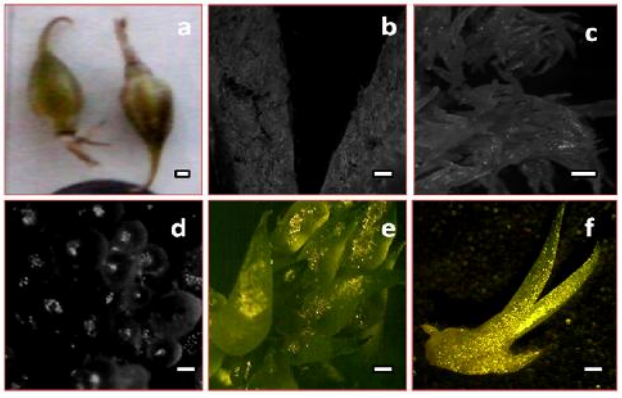

Fig. 1 a. Capsules of *Dendrobium* harvested 4 weeks after anthesis b. Longitudinally split capsule revealing fusiform microseeds c. fusiform micro seeds separated from capsules d. Primary and secondary spherules formed from micro seeds e. transition of primary and secondary spherules to protocorm like bodies f. Microshoot developed from protocorms. Bars a &b= 0.5 cms c= 200µm d,e, &f=100µm

However, sucrose concentration, which was beneficial for spherule formation that gave rise to PLBs, was 2% in half strength MS medium. There we witnessed spherule formation

Explants like leaf, nodes and internodes exhibited signs of necrosis in full strength MS media, which got minimized when cultured on half and quarter strength MS. Half and quarter strength supplemented with 1-5% sucrose concentrations did not induce any tissue blackening. Tepals and microseeds were the two explants, where no blackening was witnessed irrespective of media strength and sucrose concentration employed. Phase I and Phase II studies confirmed that the potential explants were the fusiform micro seeds, which when cultured in half strength MS fortified with 2% sucrose gave spherules which later formed PLBs. The cytokinin supplementation in half strength MS was beneficial for accelerating induction of PLBs from micro seeds (Table.2).

Fig.2. Micro shoots from PLBs in Modified half strength MS supplemented with 0.1 mg/l Zeatin a) on the 21stth day of culture b) on the 30th day of culture c) Plantlets at the 42nd day of culture (scale bar= 3cms) d) Inset: Plantlets in green house after 2nd week of hardening . Plantlets after 5th week of hardening

The spherules formed were simulating the phenomenon of direct somatic embryogenesis where the globular stage will develop a torpedo stage that will have a shoot pole and root pole. However, the number of spherules formed and their conversion to PLBs varied in distinct cytokinins and it was concentration dependent (Table.3). Neither there was a requirement of altered media supplementation, nor was a need of spherule-derived micro shoots (Fig.1.f) transfer to a separate rooting medium. ZN had a beneficial effect on hypergeneration of spherules (within 21 days) that later developed into plantlets within a month. The development of protocorm from the spherule shaped PLBs occurred which were converted to plantlets. These plantlets attained 3 cms height within 28 days and could be directly subjected to hardening processes. Plantlets were grown in plastic cups filled with equal proportions of finely ground autoclaved charcoal, coconut husk and sand and soil mixture. The percentage of survival for ZN derived plantlets was 86. The roots generated were very similar to plantlets found in the wild. There was no need of shifting the plantlets to a separate rooting medium and thus generating plantlets in a single step process.

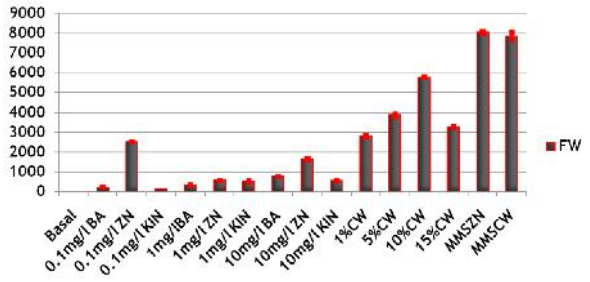

Fig.3. Fresh Weight of Tissue in mg with Protocorm like Bodies developed from spherules at the 4th week of culture at different Plant growth regulators in half strength and Modified half strength MS media . Basal medium represents half strength MS without any growth regulator supplementations

As concentrations of ZN increased, there was a substantial reduction in the number of PLB formation. 6-BAP and KIN concentrations exhibited low PLB induction and conversion when compared to ZN. 0.1 and 1 mg/l KIN and BA also induced spherules but their ability to induce PLBs where very low when relative to ZN. 1 mg/l KIN had a superior effect in generating a higher number of PLBs (48 per 10mg FW of tissue). We replaced ZN with 1-15% coconut water to understand the impact of an undefined additive that has been commonly used as PGR.

The spherule induction efficiency of coconut water enriched half strength MS media was on par (86.3%) with ZN supplemented medium (92.8%). Nevertheless, 10% CW supplementation exhibited highest conversion efficiency of spherule development from micro seeds (93%) despite high variation between replicates. Phase III trials proved that lower concentrations of cytokinins evoked hypergeneration of spherules, followed by rapid PLB formation and faster transition to plantlets within a span of 28 days. Even though 10% CW was superior to 0.1mg/l ZN in spherule induction, their transition to PLB was high (273 per 10mg FW), and consistent in ZN supplemented medium (Fig.2a & b). We replaced the major macronutrient ammonium nitrate to ammonium sulphate to study the impact of modified half strength MS fortified with 10% CW and 0.1mg/l ZN respectively. This modified half strength MS exhibited 95% of spherule induction and a fold increase in hypergeneration of PLB formation (348.6 per 10mg FW of tissue) (Figs. 2a & 2b). Their conversion efficiency to plantlets (Figs. 2c & 2d) was 91%. Thus, we have achieved non-symbiotic micro seed conversion to spherules and subsequently 348 PLBs per 10mg FW of tissue at a very low concentration of ZN (0.1mg/l ZN). 10% CW will be yet another economical and efficient substitute for this expensive cytokinin where one could generate 342 PLB per 10 mg FW of tissue.

DISCUSSION

Orchid tissue culture is challenging and always there is a pursuit of simple strategies to get consistent, reproducible mass propagation from were healthy plantlets could be obtained within a short span of time. When a perfect protocol is optimized it can be used for germplasm conservation, eco rehabilitation as opined by some studies (Wu *et al*., 2012) and long term storage like cryopreservation and synthetic seed technology (Sharma *et al*., 2012).

The basic step in orchid micropropagation would be to develop PLBs from prospective explants. The first PLBs (primary PLBs) when transferred to another medium might generate secondary PLBs and if the secondary PLBs gives rise to another set of PLBs they are termed as tertiary PLBs (Teixeira da Silva and Tanaka, 2011). However, PLB is a terminology restricted to development of rhizoid like structures from any part of the plant body other than the seeds. In the present study, there was formation of an intermediate phase, which resulted in the development of globular structures. These spherules that later transformed into secondary and tertiary spherules. The globular structures simulated globular somatic embryos that transformed into protocorms. These protocorms were called PLBs as they developed from the spherules derived from the micro seeds and not directly from micro seeds.

Stereozoom microscopic examinations exhibited the presence of secondary and tertiary PLBs. Despite the occurrence of secondary and tertiary PLBs better proliferation and superior conversion, efficiency of PLBs was witnessed in the present study. Occurrences of secondary and tertiary PLB formation can hinder the development and conversion of PLBs in *Cymbidium* species (Begum *et al*., 1994).

Basal media, especially half strength MS devoid of growth regulators are found to be beneficial for PLB differentiation and conversion (Teixeira da Silva *et al*., 2005, 2006; Teixeira da Silva and Tanaka, 2011) and this was found to be in consonance with our study. Sucrose, the carbohydrate source had a weighty impact on the generation of spherules in the present study as reported elsewhere (Desjardins *et al*., 1995). Higher sucrose concentrations retarded the PLB development in the *D. ovatum* and similar effects was seen in *Echinacea angustifolia* (Wu *et al*., 2006). It has also been reported that the addition of organic supplements at higher concentrations can cause necrosis of the plant material (Ichihashi and Islam, 1999). A similar phenomenon was observed in *D.ovatum* in our study. This could explain the beneficial effects of half strength

MS medium with low organic supplements that led to spherule induction and thereby hypergeneration of PLBs.

Our study indicated that very low concentration of 6-BAP and ZN had a beneficial effect on spherule induction and development of PLBs in the present study as previously reported in many other species (Park *et al*., 2002, Chung *et al*., 2005 and Kuo *et al*., 2005). Supplementation of CW to the basal media enhanced the possibility of early differentiation of PLBs in the current study as in *D. aphyllum* (Talukdar, 2001).

Tender CW is an amalgamation of cytokinins (Leetham, 1974) that would have contributed to the rapid development of plantlets. The choice of cytokinin was done based on the ability of the PLBs to develop into plantlets that can acclimatize to the greenhouse conditions very soon. Acclimatization efficiency was higher in plants raised in ZN and CW fortified medium of which ZN exhibited higher efficiency. ZN was reported to have a superior effect over other cytokinins in propagating *Vaccinium corymbosum* (Chandler and Draper, 1986) that is in consonance with the present study. Replacement of ammonium nitrate with ammonium sulphate further enhanced induction of PLBs. VW medium (Viacin and Went, 1949) is often considered good for PLB based orchid micropropagation notably in *Cymbidium* species as per studies elsewhere (Teixeira da Silva *et al*., 2005; 2006; 2007a,b; Teixeira da Silva and Tanaka 2011; Teixeira da Silva 2012a,b) where, ammonium sulphate is the prime macronutrient.

CONCLUSION

The macronutrient ammonium suphate have contributed to the hypergeneration of PLBs in *Dendrobium ovatum*. There was an average increase of spherule every 10 mg of FW of tissue with the modified MS medium that was supplemented with ammonium sulphate. We have optimized the asymbiotic hypergeneration of PLBs in *D. ovatum*, where plantlets develop without mycorrhizal associations. This optimized protocol has rapid, efficient conversion rate, where, formation of plantlets could be achieved within 21-25 days. The plant growing in the wild generally takes 6 months to develop; if only, the environmental factors are congenial. This protocol is useful for rapid micropropagation of any wild orchids furthermore.

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