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ASYMBIOTIC SEED GERMINATION AND ENHANCED SHOOT PRODUCTION IN DUAL PHASE CULTURE SYSTEM USING ORGANIC ADDITIVES IN *DENDROBIUM OVATUM* (L.) KRAENZL.

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Abstract

Liquid overlays culture system improves shoot proliferation during micropropagation in many plant species including orchids. The present study reports the development of a protocol for the development of enhanced shoot regeneration from embryo derived protocorms, using casein hydrolysate (CH) as an organic additive, for the *in vitro* production in a threatened tropical epiphytic orchid species, *Dendrobium ovatum*. Organic additive (CH: 0.05%) mediated dual phase culture system (DPS) was performed using MS medium and yielded a maximum of 11.60±0.10 shoots per explant. Multiplication of protocorms via this method led to high frequency formation of protocorm-like bodies (PLBs). Plantlets thus developed may be used for large scale multiplication and conservation of *D. ovatum*. Replacing the CH with 5% banana homogenate in the culture medium improved plantlet development and 89% of these survived after transplantation, under greenhouse conditions.

Introduction

ORCHIDACEAE IS one of the largest and the most evolved plant families of flowering plants comprising 29,481 species under 703 genera (POWO, 2024; WFO, 2023). Orchids are considered as ornamental elites due to their stunning flowers with diverse fragrance and colouration and varied forms and patterns; these are considered as the second most popular cut flowers in international floriculture trade across the world (Mebakerlin and Chakravorty, 2015; Prakash and Pathak, 2020, 2023). Apart from ornamental value, the orchid history started with their use for medicinal purposes. The ancient references to the medicinal herbs in India provide important information regarding the use of orchids in medicine (Hossain, 2011; Kirtikar and Basu, 1918; Pathak *et al.,* 2010; Prakash and Pathak, 2019; Tikendra *et al*., 2020). Orchids are virtually found in all parts of the world except icy Antartica and hot deserts and with greater diversity across tropical and sub-tropical regions.

The genus *Dendrobium* Sw. consists of about 1602 species and is significant amongst the orchids due to their high ornamental and medicinal values (POWO, 2024; Tikendra *et al*., 2020; Xiaohua *et al*., 2009). Most species in the genus *Dendrobium* are used in many systems of medicine in the world. Due to the immense medicinal potential and huge demand for the pharmaceutical industries, natural populations of most of the orchid species in the genus *Dendrobium* are on decline due to habitat destruction and unregulated collection for illegal trade. For effective germplasm conservation and increasing commercial demands of these species, rapid large scale propagation of these is very essential. Conventional propagation methods are very slow in response, extremely time consuming and labour-intensive. Substitution to conventional approaches of propagation of dendrobiums can be achieved through plant tissue culture techniques for commercialization and effective conservation.

Dendrobium ovatum is a threatened epiphytic herbaceous tropical orchid found in Western peninsular India especially in the Western Ghats and open deciduous forests in Southern India; the species is commonly referred as *green-lipped Dendrobium* due to the presence of green center in off white coloured flowers (Teoh, 2016). Van Rheede (1703) reported that *D. ovatum* was used for relieving all sorts of chest pain and stomach ache in his legendary book *Hortus Malabaricus* and can be used as a substitute for Jivanti in Ayurveda (Khare, 2007). The orchid seeds need specialized mycorrhizal fungus for nutritional supplementation and germination in nature. This explains the essentiality of asymbiotic approach of *in vitro* propagation of *D. ovatum* using seed culture method. *In vitro* asymbiotic seed germination rates are more than 70% which rarely exceed 5% in natural habitat conditions (Cardoso *et al*., 2020). Different *in vitro*

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culture systems for micropropagation in *D. ovatum* have been established by a few authors using seeds at both mature and immature stage (Thejaswini and Narasimhan, 2017). *Dendrobium ovatum* is a therapeutically significant species as it is known to contain bibenzyl derivatives which are found to exhibit numerous therapeutic actions such as antioxidant, anti-inflammatory, and anti-tumour properties (He *et al*., 2020). The essential method for the rapid and large scale production of the taxon requires improved *in vitro* culture system so that it may be advantageous for the production of medicinal compounds. Because of the over-exploitation for its medicinal properties, its natural populations are declining at an alarming rate. Hence, this species needs urgent conservation measures. *In vitro* propagation technique is an efficient method to increase population size of many orchid species. Some attempts have been made earlier to propagate and conserve a few orchid species using *in vitro* techniques (Anuprabha and Pathak, 2012; Arora *et al.,* 2016; Bhowmik and Rahman, 2022, 2023; Dhillon and Pathak, 2023; Kirti *et al.,* 2023; Lalduhsanga *et al.,* 2021; Mutum *et al.,* 2022; Pathak *et al.,* 2023; Sunita *et al.,* 2021; Thakur and Pathak, 2021; Tripura *et al.,* 2022; Vasundhra *et al.,* 2021; Vij and Pathak, 1989). The physical state of the culture medium system is an important factor, which plays a significant role in enhancing shoot proliferation in micropropagation. Liquid system or dual phase system (DPS) of culture medium can increase regeneration capacity with better nutrient availability in the culture system than single phase system (SPS) (de Oliveira *et al*., 2013; Roy *et al*., 2012; Thompson *et al*., 2007). Temporary liquid immersion system mediated enhanced shoot proliferation from the protocorms has drawn less attention in orchid culture and there are no such attempts in the enhanced production of *D. ovatum*. Therefore, the present paper reports the evaluation of liquid medium mediated asymbiotic seed germination and a novel approach of double phase *in vitro* culture system for the enhanced shoot production of *D. ovatum* with a view to contribute a new approach for its large scale production and conservation.

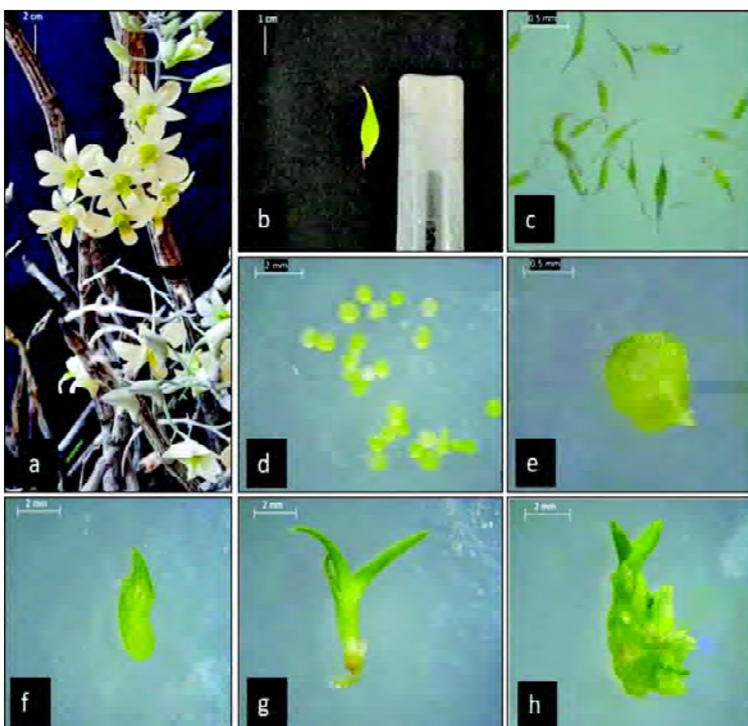


Fig. 1a-h. *In vitro* asymbiotic seed germination in *Dendrobium ovatum*: a, *Dendrobium ovatum* plant in bloom; b, Green undehisced capsule; c, Seeds at the time of inoculation; d, Spherules developed after 20 days of culture in half strength liquid MS medium; e-f, Protocorm stage after 30 days of culture; g, Seedling development in basal liquid MS medium; h, Development of PLBs (Protocorm-like bodies) after 60 days of culture.

Material and Methods

Source of Capsule and Culture Conditions

Immature seeds from undehisced green capsules (*pods*) of *Dendrobium ovatum* (Fig. 1a) were collected from Colomb, Goa. The capsules (Fig.1b) were gently rinsed with running tap water for 10 min followed by 10% Labolene (Galaxo India Ltd., Mumbai) treatment for 15 min with constant agitation. Capsules were then left under running tap water for 30 min and subjected to surface sterilization using 0.1% $HgCl_2$ (w/v) (SRL, Pvt. Ltd., India) for 10 min followed by washing in sterile distilled water for five times with 1 min time interval each; these were then dipped into 95% (v/v) of ethanol for 5 sec and passed briefly through the flame. These were then cut open aseptically inside laminar air flow hood (Klenzaids, India) and the seeds were inoculated into liquid culture medium using sterile forceps and blade. Half strength MS medium (Murashige and Skoog, 1962) with carbon source (3% Sucrose (w/v) and a pH of 5.8 was used. Medium containing pre-sterilized Erlenmeyer flasks (200 ml) were sterilised at 120°C for 18 min under 15 psi pressure. Cultures were maintained at 25±2°C under 16/8 hr photoperiod with light intensity of 2500 lux (μmol^{-2} S^{-1}) using white led tubes (Crompton Ltd., Mumbai) and 55-60% relative humidity was used.

Effect of Organic Additives on Seed Germination

The immature seeds procured from the sterilized capsules were inoculated into the liquid half strength MS medium fortified with organic additives *i.e.* casein hydrolysate (CH, 0.05%), peptone (PEP, 0.05%), yeast

extract (YE, 0.05%), and coconut water (CW, 0.05%). The coconut water was filtered before using in the liquid medium. Liquid medium devoid of additives served as control. Cultures were maintained using frequent agitation using orbital shaker (Jeio Tech Co., Ltd. Korea) at 90 rpm with optimal culture conditions for 60 days. All the developmental stages during seed germination were observed and identified under a stereomicroscope (Leica Microsystems, Germany), equipped with the software 'LAZ EZ'. These microscopic images of different stages were captured.

Effect of Organic Additives on Shoot Multiplication

In vitro derived 60 day-old protocorms served as explants for shoot multiplication. Protocorms obtained from the cultures were further inoculated on agar gelled [0.8% w/v, Himedia, India) half strength MS medium containing different organic additives *i.e.* casein hydrolysate (CH, 0.05%), peptone (PEP, 0.05%), yeast extract (YE, 0.05%), and coconut water (CW, 0.05%). Medium without organic additives served as control. The cultures were maintained in the culture room for 40 days and the data was recorded and images of shoot proliferation were captured.

Effect of Dual Phase Culture System on Shoot Proliferation

MS medium augmented with casein hydrolysate (CH, 0.05%) for shoot multiplication was selected for the evaluation of double phase culture system for enhanced shoot proliferation. Double phase system (DPS) consists of solid phase medium and a layer of liquid phase medium (3ml). *In vitro* derived protocorms were inoculated on DPS with two modifications- i) DPS- SBM +LCHM: solid basal MS medium (SBM) with a layer of liquid CH containing medium (LCHM) and ii) DPS-SCHM +LBM: solid CH containing medium with a layer of basal MS medium. DPS- SBM +LBM: DPS with basal solid and liquid medium was also used for the comparison with other systems and single phase system (SPS) served as control. Liquid layer was introduced into the system after 15 days of inoculation of explants on solid nutrient media. The data was recorded after 40 days of culture.

Effect on Plantlet Development

In vitro derived shoots were further inoculated into MS medium with different concentrations of banana homogenate (BH: 1%, 5%, and 10%) for better seedling growth and enhancement. Basal MS medium was used as control. Number of roots per plantlet, root length, number of leaves per plantlet, plantlet length, and percentage of response were recorded after 30 days of inoculation.

Hardening of In Vitro Raised Plantlets

Well developed *in vitro* raised plantlets with 3.5 cm height were taken out from culture vessels and washed thoroughly under tap water in order to remove gel traces of nutrient medium. Plantlets were treated with 0.5% bavistin for 15 min and planted in pots containing brick pieces, coconut husk, and charcoal pieces (1:2:2). The plants were covered with polythene sheet for one month to maintain higher humidity and were irrigated thrice a week.

Experimental Design and Data Analysis

The experiment was carried out in complete randomized design (CRD) with three replications. Experimental data was recorded after 60 days for germination experiment, after 90 days for shoot proliferation and DPS culture experiment and after 30 days for plantlet development experiment. Data was analysed by R software (version 4.2.0) and subjected to ANOVA and the mean values were compared using Duncan's multiple range Test (DMRT) at $p<0.05$ significance level.

Results and Discussion

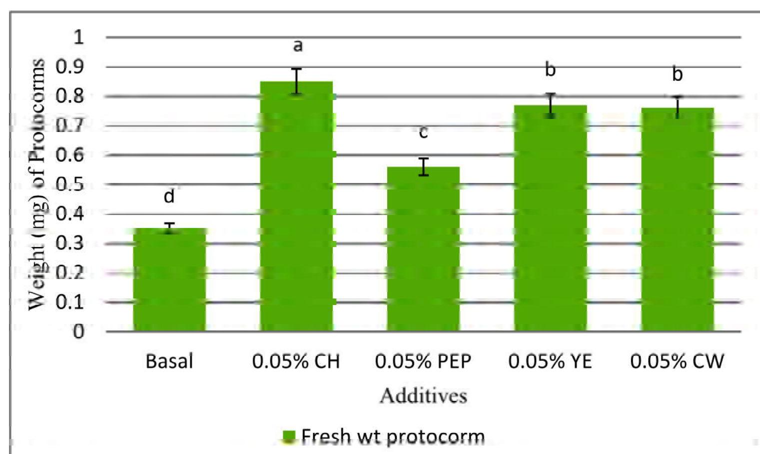
During the present studies, immature seeds from undehisced green capsules (*pods*) of *Dendrobium ovatum* (Fig. 1c) were used for the present experiments. The quantitative characters such as length and width of capsules, seeds, and embryos were observed (Table 1). The seeds were creamy white, filamentous in nature with green embryos inside the seed coats. The embryo length was almost half of the entire seed length.

Table 1. The quantitative characters studied in *Dendrobium ovatum* capsules, seeds and embryos.

Quantitative characters		Results (mean±SE)
Capsule	length	2.01±0.03 cm
	width	0.74±0.03cm
Seed	length	0.47±0.01mm
	width	0.08±0.00mm
Embryo	length	0.20±0.00mm
	width	0.05±0.00mm

Effect of Organic Additives on Seed Germination

Seed germination process was initiated *in vitro* with the swelling of embryos after 10 days of inoculation. Seeds swelled and embryos developed into spherules

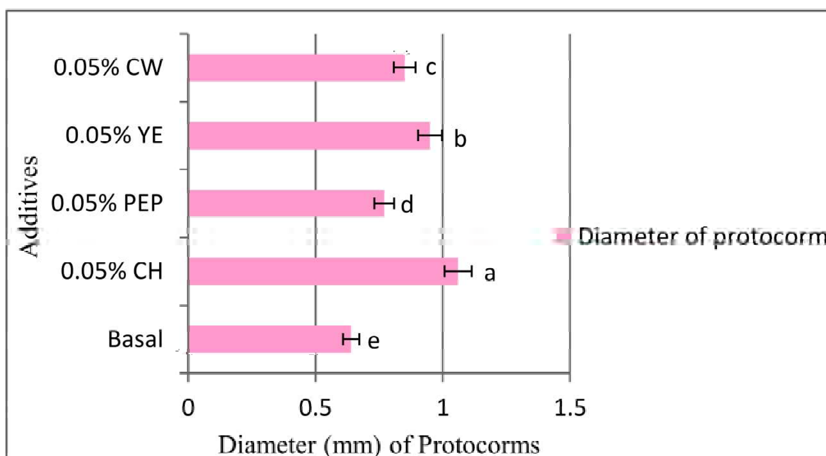


Same letters above the bars are not significantly different (p < 0.05) and vertical bars represent mean±SEs determined by DMRT.

Fig. 2. Graphical representation of varied *D. ovatum* protocorms weight in different organic additives supplemented MS medium.

in 20 days (Fig. 1c). These gradually converted into protocorms in 30 days (Fig. 1e-f). Effect of different organic additives *i.e.* casein hydrolysate (CH, 0.05%), peptone (PEP, 0.05%), yeast extract (YE, 0.05%) and coconut water (CW, 0.05%) supplemented half strength MS medium was tested for *in vitro* asymbiotic germination. Maximum germination percentage was obtained when seeds were cultured on 0.05% casein hydrolysate (75.0±1.22%) supplemented medium. Other additive [peptone (PEP, 0.05%), yeast extract (YE, 0.05%) and coconut water (CW, 0.05%)] enriched nutrient media did not favour much germination responses (Fig. 4). Maximum fresh weight (0.85±0.004 mg) and diameter of the protocorm (1.06 ± 0.009 mm) was also exhibited in 0.05% CH supplemented medium (Fig. 2 and 3). All other additives did not contribute much in protocorm fresh weight and diameter. Rapid formation of protocorm-like bodies (PLBs) was clearly visible after 60 days of culture (Fig.1h).

Inorganic and organic nutrient supplemented culture medium facilitates *in vitro* asymbiotic seed germination in dendrobiums (Teixeira da Silva *et al*., 2015a). *In vitro* asymbiotic culture of orchid seeds in general shows higher germination percentage than the symbiotic mode and serves as a powerful tool towards germplasm conservation. In the present investigation, *in vitro* asymbiotic germination of orchid seeds was achieved using organic additives as these are reported to improve regeneration rate and enhance the efficacy of related processes. Maximum germination rate of *D. ovatum* seeds was observed in CH supplemented medium indicating thereby at the exogenous supply of amino acids which



Same letters above the bars are not significantly different (p < 0.05) and vertical bars represent mean±SEs determined by DMRT.

Fig. 3. Graphical representation of varied *D. ovatum* protocorm diameter in different organic additives supplemented MS medium.

Table 2. The effect of different organic additives augmented MS medium for the enhanced shoot proliferation of *D. ovatum* protocorms.

Additives	Number of shoots per explant (Mean±SE)	Shoot length per explant (cm) (Mean±SE)	% Response (%±SE)
Basal	1.00±0.00^{d}	4.92±0.03^{c}	70.80±0.47^{e}
0.05% CH	7.50±0.12^{a}	6.38±0.02^{a}	92.00±2.00^{a}
0.05% P	3.75±0.09^{c}	4.72±0.00^{d}	88 .00±0.70^{b}
0.05% YE	5.36±0.09^{b}	5.92±0.06^{b}	81.00±0.57^{c}
0.05% CW	3.54±0.12^{c}	3.65 ± 0.00^{e}	76.00±1.15^{d}
	625.00***	3316.00***	58.35***

Means followed by same letter within a column are not significantly different (p < 0.05) as determined by DMRTNS- Non significant; *** F value is highly significant at p < 0.001; ** significant at p < 0.01; *significant at p < 0.05.

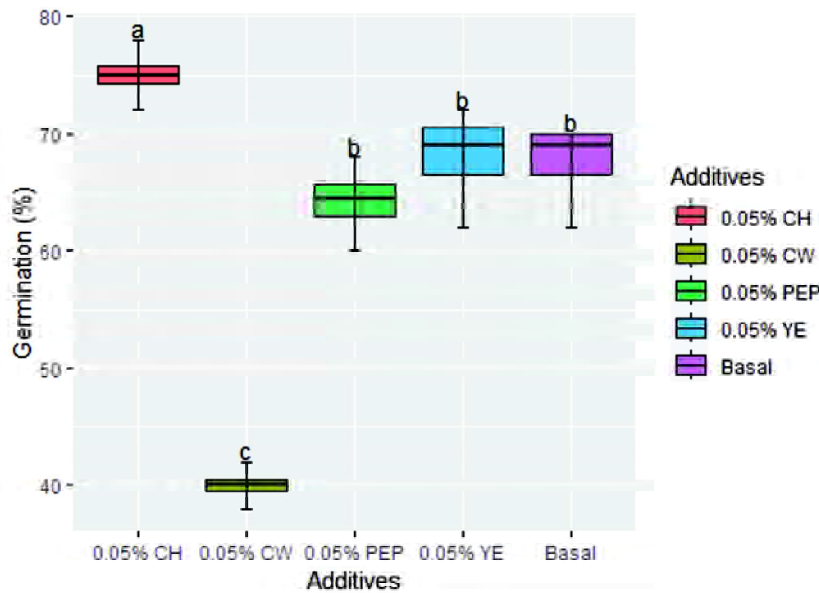


Fig. 4. Graph showing varied seed germination percentage in *D. ovatum* using different organic additives supplemented MS medium.

improved seed germination and protocorm development. CH is a complex organic mixture of vitamins, 18 amino acids, low molecular weight proteins, and growth-stimulating agents which facilitate nitrogen availability and ensure plant development (Bhatia *et al.*, 2015). Plant cells exhibit higher ability to metabolize and transfer nitrogen from organic rather than inorganic sources. CH holds nitrogen, calcium, phosphorous, and several microelements which improve the efficiency of plant growth (Sridhar *et al.*, 2014). Zeatin mediated enhanced seed germination was also earlier reported by Pujari *et al.* (2021a). However, in the present investigation, seeds exhibited very low germination percentage in CW supplemented medium as compared to the other nutritional combinations; this may be related to higher activity of its natural cytokinins and gibberellins.

A single mature capsule of *Dendrobium* can hold 2-3 million minute seeds (Teixeira da Silva *et al.*, 2015a). Seed culture resulting in the formation of spherules and protocorms was also earlier reported by Pujari *et al.* (2021b). Protocorm formation which was observed in *D. ovatum* is a part of zygotic embryogenesis, while in tissue culture, apart from the protocorm development, multiple protocorm-like structures termed as protocorm-like bodies (PLBs) also generated from the pre-existing protocorms. The generated PLBs are different from protocorms in their ontogeny; they are a sequel of somatic embryogenesis, since they developed from the meristemoid regions of somatic tissues of germinated protocorms (Pujari *et al.*, 2021a). Thus, zygotic and somatic embryogenesis is witnessed in *in vitro* asymbiotic seed culture of orchids.

Effect of Organic Additives on Shoot Multiplication

After 60 days of inoculation, developed protocorms were sub-cultured on to the same medium composition gelled with agar (0.8%, w/v) so as to analyse the effects of additives on shoot proliferation enhancement. All the organic supplements showed above 75% of response with rapidly formed PLBs within 20 days of culture and subsequent shoot formation from each PLB. Transferred PLBs in the medium containing CH (0.05%) resulted in the formation of maximum shoots (7.50±0.12) and shoot length (6.38±0.02) per explant with a maximum of 92% of response. All the other additives used showed comparatively lower effect (Table 2). PLBs multiplied rapidly and developed enormous number of shoots without root formation (Fig. 5a-c).

Table 3. The effect of different culture systems on shoot regeneration of *D. ovatum* protocorms.

Culture systems	Number of shoot per explant (Mean±SE)	Shoot length per explant (cm) (Mean± SE)	Number of roots per explant (Mean ±SE)	Root length per explant (cm) (Mean± SE)	Response (%)
SPS	1.00 ±0.00c	4.95 ±0.03c	1.11 ±0.06b	0.68 ±0.00a	67.50 ±1.89b
SCHM	7.50 ±0.12b	6.38 ±0.02b	0.00 ±0.00a	0.00 ±0.00b	90.00 ±1.83a
DPS-SBM +LBM	1.11±0.06c	4.92±0.03c	1.25 ±0.12b	0.67±0.03a	70.50 ±0.50b
DPS- SBM +LCHM	11.40 ±0.09a	6.41±0.03b	1.14 ±0.15a	0.67±0.01a	91.50 ±0.95a
DPS- SCHM +LBM	11.60±0.10a	8.72 ±0.00a	1.29 ±0.11a	0.67±0.00a	92.50 ±1.26a
	3580***	3130***	25.6***	355***	78.39***

Means followed by same letter within a column are not significantly different ($p < 0.05$) as determined by DMRT NS- Non significant; *** F value is highly significant at $p < 0.001$; ** significant at $p < 0.01$; *significant at $p < 0$.

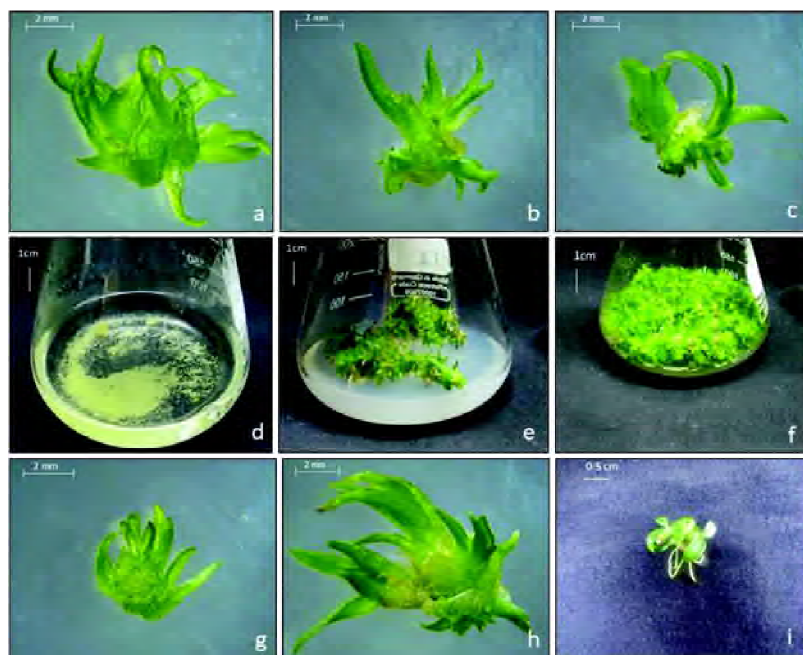


Fig. 5a-i. Effect of dual phase culture system on shoot proliferation in *D. ovatum*: a-c, Multiple shoots produced on MS medium containing CH (0.05%); YE (0.05%); PEP (0.05%), respectively; d, Development of spherules in liquid medium after 20 days of culture; e, Shoot proliferation in medium supplemented with SCHM; f, Enhanced shoot proliferation in DPS-SCHM + LBM; g, Shoot proliferation after 20 days of culture in DPS-SCHM + LBM; h, Multiple shoots developed from MS medium containing DPS-SCHM + LBM; i, Multiple shoots with roots after 35 days.

Hypergeneration of PLBs in *D. ovatum* using Zeatin supplemented medium was already reported by Pujari *et al*. (2021a). However, CH mediated enhanced production of PLBs was reported in the species for the first time. CH was reported earlier as an excellent additive for *in vitro* shoot regeneration from different explants (Samiei *et al*., 2021). Shoot proliferation enhances in the presence of amino acids which are supplemented by CH in the medium. This also improves the regeneration efficiency of protocorms, which results in hyper generation of PLBs from pre-existing protocorms

Effect of Dual Phase Culture System on Shoot Proliferation

Proliferating protocorms produced after 60 days in liquid medium were transferred to solid MS medium supplemented with CH (0.05%). After 15 days of culture, a thin layer of liquid medium (3 ml) was introduced into the culture [termed as dual phase culture system (DPS)]. Two types of DPS were studied for shoot proliferation (DPS- SBM+LCHM and DPS-SCHM +LBM) experiments within a time limit of 40 days. DPS- SBM+LBM and SCHM were also set for the comparison of the experiments and SPS served as control. DPS- SCHM +LBM culture system exhibited maximum shoot proliferation (11.60±0.10) along with root formation (1.29±0.11) in each developed shoot with 92.50% of response while the other systems produced comparatively less shoot proliferation (Table 3) (Fig. 5f-i). DPS enhanced more root proliferation from the developed multiple shoots than SCHM which inhibited root proliferation and enhanced only shoot development.

According to Teixeira da Silva *et al*. (2015a,b), MS medium is the most favourable medium for *Dendrobium* micropropagation with agar as gelling agent and for acquiring positive carbon balance, sucrose has always been the excellent choice as carbohydrate source (Ferreira *et al*., 2011) which was also employed in the present study. Protocorm proliferation and regeneration of enormous PLBs can be obtained through half strength MS basal medium. An optimal sucrose concentration of 3% was used in the medium for rapid PLB formation, asit plays a significant role in the regulation of cell osmolarity.

Bridge or paper raft used liquid culture systems are common in micropropagation. DPS system of culture

Table 4. Effect of different concentrations of BH on plantlet development in MS medium.

Culture systems	Number of roots per plantlet (Mean±SE)	Root length per plantlet (cm) (Mean±SE)	Number of leaves per plantlet (Mean±SE)	Plantlet length (Mean±SE)	Response (%)
Basal	1.50 ±0.14c	0.51 ±0.01c	3.54 ±0.03b	2.72 ±0.08c	67.5 ±1.89b
1%BH	2.21 ±0.09b	0.67 ±0.00a	5.21 ±0.09a	3.45 ±0.02b	92.00 ±2.00a
5%BH	3.25 ±0.12a	0.72 ±0.01a	5.18 ±0.25a	3.70±0.00a	91.5 ±0.95a
10%BH	1.68 ±0.14c	0.58 ±0.04b	3.25 ±0.12b	3.58±0.07ab	91.5 ±0.95a
	36.89***	16.2***	50.4***	72.54***	62.04***

Means followed by same letter within a column are not significantly different ($p < 0.05$) as determined by DMRT NS- Non significant; *** F value is highly significant at $p < 0.001$; ** significant at $p < 0.01$; *significant at $p < 0.05$.

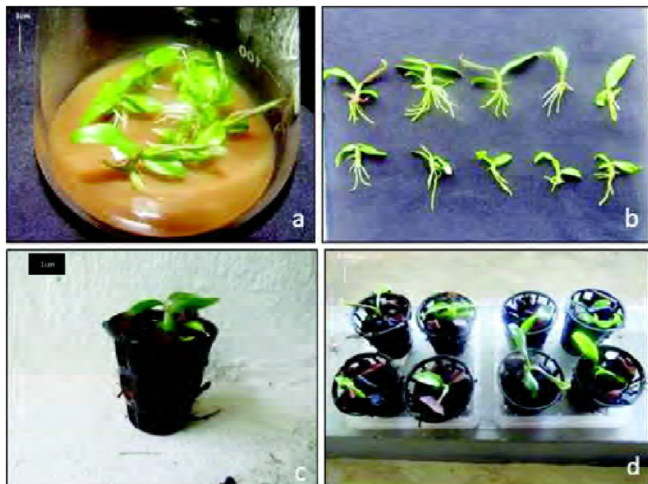


Fig. 6a-d. Effect of banana homogenate (BH) on growth and development of *D. ovatum* plantlets: a, Plantlets cultured on MS half strength medium augmented with 5% BH after 30 days; b, Plantlets produced after 120 days; c, Acclimatised plantlet; d, Plantlets ready for transfer to green house.

uses solid phase basement for explant anchorage with nutrient supply and liquid phase provide replenishment of plant growth regulators and nutrients which enhances shoot proliferation (Sandhyarani *et al.*, 2011). DPS ensures subculture reduction, nutrient availability in higher quantity which serves proper nutrient supplement and easier maintenance of the culture for several months (Scherwinski-Pereira *et al.*, 2012). This system was widely used for the enhanced production of many crop species such as banana (Alvard *et al.*, 1993; Levin *et al.*, 1996), sugarcane (Lorenzo *et al.*, 1998), pineapple (Escalona *et al.*, 1999; Feuser *et al.*, 2003), and potato (Pereira and Fortes, 2003).

DPS system mediated orchid culture was also reported as the most appropriate method for the induction of healthy plantlets from various explants (de Oliveira *et al.*, 2013; Roy *et al.*, 2012). In the present study, DPS enhanced the shoot proliferation more than the conventional methods of shoot regeneration using zeatin (Pujari *et al.*, 2021b) and thin cell layer culture using TDZ (Pyati, 2022). CH is an excellent source of amino acids which augments the nitrogen supply to the medium, which is important for the synthesis of cytokinins and thereby induces plant regeneration. Endogenous cytokinin accumulation and (or) synthesis induced rapid cell division is responsible for this enhanced shoot organogenesis (Tao *et al.*, 2011). TDZ involving DPS mediated enhanced shoot proliferation in *Cymbidium giganteum* was reported earlier by Roy *et al.* (2012). Similarly, the present work demonstrated that the addition of CH in DPS either in solid or liquid form also favours shoot regeneration by enhanced endogenous cytokinin production and concentration specific balancing in the ratio of endogenous growth regulators which favours rooting in the same medium in *D. ovatum* (Tao *et al.*, 2011).

Micropropagation protocol for many *Dendrobium* species has been reported by a few researchers using seed derived protocorms in single phase culture system for the enhanced production of shoots (Lin *et al.*, 2020; Nugroho *et al.*,2019; Parthibhan *et al.*, 2015; Tikendra *et al.*, 2019; Utami *et al.*, 2017). In *D. ovatum,* different solid phase propagation systems were introduced using seeds, seed derived protocorms and thin cell layers for large scale production (Pujari *et al.*, 2021a,b; Pyati, 2022; Thejaswini and Narasimhan, 2017). However, none of these culture systems included testing the efficiency of the DPS for enhanced shoot proliferation in seed derived hypergenerating protocorms. The physical state of the culture medium is an important factor which plays a significant role in enhancing shoot proliferation in micropropagation of orchid species (de Oliveira *et al.*, 2013). Roy *et al.* (2012) asserted that a layer of liquid medium upon solid medium enhances the shoot proliferation. Therefore, improved culture system is required for enhanced shoot proliferation of orchids. This improved system can be used as a reliable system for the rapid and enhanced seedling production of *D. ovatum* as compared to earlier established protocols. Moreover, lack of information about the significant average shoot production per protocorm highlights the importance of this novel *in vitro* technique.

Effect of Banana Homogenate (BH) on Plantlet Development

Shoots proliferated from SCHM were separated and cultured on basal medium for proper root production since developed shoots only possessed emergence of root initials. After the root formation, the plantlets from all culture systems were transferred to medium supplemented with banana homogenate (BH) at different concentrations (1%, 5%, and 10%) (Fig. 6a). Maximum plantlet growth with healthy thick roots was observed on half strength MS medium augmented with 5% BH (Fig. 6b; Table 4). Maximum plantlet growth exhibited in BH supplemented medium is due to its pH stabilising capacity and cytokinin content that stimulates plant growth (Bhatia *et al.*, 2015). Higher concentration of BH did not show much growth in the developed plantlets.

Hardening and Establishment of Developed Plantlets

In vitro raised plantlets (2 cm length) were deflasked and washed thoroughly using tap water followed by 15 min Bavistin treatment. These were then planted in pots containing brick pieces, coconut husk, and charcoal

pieces (1:2:2) (Fig. 6c,d). The planted pots were transferred to greenhouse for acclimatization. The plantlets produced from both the culture systems showed 90% survival rate which is almost similar to the reported survival rate of 89.0% for *D. ovatum* plants produced from longitudinal thin layer cell culture technique (Pyati, 2022).

Conclusion

Organic additive mediated *in vitro* asymbiotic seed germination and shoot proliferation was achieved during the present study. Rapid production of plantlets from protocorms was achieved using cost effective and reproducible method that can be employed for other orchids as well. This efficient method of CH mediated DPS can be used for the enhanced production of PLBs from *in vitro* derived protocorms, which ensures the conservation and rapid production of *D. ovatum,* a medicinally important threatened orchid species from the Western Ghats, India.

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