In vitro asymbiotic seed germination and micropropagation of Dendrobium heyneanum Lindl. – an endemic orchid of Western Ghats, India

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Research Article

Keywords: Asymbiotic seed germination, Endemic Orchid, Protocorms, Kinetin, In vitro flowering

Posted Date: December 21st, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3768530/v1

Version of Record: A version of this preprint was published at Plant Cell, Tissue and Organ Culture (PCTOC) on April 18th, 2024. See the published version at https://doi.org/10.1007/s11240-024-02758-w.

Abstract

Dendrobium heyneanum Lindl. or Heyne's Dendrobium is an endemic epiphytic orchid of Western Ghats categorized as a threatened taxon. The present investigation was aimed for conservational strategy using in vitro regeneration methods. In this investigation, mature pods of the *D. heyneanum* were collected from the field, and were inoculated aseptically on various nutrient media. Half-strength macro-MS media was found to be an efficient asymbiotic seed germination with 86.70% in 12 days old culture. Different stages (I-VI) of morphogenic characters were observed during entire studies; however, 20.84% of seeds produced young seedlings with roots on half-strength macro-MS media. Micropropagation protocol of *D. heyneanum* was established by using the protocorms (Stage IV) from the asymbiotic germinated seeds. The metamorphosis was observed on half-strength macro-MS fortified with 0.1 mg/L to 2.0 mg/L of Thidiazuron (TDZ), Benzyl amino purine (BAP) and Kinetin (KN). Higher frequency of protocorms (90.20%) were found in 1.0 mg/L KN with 95.56% of proliferation, shoot number (6.56) and shoot length (1.13 cm). Different frequency of in vitro flower buds was observed in protocorm at 0.5 mg/L (30.2%) and 1.0 mg/L (22.37%); and callus induction at 2.0 mg/L (20.67%) of BAP. Synergistic effect of plantlets were assessed with different concentration of combination of KN (1.0 mg/L) and auxins - α- Naphthaleneacetic acid (NAA), Indole- 3- acetic acid (IAA), and Indole- 3- butyric acid (IBA) from 0.1 mg/L to 1.0 mg/L). Combination of KN + IAA (1.0 mg/L) induced elongation, 0.92 cm long pseudobulbs and 0.74 cm rooting. The plantlets were then subsequently acclimatized and hardened on pots containing cocopeat and brick pieces. The survival rate was 52.73%. The present study results finding introduced a protocol for in vitro propagation of *Dendrobium heyneanum* Lindl. and thus, can be implemented as an ex-situ conservation.

Introduction

Orchids are one of the largest and most diverse groups of monocots consisting of nearly 28,000 species under more than 763 genera (Christenhusz and Byng 2016) with cosmopolitan distribution occurring in almost every habitat. Many members of this family are commercially known for their aromatic properties and decorative purposes and are used in the perfumery, cosmetics, and horticulture industries. Several others have cultural significance, are used as traditional folklore medicines to cure various ailments, and are even used as food. Some of the orchid species are confined and restricted to certain geographical areas and cannot be found elsewhere, such species are referred to as endemic orchids and these species provide an important insight into the biogeography of that region and also into the centers of diversity and adaptive evolution of the floristic components of that region (Nayar 1996). In India, the Peninsular region consisting of Western Ghats, Eastern Ghats, and Deccan plateau has a high degree of endemism of about 130 species under 38 genera (Jalal and Jayanthi 2012). The endemic orchids of the peninsular region are however facing various kinds of localized threats like livestock grazing, natural disasters (namely forest fire, landslides, etc.), and industrialization (mining and agricultural expansions, etc.). Apart from that, orchid seeds are minute and less dense; they require the right mycorrhizal association and suitable host or substratum for germination to take place. Conventional breeding techniques cannot be implemented for orchid propagation. Ex-situ conservation strategies like tissue

culture and cryopreservation techniques are widely employed to maintain the existence of these plant groups to prevent their overexploitation.

Dendrobium heyneanum Lindl. is an endemic epiphytic orchid of the Western Ghats spreading in parts of Karnataka, Tamil Nadu, and Kerala. The plant can be distinguished from other species by its unique flowers with the following characteristics- Flowers white, ca 1.5 cm across; lip obovate, 3-lobed; lateral lobes erect, apex at acute; middle lobe ovate, apex at acute, margin crenate, disc with channeled ridge and fleshy callus. The previous reports were made solely on the taxonomic identification, and distribution and also suggested possible threats on these plants while they are categorized as Endangered by IUCN (CAMP 2001) and the current status is 'Not Evaluated' (Saleem et al. 2022). Since there is a huge risk factor for the existence of these plants in the near future, an immediate and efficient approach needs to be taken in the conservational aspect, and what better way to do this by in vitro propagation techniques. In vitro, asymbiotic seed germination; subsequent micropropagation from protocorms, and hardening of these plants, have been successfully implemented recently in several orchid species like *Vanda cristata* Wall. (Pathak et al. 2023), and *Orchis simia* Lam. (Fatahi et al. 2022) etc. The present investigation aims to identify the suitable nutrient media for asymbiotic seed germination of *D. heyneanum* and observe the various stages of seed development; perform micropropagation from protocorm on ½ macro-MS supplemented with various cytokinin (alone and in combination with auxins) followed by hardening the in vitro plantlets on the appropriate substratum.

Materials and Methods

Plant material collection and explant preparation for asymbiotic seed germination

Mature green pods of *Dendrobium heyneanum* Lindl. was collected from Vellingiri Hills, Coimbatore, Tamil Nadu of India (10.9842933 N, 76.6945818 E; Altitude − 1559 m). The pods were surface sterilized in 0.1% $HgCl_2$ for 3 minutes and rinsed thoroughly 3–4 in sterile distilled water. The pods were also dipped in 70% ethanol for 30 seconds followed by flaming off for 2–3 seconds. A longitudinal slit was made on the pod using a sterile blade and healthy aseptic seeds were carefully scooped out and dusted over the media surface. Seed viability was conducted using 2,3,5-triphenyl tetrazolium chloride (TTC) prior to inoculation (Vujanovic et al. 2000).

In vitro asymbiotic seed germination

The viable seeds were aseptically inoculated on 6 basal nutrient media namely Vacin and Went medium (VW, Vacin and Went 1949); Knudson C medium (KC, Knudson C 1946)); Lindemann Orchid medium (LO, Lindemann 1970); Murashige and Skoog medium (MS, Murashige and Skoog 1962) and MS with two modifications i.e., one with half strength concentration of macronutrients and 2% sucrose (½ macro-MS), and other with MS vitamin replaced by B5 vitamin (MS_{B5}, Gamborg 1968). The pH of the culture media was adjusted according to the specific formulations prescribed by the authors prior to autoclaving for 20

min at 15 lbs pressure and 121°C. The initial percentage of response and nature of seeds in each media were noted and the germination responses to various stages were also recorded after 120 days of culture based on characteristic features mentioned in Table 1.

Table 1
Stage of orchid seed germination

Stages	Characteristic features
0	No germination
1	The imbibed embryo in testa
2	Enlarged embryo with half ruptured testa
3	Protocorms with pointed initial
4	Protocorms with the first leaf
5	Protocorm with elongated leaves
6	Young seedlings with shoot and developed root

Additionally, a study was also conducted for asymbiotic seed germination on ½ macro-MS media supplemented with PGR namely, Abscisic acid (ABA), 2,4-Dichlorophenoxyacetic acid (2,4-D), and Thidiazuron (TDZ) in the concentration 0.1 mg/L and 0.5 mg/L.

Effect of cytokinin on protocorm development

Protocorm (stage IV) initiated from in vitro asymbiotic seed germination experiment was used as explant source and was inoculated on ½ macro-MS media supplemented with cytokinins such as Thidiazuron (TDZ), 6-Benzylaminopurine (BAP) and Kinetin (KN) ranging from the concentration 0.1 mg/L to 2.0 mg/L. The frequency of response of protocorms; response to callus induction and shooting; average height and number of multiple shoot buds was recorded after 100 days of culture.

Effect of kinetin and auxins on in vitro plantlet development

The developing plantlets from the above cultures were then transferred to ½ macro-MS with 1.0 mg/L KN supplemented with various auxins such as α- Naphthaleneacetic acid (NAA), Indole-3-acetic acid (IAA) and Indole-3-butyric acid (IBA) in the concentration ranging from 0.1 mg/L to 1.0 mg/L. The frequency of response; the average number of shoots per explant, leaf, and root as well as the average length of pseudobulb, leaf, and root were recorded after 85 days of culture.

Acclimatization and hardening in vitro raised plantlets

The developing plantlets were sequentially hardened by subculturing to hormone-free basal media (½ macro-MS) followed by minimal media (MS with ¼ strength macronutrient and 1.5% sucrose). The in vitro developed plantlets were washed thoroughly in distilled water to remove agar and then hardened to

pots containing sterilized cocopeat and brick pieces in a ratio of 1:1 and their survival rate was observed after 50 days.

Culture maintenance and Statistical analysis

The cultures were maintained in a culture room at 24 ± 2°C, 16 h light and 8 h dark photoperiod using cool-white fluorescent lamps with a light intensity of 3000 lux and 65% humidity; a minimum of 15 replicates were kept and the experiment was repeated thrice. All the chemicals used in the experiments were bought from HiMedia, Mumbai, India. The numerical data are represented as mean ± standard error and statistical analysis was performed using one-way analysis of variance (ANOVA) at $p < 0.05$ and means compared Duncan's multiple range test (DMRT) at the probability level of 5% (Duncan 1955) using SPSS software version 26 (IBM SPSS Statistics 26).

Results and Discussion

In vitro asymbiotic seed germination

Effect of hormone-free media on seed germination

In vitro asymbiotic seed germination and seedling development of orchids are the most effective and practical methods for their propagation and conservation as conventional breeding techniques cannot be applied in these groups of plants. The mature green pods of *Dendrobium heyneanum* Lindl. containing 83.56% viable seeds after performing TTC test when inoculated on various basal media gave different responses on days taken for initiation and color of developing seeds with respect to the composition of each media (Table 2). The ½ macro-MS media gave the highest percentage of seed germination i.e., 86.70% of green seeds germinated within 12 days of inoculation, while only 71.56% of yellow-green germinating seeds were formed on VW medium after 20 days. The experiment then continued to identify various stages (Fig. 1) of asymbiotic seed germination of *D. heyneanum* and the data recorded after 120 days of inoculation is represented in Table 3. Seeds inoculated on ½ macro-MS media developed 20.84% young seedlings (stage VI), followed by 18.67% on MS media and 6.64% on MS_{B5}. Half macro-MS proved to be ideal for asymbiotic seed germination for the present study which was also similar to the reports on *Dendrobium aqueum* (Parthibhan et al. 2015). The use of MS_{B5} media in the present study did not provide a promising result when compared to full-strength MS and ½ macro-MS. This indicates that the vitamin supplemented can act as a limiting factor for seedling development. KC media, LO media, and VW media failed to produce stage VI seedlings, this may be due to the lack of essential growth-inducing macro and micro elements in their composition when compared to MS nutrients. The previous study on *Dendrobium tosaense* by Lo et al. 2004 suggested that KC and VW media cannot develop protocorms which is in contrast to the current study where seeds inoculated on VW media gave protocorms with shoot initials (14.17%) and that on KC media gave both protocorms stage IV (18.04%) and stage V (5.38%).

Table 2
The initial response of asymbiotic seed germination of *Dendrobium heyneanum* Lindl. on different basal medium

Media	Days taken for germination	Percentage of germination (%)	Color of the developing seeds
½ macro-MS	12	86.70 ± 0.46 a	Green
MS	12	84.44 ± 0.56 b	Green
MS_{B5}	12	84.09 ± 0.09 b	Green
KC	15	76.46 ± 0.32 d	Light Green
LO	15	79.41 ± 0.96 c	Light Green
VW	20	71.56 ± 0.57 e	Yellow Green
Values represent means ± S.E. Values followed by the same letter are not significantly different at p ≤ 0.05			
according to DMRT			

Table 3
Asymbiotic seed germination response of *Dendrobium heyneanum* Lindl. to various stages on basal medium after 120 days of culture

Media	Percentage of response in different media after 120 days					
	Stage I	Stage II	Stage III	Stage IV	Stage V	Stage VI
½ macro-MS	0.00 ± 0.00 b	0.34 ± 0.19 d	19.42 ± 0.27 e	37.62 ± 0.24 b	22.07 ± 0.00 c	20.84 ± 0.44 a
MS	0.01 ± 0.00 b	0.03 ± 0.02 d	11.24 ± 0.00 f	39.85 ± 0.25 a	30.65 ± 0.02 a	18.67 ± 0.23 b
MS_{B5}	0.01 ± 0.00 b	0.04 ± 0.02 d	30.00 ± 0.06 d	35.11 ± 0.06 c	28.50 ± 0.27 b	6.64 ± 0.23 c
KC	0.08 ± 0.02 b	5.32 ± 0.12 c	69.54 ± 0.27 b	18.04 ± 0.02 d	5.38 ± 0.02 d	0.04 ± 0.02 d
LO	0.22 ± 0.09 b	10.27 ± 0.01 b	72.63 ± 0.06 a	15.63 ± 0.04 e	0.06 ± 0.02 e	0.00 ± 0.00 d
VW	0.63 ± 0.22 a	25.06 ± 0.02 a	59.81 ± 0.03 c	14.17 ± 0.01 f	0.00 ± 0.00 e	0.00 ± 0.00 d
Values represent means ± S.E. Values followed by the same letter are not significantly different at p ≤ 0.05						
according to DMRT						

Effect of PGR on seed germination

Seeds when dusted over ½ macro-MS supplemented with 0.1 mg/L and 0.5 mg/L of ABA, 2.4-D, and TDZ took a prolonged time for initiation compared to hormone-free media (Table 4). Abscisic acid is mostly used to break seed dormancy, 2,4-D for callus initiation, and TDZ for high frequency of shooting. The response (Table 5) of seeds after 120 days of culture proves that the addition of PGR on nutrient media alters the normal physiology of seed and in fact, influences seedling development. Among these three hormones, ABA was effective as the seeds developed into stage V protocorms even though the majority of the protocorms were turning brown. 2,4-D however induced seed to develop into stage IV callusing protocorms with numerous adventitious rhizoids while comparatively smaller-sized protocorms were developed on TDZ augmented media (Fig. 2). All these findings suggest that PGR does not promote effective seed germination and seedling development instead has endorsed browning and arrested growth (Yao et al. 2021and Hossain et al. 2013).

Table 4
The initial response of asymbiotic seed germination of *Dendrobium heyneanum* Lindl. on ½ macro-MS media supplemented with PGR.

Media	Days taken for germination	Percentage of germination (%)	Color of the developing seeds
½ macro-MS + 0.1 mg/L ABA	20	61.22 ± 0.23 a	Light Green
½ macro-MS + 0.5 mg/L ABA	25	57.51 ± 0.94 b	Light Green
½ macro-MS + 0.1 mg/L 2,4-D	21	51.67 ± 0.37 c	Light Green
½ macro-MS + 0.5 mg/L 2,4-D	25	49.59 ± 0.39 d	Light Green
½ macro-MS + 0.1 mg/L TDZ	28	32.74 ± 0.25 e	Light Green
½ macro-MS + 0.5 mg/L TDZ	30	31.53 ± 0.36 e	Yellow Green
Values represent means ± S.E. Values followed by the same letter are not significantly different at p ≤ 0.05			
according to DMRT			

Table 5
Asymbiotic seed germination response of *Dendrobium heyneanum* Lindl. to various stages on ½ macro-MS media supplemented with PGR after 120 days of culture

Media	Percentage of response in different media after 120 days					
	Stage I	Stage II	Stage III	Stage IV	Stage V	Stage VI
½ macro-MS + 0.1 mg/L ABA	0.00 ± 0.00 a	0.02 ± 0.02 c	47.36 ± 0.04 d	31.57 ± 0.00 b	21.04 ± 0.00 a	0.03 ± 0.03 b
½ macro-MS + 0.5 mg/L ABA	0.00 ± 0.00 a	0.06 ± 0.03 c	63.65 ± 0.00 b	20.17 ± 0.01 c	11.15 ± 0.06 b	0.00 ± 0.00 b
½ macro-MS + 0.1 mg/L 2,4-D	0.00 ± 0.00 a	0.05 ± 0.02 c	59.23 ± 0.06 c	33.30 ± 0.09 a	7.41 ± 0.08 c	0.47 ± 0.25 b
½ macro-MS + 0.5 mg/L 2,4-D	0.01 ± 0.00 a	0.03 ± 0.02 c	67.64 ± 0.27 a	15.63 ± 0.01 d	5.28 ± 0.04 d	0.53 ± 0.26 a
½ macro-MS + 0.1 mg/L TDZ	0.00 ± 0.00 a	20.44 ± 0.30 b	46.34 ± 0.05 e	33.55 ± 0.30 a	0.02 ± 0.01 e	0.43 ± 0.22 b
½ macro-MS + 0.5 mg/L TDZ	0.27 ± 0.00 a	38.02 ± 0.45 a	42.11 ± 0.01 f	19.94 ± 0.05 c	0.04 ± 0.02 e	0.03 ± 0.01 b
Values represent means ± S.E. Values followed by the same letter are not significantly different at p ≤ 0.05						
according to DMRT						

Effect of cytokinin on protocorm development

The protocorms consist of highly meristematic cells and can metamorphize into any cells, tissue, or organs. In this experiment, protocorm (stage IV) were inoculated on ½ macro-MS augmented with various cytokinin developed into callus, shoots with multiple buds, and even shoots with inflorescences (Fig. 3) irrespective of type and concentration of cytokinin used. The frequency of protocorm response, callus induction, shooting and inflorescence development, and callus nature and number of multiple shoots from the study are represented in Table 6. The highest response rate of 90.20% active protocorms was found to be on 1.0 mg/L Kinetin.

Table 6
Response of protocorm to callus and shoot proliferation on ½ macro-MS media supplemented with cytokinin after 100 days of culture.

PGR	Conc. (mg/L)	Response of Protocorm on ½ macro-MS supplemented with cytokinin after 100 days					
		Freq. of protocorm response (%)	Percentage of Callus induction (%)	Type of Callus	Percentage of Shooting (%)	Av. height of shoots (cm)	Av. Number of shoots per explant
TDZ	0.1	65.21 ± 0.06 l	0.01 ± 0.00 f	-	56.62 ± 0.01 j	0.81 ± 0.01 de	0.71 ± 0.00 n
	0.5	73.11 ± 0.06 j	2.43 ± 0.01 e	Pale green, Friable	72.35 ± 0.00 g	0.83 ± 0.00 d	1.04 ± 0.01 m
	1.0	85.53 ± 0.00 d	5.04 ± 0.01 d	Pale green, Friable	85.24 ± 0.06 c	1.03 ± 0.01 b	1.10 ± 0.00 l
	1.5	86.95 ± 0.02 c	7.14 ± 0.02 b	Pale green, Friable	80.15 ± 0.03 e	0.94 ± 0.00 c	1.13 ± 0.01 l
	2.0	78.85 ± 0.10 h	6.23 ± 0.03 c	Pale green, Friable	42.39 ± 1.50 k	0.77 ± 0.01 e	2.72 ± 0.03 g
BAP	0.1	79.58 ± 0.22 g	0.01 ± 0.00 f	-	73.47 ± 0.12 fg	0.58 ± 0.01 h	1.26 ± 0.02 k
	0.5	86.86 ± 0.00 c	0.04 ± 0.01 f	-	78.97 ± 0.01 e	0.64 ± 0.01 g	2.13 ± 0.03 i
	1.0	82.50 ± 0.25 f	0.09 ± 0.00 f	-	84.48 ± 0.28 c	0.77 ± 0.01 e	3.22 ± 0.01 e
	1.5	75.12 ± 0.06 i	6.46 ± 0.02 c	Green, Friable	65.51 ± 0.24 h	0.65 ± 0.02 fg	2.65 ± 0.03 h
	2.0	70.49 ± 0.28 k	20.67 ± 0.33 a	Green, Friable	57.89 ± 0.06 i	0.51 ± 0.02 i	1.53 ± 0.00 j
KN	0.1	83.28 ± 0.00 e	0.14 ± 0.12 f	-	84.10 ± 0.58 c	0.70 ± 0.01 f	2.95 ± 0.01 f
	0.5	89.50 ± 0.31 b	0.13 ± 0.08 f	-	93.75 ± 0.01 b	0.94 ± 0.04 c	3.81 ± 0.01 d

Values represent means ± S.E. Values followed by the same letter are not significantly different at p ≤ 0.05

according to DMRT

1.0	90.20 ± 0.20 a	0.01 ± 0.00 f	-	95.76 ± 0.06 a	1.13 ± 0.00 a	6.56 ± 0.00 a
1.5	82.44 ± 0.02 f	0.00 ± 0.00 f	-	82.01 ± 0.00 d	1.05 ± 0.02 b	6.02 ± 0.03 b
2.0	79.05 ± 0.03 h	0.00 ± 0.00 f	-	74.64 ± 0.34 f	0.78 ± 0.00 de	5.14 ± 0.01 c

Values represent means ± S.E. Values followed by the same letter are not significantly different at p ≤ 0.05

according to DMRT

Effect of cytokinin on callus induction

The protocorms initiated with friable callus when cultured on TDZ and BAP augmented media. BAP 2.0 mg/L induced 20.67% green color callus on the other hand only 6.46% was formed on 1.5 mg/L of the same hormone after 100 days of culture. Pale green color callus was developed from TDZ hormone with less frequency compared to BAP-induced callus. Similarly, BAP and TDZ proved to be suitable for callus induction and PLB regeneration in *D. chrysotoxum* (Roy et al. 2003) and BAP alone in *D. barbatulum* (Pyati 2020).

Effect of cytokinin on shoot proliferation

Maximum response of 95.76% and 93.75% protocorms developed to shoots on 1.0 mg/L and 0.5 mg/L of Kinetin augmented media respectively. This study also revealed that kinetin 1.0 mg/L responded with 6.56 shoot buds for about 1.13 cm and kinetin 1.5 mg/L can generate 6.02 shoot buds with an average height of about 1.05 cm. Kinetin exhibited similar kind of promising effect of shoot proliferation in many *Dendrobium sp.* (Martin and Madassery 2006; Luo et al. 2009; Asghar et al. 2011). Maharjan et al. (2020) reports on *D. chryseum* protocorms cultured on half-strength MS containing 2.0 mg/L kinetin and 10% coconut water with maximum multiple shoots are all some of the significant reports corresponding to the present study.

Effect of cytokinin on in vitro flowering

Dendrobium species are valued for their cut flowers for ornamental purposes. In the present study in vitro flowering in juvenile plants which is quite a rare and unique phenomenon was achieved from the developing seedlings cultured on BAP-augmented media. Around 30.2% and 22.37% of the seedlings-initiated bud for inflorescence after 75 days of culture on 0.5 mg/L and 1.0 mg/L BAP respectively. Subsequent transferring of cultures to hormone-free basal media favored inflorescence maturation and ultimately blooming flowers in all these cultures. The various stages of in vitro flowering are shown in Fig. 4. BAP-induced in vitro flowering was also observed in *Dendrobium wangliangii* (Zhao et al. 2013); *Dendrobium nobile* (Wang et al. 2009); *Dendrobium* Sonia 17 (Tee et al. 2008); *Dendrobium* Chao Praya Smile (Hee et al. 2007) and *Dendrobium* Madame Thong-In (Sim et al. 2007). Other reports also conclude

cytokinin alone or along with natural additives can promote in vitro flowering in different orchids (Wang et al. 2006; Duan and Yazawa 1994; Zhao et al. 2013).

Synergistic effect of kinetin with auxins

The developing plantlets brought slight elongation and prominent rooting in all the cultures when transferred to nutrient media supplemented with 1.0 mg/L kinetin and different auxins (Table 7). Cultures on 1.0 mg/L IAA recorded the highest response rate of 74.14%, 0.92 cm long pseudobulbs, 4.72 number of roots, and 0.74 cm long roots. Pant and Thapa (2011) also suggested that 0.5 mg/L and 1.0 mg/L of IAA were effective on root proliferation in *D. primulinum*. *D. aphyllum* when cultured on 0.5 mg/L IAA provided a strong and stout rooting system (Hossain et al. 2013). Maharjan et al. (2020) report on *Dendrobium chryseum* also showed that IAA (1.5 mg/L) was suitable for rooting with or without any natural additives. The combined action of Kinetin and IAA however encouraged developed rooting in the present investigation which was also evident in the similar studies made by Dutta et al. (2011) on *D. aphyllum* where KN + IAA has given a higher shoot and root length. Half MS supplemented with KN (5 mM) + IAA (10 mM) was the best-suited media for seed germination of *Paphiopedilum insigne* according to Diengdoh et al. (2017) and identified the combined effect of these hormones can be beneficial for the in vitro plant development.

Table 7
Response of developing plantlets on ½ macro-MS with 1.0 mg/L KN supplemented with auxins after 85 days of culture.

Auxins	Conc. (mg/L)	Response of developing plantlets on ½ macro-MS with 1.0 mg/L KN supplemented with auxins after 85 days						
		Freq. of response (%)	Av. number of shoots per explant	Av. length of pseudobulbs (cm)	Av. number of leaves	Av. length of leaves (cm)	Av. number of roots	Av. length of roots (cm)
NAA	0.1	58.18 ± 0.02 i	1.86 ± 0.12 c	0.48 ± 0.00 e	4.15 ± 0.00 c	0.93 ± 0.00 d	2.27 ± 0.03 g	0.40 ± 0.02 g
	0.5	62.34 ± 0.01 g	2.99 ± 0.01 a	0.44 ± 0.00 g	3.34 ± 0.00 g	0.82 ± 0.01 e	1.22 ± 0.01 h	0.65 ± 0.00 de
	1.0	60.15 ± 0.01 h	2.01 ± 0.00 b	0.41 ± 0.00 h	3.17 ± 0.02 h	0.77 ± 0.01 f	1.13 ± 0.00 i	0.54 ± 0.00 f
IAA	0.1	67.98 ± 0.00 e	1.20 ± 0.01 e	0.56 ± 0.00 c	4.45 ± 0.02 b	1.17 ± 0.00 a	3.02 ± 0.01 e	0.50 ± 0.02 f
	0.5	73.26 ± 0.02 c	1.52 ± 0.01 d	0.59 ± 0.00 b	4.65 ± 0.01 a	1.19 ± 0.00 a	3.46 ± 0.01 d	0.61 ± 0.00 e
	1.0	74.14 ± 0.00 b	1.60 ± 0.01 d	0.92 ± 0.01 a	3.60 ± 0.01 e	1.14 ± 0.00 b	4.72 ± 0.01 a	0.74 ± 0.01 b
IBA	0.1	65.12 ± 0.01 f	1.29 ± 0.01 e	0.51 ± 0.00 d	3.37 ± 0.01 g	1.12 ± 0.00 b	2.42 ± 0.01 f	0.81 ± 0.00 a
	0.5	72.15 ± 0.01 d	0.97 ± 0.00 f	0.46 ± 0.00 ef	3.42 ± 0.01 f	0.99 ± 0.00 c	4.42 ± 0.00 c	0.68 ± 0.00 cd
	1.0	75.62 ± 0.01 a	0.90 ± 0.00 f	0.45 ± 0.00 fg	3.92 ± 0.01 d	0.93 ± 0.00 d	4.52 ± 0.01 b	0.71 ± 0.00 bc

Values represent means ± S.E. Values followed by the same letter are not significantly different at p ≤ 0.05

according to DMRT

Acclimatization and hardening

When the hormone-treated plantlets were gradually subcultured to hormone-free media and then finally cultured on minimal media generated around 75.21% active response after 75 days of culture. The average length of roots increased to 0.93 cm while the number of multiple shoots dropped to 3.33. When these cultures were acclimatized and hardened in pots containing a mixture of cocopeat and brick

pieces (in a ratio of 1:1), the survival rate was only 52.73%. The various stages of *D. heyneanum* protocorm development from micropropagation to hardening the plantlets are represented in Fig. 5.

Conclusion

In vitro propagation techniques have been widely used for the conservation of many threatened taxon. The present investigation on *Dendrobium heyneanum* Lindl. attempts to deduce a reproducible protocol for the asymbiotic seed germination and micropropagation from protocorm as an initiative step in the preservation of this species. The mature pods were the only source of plant material collected from the field to prevent the disruption of its natural population. The seeds were aseptically inoculated on various nutrient media and among them, ½ macro-MS media proved to be more efficient for asymbiotic seed germination. The protocorms developed from the seeds when transferred to ½ macro-MS supplemented with cytokinin showed different responses irrespective of the hormone nature. Protocorms developed into callus in BAP (2.0 mg/L); shooting and multiple bud induction occurred in KN (1.0 mg/L and 1.5 mg/L) and TDZ (1.5 mg/L); and flower bud induction in BAP (0.5 mg/L and 1.0 mg/L). The developing plantlets were then subjected to combined treatment of KN and auxins, among which IAA (1.0 mg/L) was found to be more efficient in elongation and rooting. However, the survival rate of the hardened plant was only 52.73%, the study demonstrated that *Dendrobium heyneanum* can be raised via in vitro conditions and the resultant protocol as such or with slight modification can be applied to other related species and even for epiphytic orchids that are at the verge of getting threatened. Furthermore, application-oriented and advanced studies such as elucidating the phytochemical constituents, nanoparticle synthesis, etc. can be carried out from the in vitro established plants to reduce over-exploitation of the plant in the wild and its impacts on the balance of nature.

Abbreviations

IUCN
International Union for Conservation of Nature
TTC – 2,3,5
Triphenyl tetrazolium chloride
ABA
Abscisic acid
BAP – 6
Benzylaminopurine
IAA
Indole-3-acetic acid
IBA
Indole-3-butyric acid
TDZ
Thidiazuron
2,4

D – 2,4-Dichlorophenoxyacetic acid
NAA
α-Naphthaleneacetic acid
KN
Kinetin
VW
Vacin and Went medium
KC
Knudson C medium
LO
Lindemann Orchid medium
MS
Murashige and Skoog medium

Declarations

Acknowledgement –. The authors sincerely acknowledge the DST-PURSE Phase 2 (Grant No. SR/PURSE Phase 2/16), UGC-SAP DRS-II (F.5–13/2018/DRS-II) and RUSA 2.0 (Biological Sciences) (Ref No.02 BDU/RUSA 2.0/TRP/BS dated 22/04/2021) support to Department of Botany, Bharathidasan University, Tiruchirapalli-620 024, Tamil Nadu, India.

Authors contributions - SK and TSK conceived the idea. SK performed the experiments. SK and AR analyzed the data. SK wrote the primary draft, which was further augmented, edited and improved by MM and TSK. RC collected and identified the plant. All the authors read and approved this article for publication

Funding- The research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Data availability – Data is available upon reasonable request.

Statements and Declaration - The authors declare that they have no competing interests.

Consent to participate - Not applicable.

Ethical approval - Not applicable.

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Figures

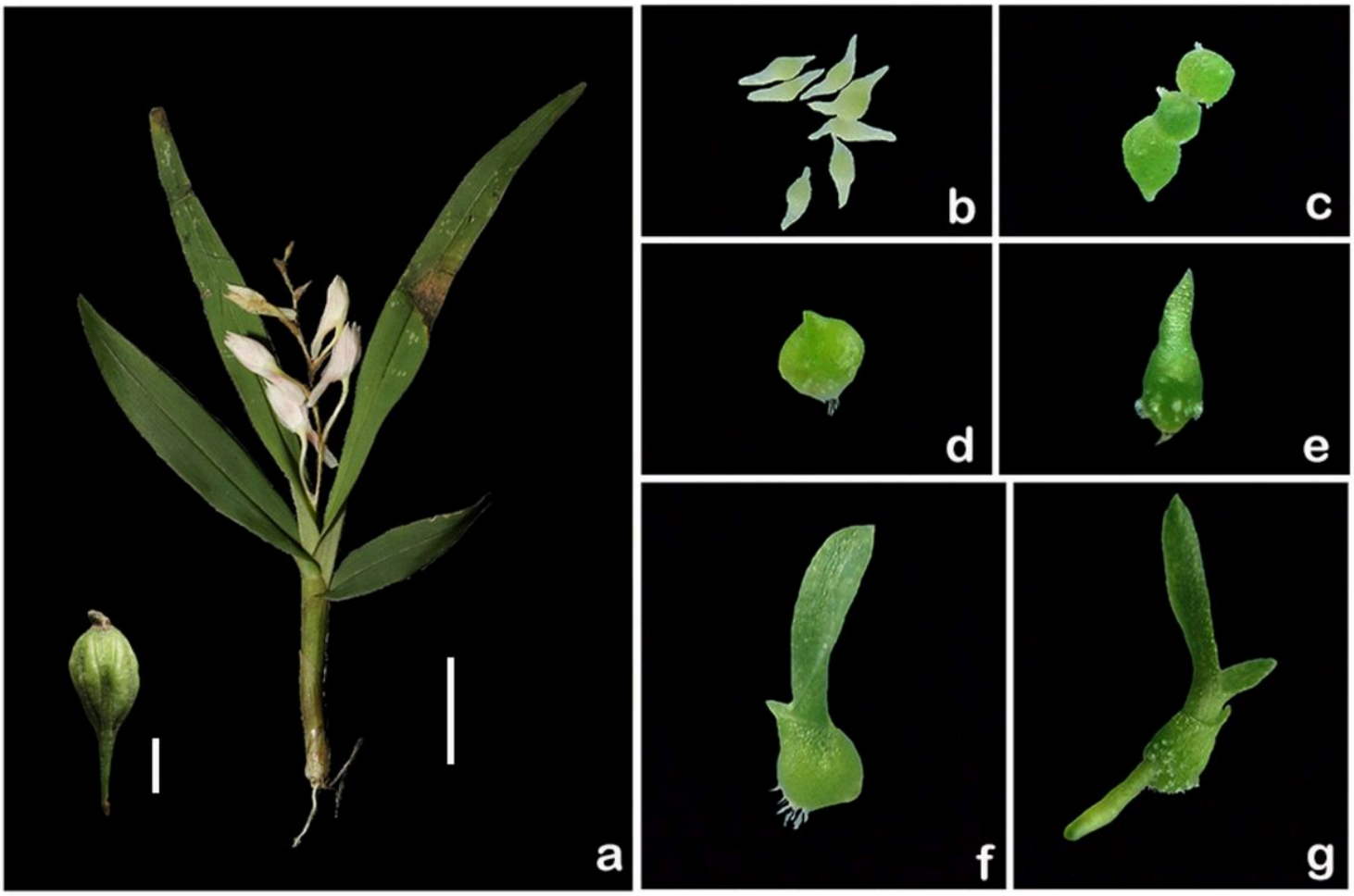

Figure 1

Stage of asymbiotic seed germination of *Dendrobium heyneanum* Lindl. - a) Plant habit (scale: 1 inch) with the mature pod on lhs (scale: 1 cm); b) Imbibed embryo in testa; c) Enlarged embryo with half ruptured testa; d) Protocorm with pointed initials; e) Protocorm with the first leaf; f) Protocorm with elongated leaves; g) Young seedlings with shoot and developing root.

Figure 2

Asymbiotic seed germination of Dendrobium heyneanum Lindl. on different medium after 120 days of culture – a) Seedlings on ½ macro-MS media (basal) media; b) Seedlings on MSB5 (basal) media; c) Protocorms on ½ macro-MS media supplemented with 0.1 mg/L ABA; d) Protocorms on ½ macro-MS media supplemented with 0.1 mg/L 2,4-D; e) Protocorms on ½ macro-MS media supplemented with 0.1 mg/L TDZ.

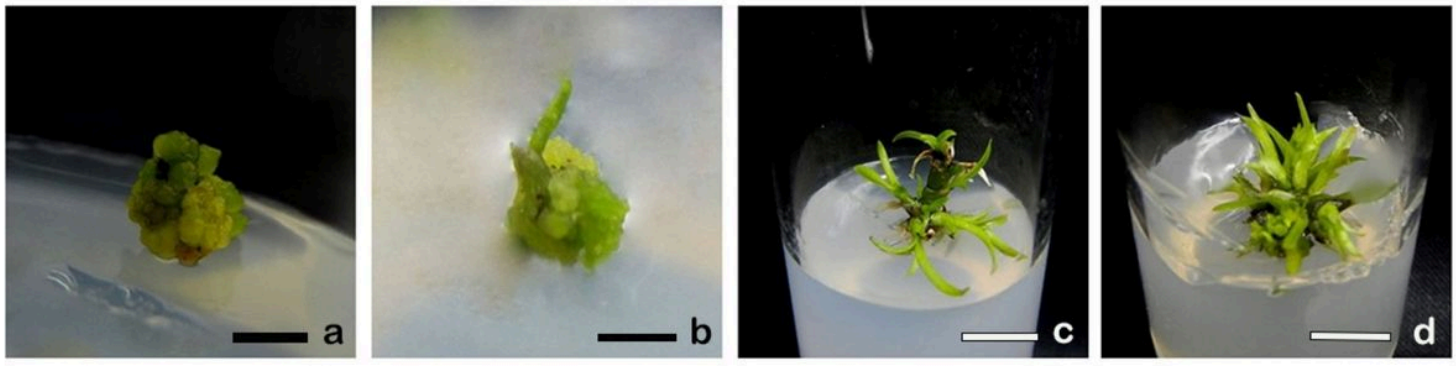

Figure 3

Response of protocorms on ½ macro-MS media supplemented with various cytokinin – a) callus induction on 2.0 mg/L BAP; b) Callus induction on 1.5 mg/L TDZ; c) Shoot proliferation on 1.5 mg/L KN; d) Shoot proliferation on 1.0 mg/L KN.

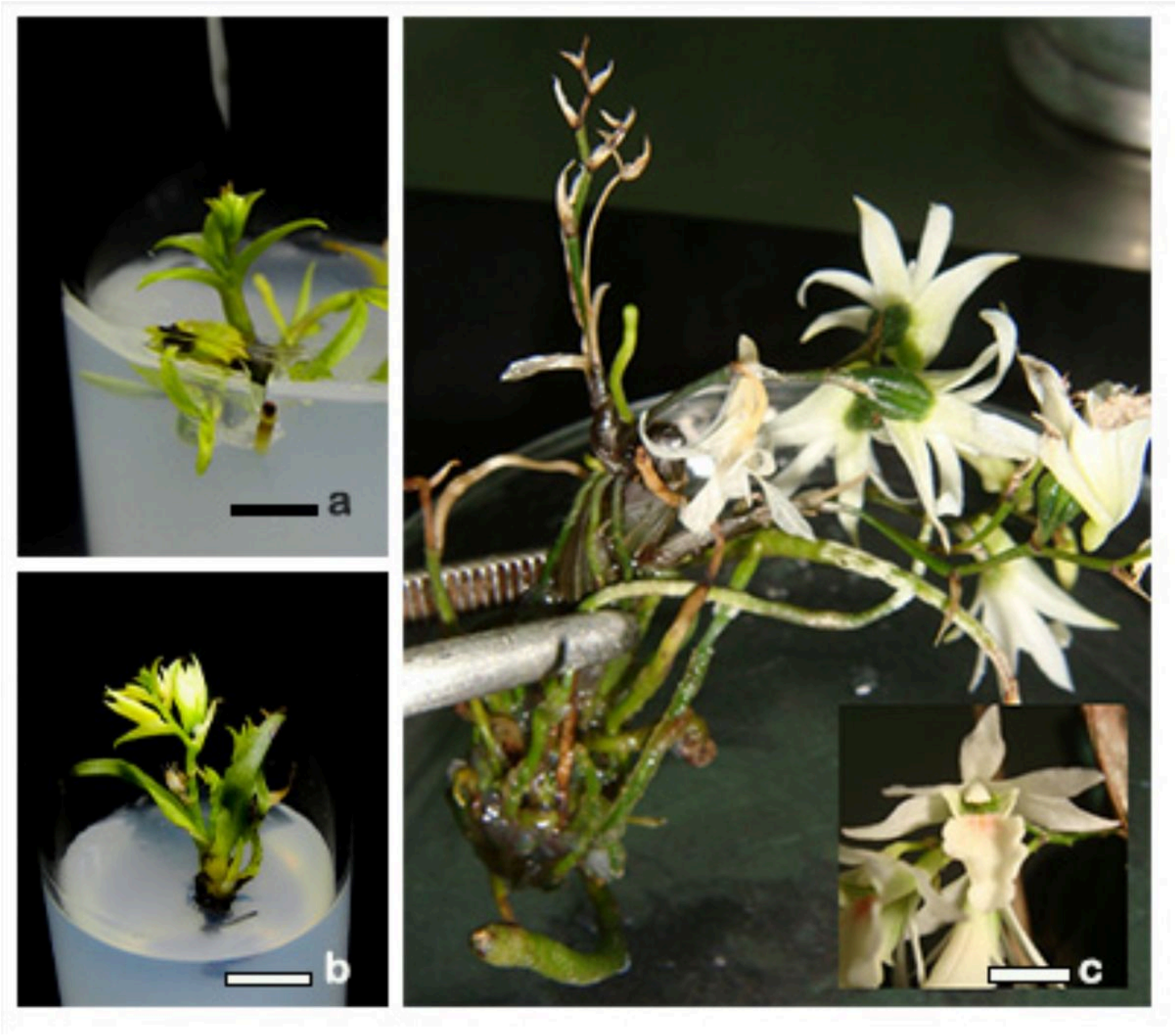

Figure 4

Invitro flowering from protocorm- a) In vitro flower bud induction on 0.5 mg/L BAP; b) Formation of Inflorescence on ½ macro-MS (basal) media; c) A matured Inflorescence bearing flowers.

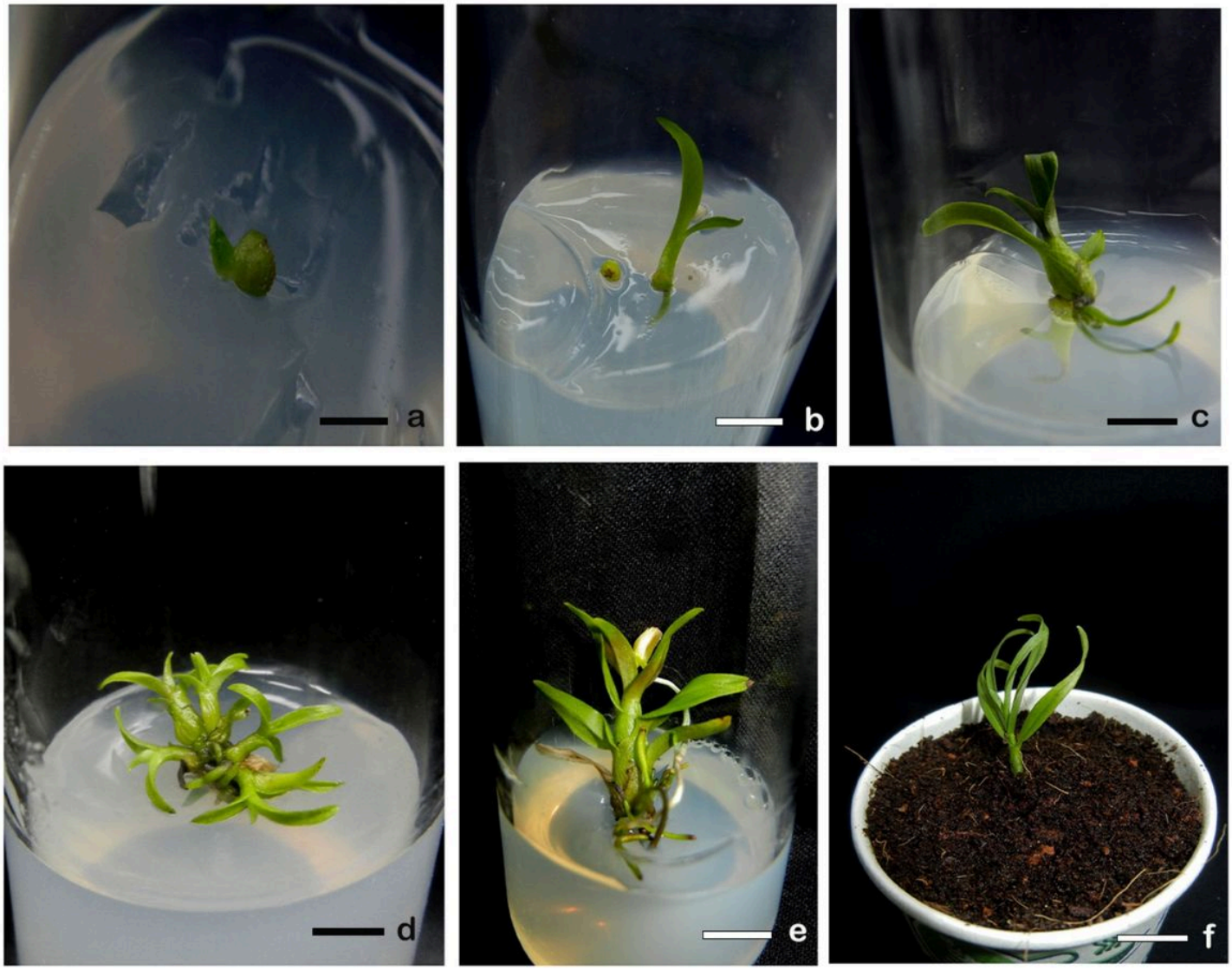

Figure 5

Micropropagation of *Dendrobium heyneanum* Lindl. from protocorms – a) Protocorm (Stage IV); b) Seedling formation from protocorms; c) Shoot with developing pseudobulb and roots; d) Multiple shoot bud formation; e) Elongation of Pseudobulb and roots; f) Hardened plantlet.