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ORIGINAL ARTICLE

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

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Abstract

Dendrobium heyneanum Lindl. or Heyne's Dendrobium is an endemic epiphytic orchid of Western Ghats, with a height of approximately 3 - 4 inches. The present study aimed to establish a conservation strategy using in vitro regeneration methods for this endangered taxon. Mature pods of the *D. heyneanum* were collected from the field, and aseptically inoculated on various nutrient media. Asymbiotic seed germination was most successful on half-strength macro-MS media, yielding an 86.70% germination rate within 12 days. Different morphogenic stages (I-VI) were observed, with 20.84% of seeds producing young seedlings with roots on half-strength macro-MS media. The micropropagation protocol of *D. heyneanum* was established by using the protocorms (Stage IV) from the asymbiotic germinated seeds, with the highest frequency (90.20%) observed in 1.0 mg/L Kinetin (KN). In vitro flower buds were observed at 0.5 mg/L and 1.0 mg/L 6-Benzyl amino purine (BAP) and callus induction at 2.0 mg/L of BAP. The synergistic effect of KN combined with auxins - α- Naphthaleneacetic acid (NAA), Indole- 3- acetic acid (IAA), and Indole- 3- butyric acid (IBA) plantlets were assessed, with KN + IAA (1.0 mg/L) inducing pseudobulb elongation (0.92 cm) and rooting (0.74 cm). The plantlets were subsequently acclimatized and hardened on pots containing cocopeat and brick pieces resulting in a survival rate of 52.73%. This study presents a comprehensive protocol for in vitro propagation of *Dendrobium heyneanum* Lindl., offering a viable method for ex-situ conservation efforts.

Key message

The present investigation reported first in vitro studies such as asymbiotic seed germination, micropropagation from protocorm, and in vitro flowering of *Dendrobium heyneanum* Lindl. an endemic species of Western Ghats, India.

Keywords Asymbiotic seed germination · Endemic Orchid · Protocorms · Phytohormones · Micropropagation

Abbreviations

IUCN	International Union for Conservation of Nature
TTC	2,3,5-Triphenyl tetrazolium chloride
ABA	Abscisic acid
BAP	6-Benzyl amino purine
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
Pa	Pascal

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Introduction

Orchids represent one of the largest and most diverse groups of monocots, comprising more than 30,000 species (Hassler 2024; Tiwari et al. 2024) with cosmopolitan distribution spanning nearly every habitat (Sarmah et al. 2024). Many members of this family are commercially known for their aromatic properties and decorative purposes, utilized in industries such as perfumery, cosmetics, and horticulture (Kanlayavattanakul and Lourith 2022, Sharma and Pathak 2020). Additionally, some orchid species hold cultural significance, serving as traditional folklore medicines for various ailments, and even as food sources (Xi et al. 2024). Certain 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 valuable insight into the regional biogeography, centers of diversity, and adaptive evolution of the flora (Gale et al. 2018). For instance, in India, regions such as the North-Western Himalayas, North-East, and Western and Eastern Ghats, exhibit a high degree of orchid endemism, encompassing approximately 311 species (Tomar et al. 2022).

However, the endemic orchids face numerous localized threats, including natural causes such as over-grazing, forest fire, and landslides, as well as anthropogenic activities like mining, agricultural expansions, and illegal trade (Kumar et al. 2024; Yudaputra et al. 2024). Moreover, orchid seeds, being minute and less dense, require the right mycorrhizal association and suitable substrates for successful germination to take place (Nongdam et al. 2023; Chen et al. 2022). Conventional breeding techniques are often ineffective for orchid propagation (Li et al. 2021), leading to the adoption of ex-situ conservation strategies such as tissue culture and cryopreservation to safeguard these species and preserve their genetic traits (Guo et al. 2024).

Dendrobium heyneanum Lindl. is an endemic epiphytic orchid found in the Western Ghats region, spanning parts of Karnataka, Tamil Nadu, and Kerala (Sulaiman et al. 2022; Rao and Krishnamurthy 2021; Ravichandran and Karuppusamy 2016). This species is distinguished by its unique flowers characterized by white petals approximately 1.5 cm across, with a 3-lobed obovate lip exhibiting erect lateral lobes and an ovate middle lob with crenate margin, a channeled ridge, and fleshy callus on the disc. While previous reports primarily focused on the taxonomic identification, and distribution, they also suggested possible threats to these plants leading to their categorization as Endangered by the IUCN (CAMP report, Kumar et al. 2001) with the current status listed as 'Not Evaluated' (Saleem et al. 2022). Given the significant risk to the continued existence of these plants, urgent and effective conservational efforts are imperative, with in vitro propagation techniques presenting a promising approach.

In recent years, in vitro asymbiotic seed germination, subsequent micropropagation from protocorms, and hardening of resulting plantlets, have been successfully implemented in various orchid species including *Pterostylis montana* and *P. paludosa* (Walt et al. 2024), *Vanda cristata* (Pathak et al. 2023), and *Orchis simia* (Fatahi et al. 2022). The aim of the present study was to establish a micropropagation protocol for *D. heyneanum* through asymbiotic seed germination and, observing seed development stages, propagating plantlets directly from protocorm explant with the exogenous supply of phytohormones without intermediate callus formation.

Materials and methods

Plant material collection and explant preparation for asymbiotic seed germination

Mature green unopened 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 approximately 2.0–2.5 cm in length were surface sterilized in 0.1% $HgCl_2$ for 3 min and rinsed thoroughly 3–4 in sterile distilled water. The pods were also dipped in 70% ethanol for 30 s followed by flaming off for 2–3 s. 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 and St-arnund 2000, Hosomi et al. 2011).

In vitro asymbiotic seed germination

The seeds were aseptically inoculated on 6 basal nutrient media namely Vacin and Went medium (VW, Vacin and Went 1949); Knudson C medium (KC, Knudson 1946)); Lindemann Orchid medium (LO, Lindemann and Gunckel 1970); Murashige and Skoog medium (MS, Murashige and Skoog 1962) and MS with two modifications i.e., one with half strength concentration of macronutrients (½ macro-MS), and other with MS vitamin replaced by B5 vitamin (MS_{B5}, Gamborg et al. 1968). The pH of the culture media was adjusted between 5.6 ± 0.2 according to the specific formulations prescribed by the authors prior to autoclaving for 20 min at 103421.35 Pa 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 by Teixeira da Silva et al. (2015) 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 Phytohormones 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 one-week apart from the treatments mentioned above using the same unopened pods.

Effect of cytokinin on protocorm development

Protocorm with the first leaf primordia (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-Benzyl amino purine (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. The plants were kept in a well-maintained greenhouse environment, ensuring adequate moisture, ventilation, and supplemental light. Regular misting with water containing trace amounts of MS macro-nutrient salts was carried out to support their growth.

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 lx and 65% humidity; a minimum of 15 replicates were kept and the experiment was repeated three times. 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

Effect of hormone-free media on in vitro asymbiotic seed germination

In vitro asymbiotic seed germination and subsequent seedling development present an efficient and practical approach for both propagating and conserving orchid species, particularly considering the contamination risks associated with symbiotic seed germination involving mycorrhizal or endophytic microbe (Tinoammini et al. 2024). The mature green pods of *Dendrobium heyneanum* Lindl. containing 83.56% viable seeds confirmed by the TTC test, displayed various responses when inoculated on different basal media in terms of initiation time and seed color development (Table 2). Notably, ½ macro-MS media yielded the highest seed germination rate, with 86.70% of green seeds germinating within 12 days, whereas only 71.56% of yellow-green seeds germinated on VW medium after 20 days. Subsequently, the experiment progressed to identify various stages (Fig. 1) of asymbiotic seed germination of *D. heyneanum* with data recorded after 120 days of inoculation presented in Table 3. On ½ macro-MS media, 20.84% of seeds developed into young seedlings (stage VI), followed by 18.67% on MS media and 6.64% on MS_{B5}. This underscores the suitability of ½ macro-MS for asymbiotic seed germination, consistent with the earlier findings on *Dendrobium aqueum* (Parthibhan

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 $p \leq 0.05$ according to DMRT

et al. 2015), thereby suggesting halving macro salts concentration is adequate for germinating seeds of some selected orchids (Diengdoh et al. 2017) primarily due to its balanced Nitrogen and Potassium levels.

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, implying that the vitamin supplements can impede seedling development. Media formulations such as KC, LO, and VW failed to produce stage VI seedlings, potentially attributed to their inadequacy in essential growth-inducing macro and micro elements compared to MS nutrients. Additionally, sucrose, serving as a crucial carbohydrate source for energy during growth and development (Hashemi et al. 2024), was found to be comparatively lower in LO, KC, and VW media formulation. Interestingly, previous studies on *Dendrobium tosaense* by Lo et al. (2004) indicated that KC and VW media were incapable of developing protocorms, in contrast to the current study where VW media produced protocorms with shoot initials (14.17%) and KC media yielded both protocorms stage IV (18.04%) and stage V (5.38%) protocorms. These disparities underscore the nuanced response of different orchid species to varied culture media formulations and highlight the importance of optimizing growth conditions tailored to specific species requirements.

Effect of phytohormones on in vitro asymbiotic seed germination

The study observed the effects of three different phytohormones - abscisic acid (ABA), 2,4-dichlorophenoxyacetic acid (2,4-D), and thidiazuron (TDZ)- on seed germination and seedling development. These PGRs are known to play specific role in plant development, with ABA promoting

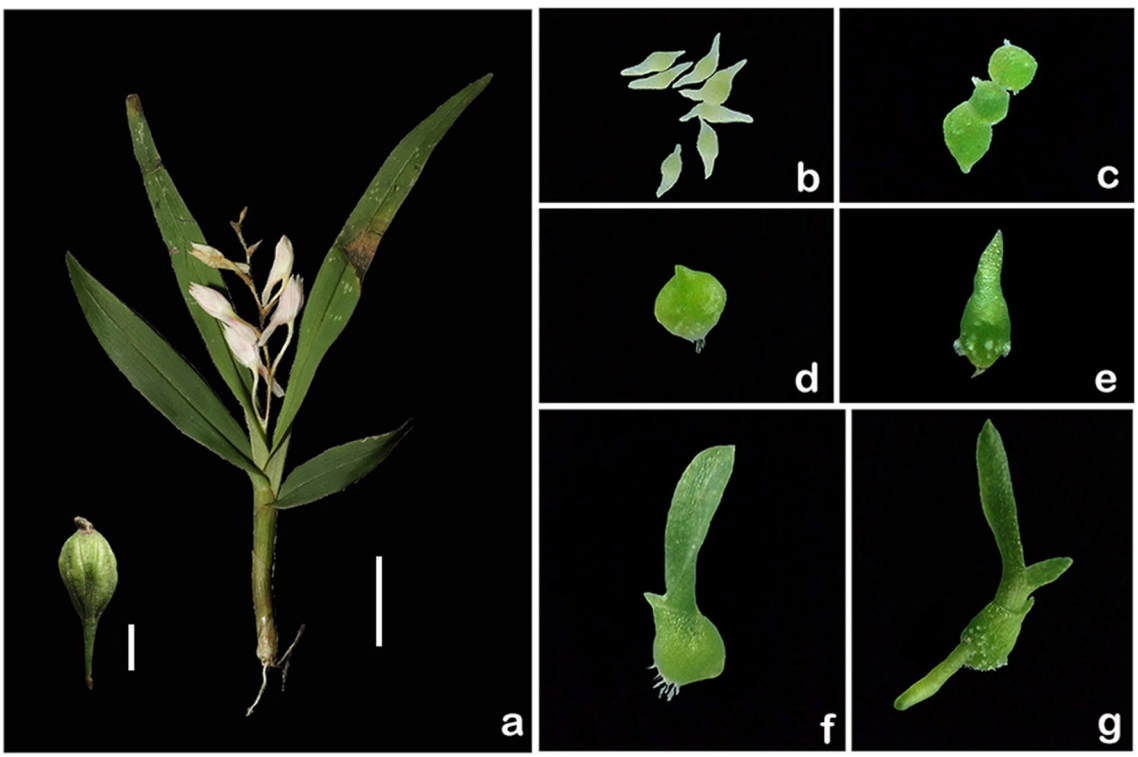

Fig. 1 Stage of asymbiotic seed germination of *Dendrobium heyneanum* Lindl. -**a**) Plant habit (scale: 1 inch) with the mature pod on lhs (scale: 1 cm); **b**) Imbibed embryo in testa; **c**) Enlarged embryo with half ruptured testa; **d**) Protocorm with pointed initials; **e**) Protocorm with the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

adaptive response to stressed conditions, 2,4-D inducing callus formation, and TDZ promoting shoot regeneration. Contrary to expectation, the addition of PGRs to the growth medium resulted in prolonged initiation time and decreased germination rates compared to hormone-free media (Table 4). Additionally, the response (Table 5) of seeds to various stages over 120 days indicated that the presence of PGRs induced some seeds to enter a dormant phase prematurely. This alteration in seed physiology and morphogenesis was evident from the variation observed in seed development in each culture.

Among the three hormones, ABA was found to be somewhat effective, as it led to the development of seeds into stage V protocorms, although many of these protocorms turned brown, suggesting that they were dead. 2,4-D induced the development of seeds into stage IV callusing protocorms with numerous adventitious rhizoids, while TDZ resulted in the development of smaller-sized protocorms (Fig. 2). Overall, the findings of the study suggest that the addition of PGRs did not promote effective seed germination and seedling development. Instead, it led to the browning of protocorms and arrested growth which were similar to the observations in previous studies by Yao et al. (2021) and Hossain et al. (2013).

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 $p \leq 0.05$ according to DMRT

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 $p \leq 0.05$ 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 $p \leq 0.05$ according to DMRT

Effect of cytokinin on protocorm development and shoot proliferation

In the seed germination, it was noted that the transition of young seedlings into adult plantlets was hindered due to the absence of external phytohormones particularly cytokinins which play a vital role in plant development. To address this, protocorms at stage IV, which are composed of highly meristematic cells capable of developing into various cell types, tissues, or organs were utilized. These protocorms were cultured on ½ strength macro-MS augmented with different concentrations of cytokinin. Remarkably, regardless of the type or concentration of cytokinin used, the protocorms demonstrated a versatile response, giving rise to callus formation, shoots bearing multiple buds, and even shoots with inflorescences. The results, detailed in Table 6, depict the frequencies of protocorm response, callus induction, shooting, inflorescence development, as well as callus nature and multiple shoots produced. Notably, the highest response rate of 90.20% active protocorms was observed when 1.0 mg/L Kinetin was applied. This finding is consistent with the observations made by Nongdam and Tikendra (2014) in *D. chrysotoxum,* where KN-supplemented media facilitated the maximum conversion of seedlings. In essence, the experiment underscores the remarkable plasticity and responsiveness of protocorms to cytokinin treatment, highlighting their potential for efficient regeneration and proliferation in vitro.

In this study, it was found that media augmented with different concentrations of Kinetin had a significant effect on the development of protocorm into shoots. Specifically, a maximum response of 95.76% was observed when using 1.0 mg/L of Kinetin, while a slightly lower response of 93.75% was noted with 0.5 mg/L of Kinetin. Furthermore, the study revealed that using 1.0 mg/L of Kinetin resulted in the development of 6.56 shoot buds with an average height of approximately 1.13 cm. Similarly, employing 1.5 mg/L of Kinetin led to the generation of 6.02 shoot buds, each with an average height of about 1.05 cm. The Fig. 3. illustrates the multiple shoot bud formation in different Kinetin concentrations. These findings are consistent with previous research on various species of *Dendrobium*, as documented by Martin and Madassery 2006, Luo et al. 2009, and Asghar et al. 2011. Additionally, Maharjan et al. (2020) reported significant results in their study on *D. chryseum* protocorms cultured in half-strength MS medium supplemented with 2.0 mg/L of Kinetin and 10% coconut water, achieving maximum multiple shoots.

Effect of cytokinin on callus induction

When cultured on media containing TDZ and BAP, (Fig. 3) the protocorms initiated growth with green friable callus. BAP at the concentration of 2.0 mg/L resulted in the formation of 20.67% of callus, while only 6.46% was formed on 1.5 mg/L after 100 days of culture. Callus with a pale green color hue was less frequently observed when treated with TDZ compared to callus induced by BAP. Previous studies, such as Roy et al. (2007) and Pyati (2020), have shown that both BAP and TDZ are effective in inducing callus formation and subsequent regeneration of plantlets in *D. chrysotoxum* and BAP alone in *D. barbatulum*. Cytokinins including BAP and TDZ, have been found to reduce lignification of cell walls, which promotes the initiation of callus (Dar et al. 2021). Additionally, it is speculated that the abnormal callus formation induced by BAP and TDZ alone may be due to the stress exerted on meristematic cells, as suggested by Dinani et al. (2018), whereas typically, a combination of cytokinins and auxins is known to trigger callus formation.

Effect of cytokinin on in vitro flowering

Dendrobium species are valued for their cut flowers for ornamental purposes. In the present study, an unusual and noteworthy occurrence of in vitro flowering in juvenile *Dendrobium* plants was achieved through their cultures on media augmented with BAP. Remarkably, approximately 30.2% and 22.37% of the seedlings showed initiation of bud

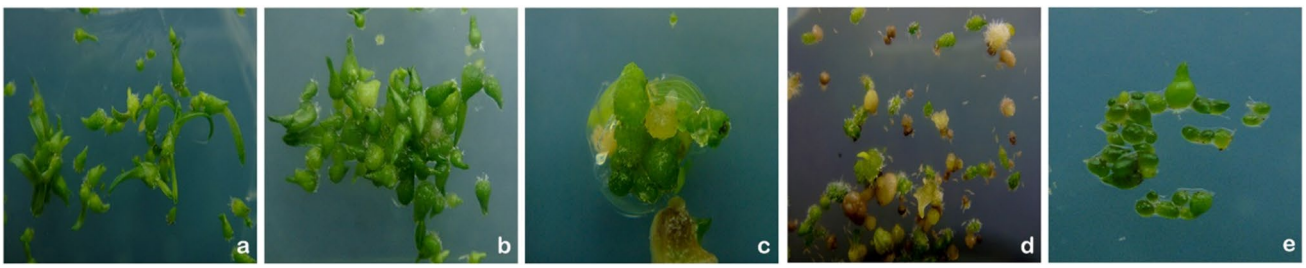

Fig. 2 Asymbiotic seed germination of *Dendrobium heyneanum* Lindl. on different medium after 120 days of culture – **a**) Seedlings on ½ macro-MS media (basal) media; **b**) Seedlings on MSB5 (basal) media; **c**) Protocorms on ½ macro-MS media supplemented with 0.1 mg/L ABA; **d**) Protocorms on ½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

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}
	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 $p \leq 0.05$ according to DMRT

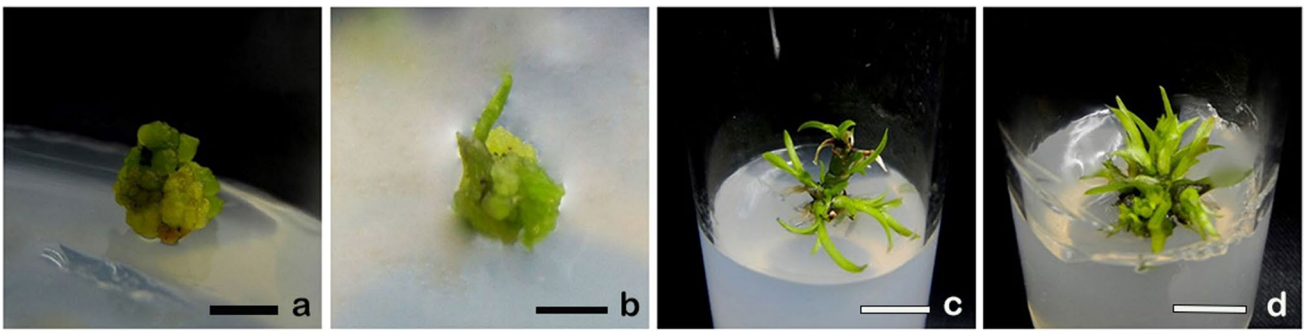

Fig. 3 Response of protocorms on ½ macro-MS media supplemented with various cytokinin – **a**) callus induction on 2.0 mg/L BAP; **b**) Callus induction on 1.5 mg/L TDZ; **c**) Shoot proliferation on 1.5 mg/L KN; **d**) Shoot proliferation on 1.0 mg/L KN

formation for inflorescence after 75 days of being cultured on media containing 0.5 mg/L and 1.0 mg/L BAP respectively. It was observed that the presence of cytokinin in the media played a pivotal role in floral meristem development (Nadal et al. 2023). Subsequently, transferring cultures to hormone-free basal media facilitated the maturation of inflorescence, ultimately leading to the blooming flowers in all these cultures. The various stages of in vitro flowering are depicted in Fig. 4. The phenomenon of BAP-induced in vitro flowering was also reported in other Dendrobium species such as *Dendrobium wangliangii*, *Dendrobium nobile*, *Dendrobium* Sonia 17, *Dendrobium* Chao Praya Smile, and *Dendrobium* Madame Thong-In (Zhao et al. 2013; Wang et al. 2009; Tee et al. 2008; Hee et al. 2007; Sim et al. 2007). Additionally, other studies have concluded that cytokinin, either alone or in conjunction with natural additives, can promote in vitro flowering in various orchid species (Wang et al. 2006; Duan and Yazawa 1994). Although the exact criterion responsible for the initiation of in vitro flower buds in the present study remains unknown (Pujari and Babu 2022), it is hypothesized that the abiotic stress induced by the culture conditions might have prompted the plants to progress through their life cycle. This activation of the reproductive phase could potentially be attributed to the synthesis of ethylene, a natural inducer of flower senescence (Ranganatha et al. 2023).

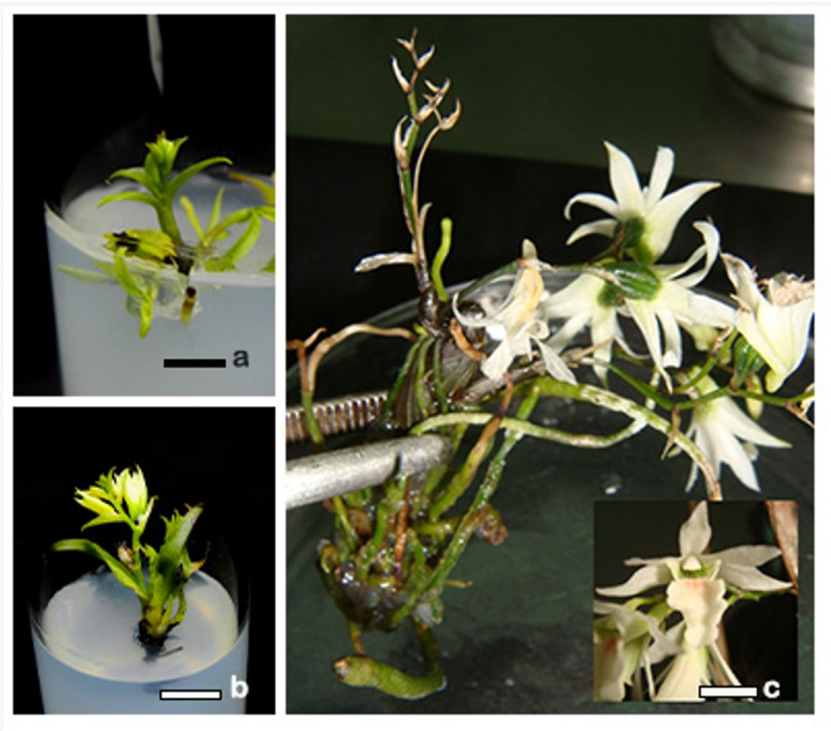

Fig. 4 Invitro flowering from protocorm- **a**) In vitro flower bud induction on 0.5 mg/L BAP; **b**) Formation of Inflorescence on ½ macro-MS (basal) media; **c**) A matured Inflorescence bearing flowers

Synergistic effect of kinetin with auxins

The balance between cytokinin and auxins in culture media is crucial for the overall growth of plants, as their synergic action can enhance cell division, as noted by Singh and Sinha (2017). When the developing plantlets were transferred to a nutrient medium containing 1.0 mg/L kinetin along with various auxins, they exhibited slight elongation and prominent rooting in all the cultures, as shown in Table 7. Notably, cultures supplemented with 1.0 mg/L IAA recorded the highest response rate of 74.14%, along with pseudobulbs measuring 0.92 cm in length, an average of 4.72 roots with 0.74 cm in length. These findings align with Pant and Thapa (2012) observations which suggested that IAA concentrations of 0.5 mg/L and 1.0 mg/L effectively promoted root proliferation in *D. primulinum*. Similarly, Hossain et al. (2013) reported that 0.5 mg/L IAA resulted in a robust rooting system in *D. aphyllum*. Maharjan et al. (2020) study on *Dendrobium chryseum* also supported these findings, indicating that IAA (1.5 mg/L) was suitable for rooting with or without any natural additives. The combined action of Kinetin and IAA, as observed in the present investigation, significantly promoted root development, which is consistent with findings made by Dutta et al. (2011) on *D. aphyllum* where the combination of KN + IAA resulted in increased shoot and root length. It is noted that a low concentration ratio of these two PGRs in the growth media can stimulate rooting, as suggested by Kotov and Kotova 2018. Moreover, Diengdoh et al. 2017 found that Half MS supplemented with KN (5 mM) + IAA (10 mM) was optimal for seed germination of *Paphiopedilum insigne* and they identified that the synergistic effect of these hormones can be beneficial for the in vitro plant development.

Acclimatization and hardening

When the hormone-treated plantlets were gradually subcultured to hormone-free media and eventually on minimal media, approximately 75.21% of them showed active growth after 75 days. During this process, the average root length increased to 0.93 cm, while the number of multiple shoots decreased to 3.33. This signifies that the absence of PGR in the media influences the development of plants, leading to less favorable outcomes than expected. Upon acclimatization and hardening in pots filled with a mix of cocopeat and brick pieces (in a ratio of 1:1), the survival dropped to 52.73%. The correct mycorrhizal partnership plays a crucial role in promoting optimal plant growth in vivo (Aucencia and Anne 2024), given that the plants are solely acclimatized in a sterile substratum, it is reasonable to anticipate that the symbiotic advantage of such a relationship may not have occurred, consequently reflecting in lower survival rates. The various stages of *D. heyneanum* protocorm development from micropropagation to hardening the plantlets are represented in Fig. 5.

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 $p \leq 0.05$ according to DMRT

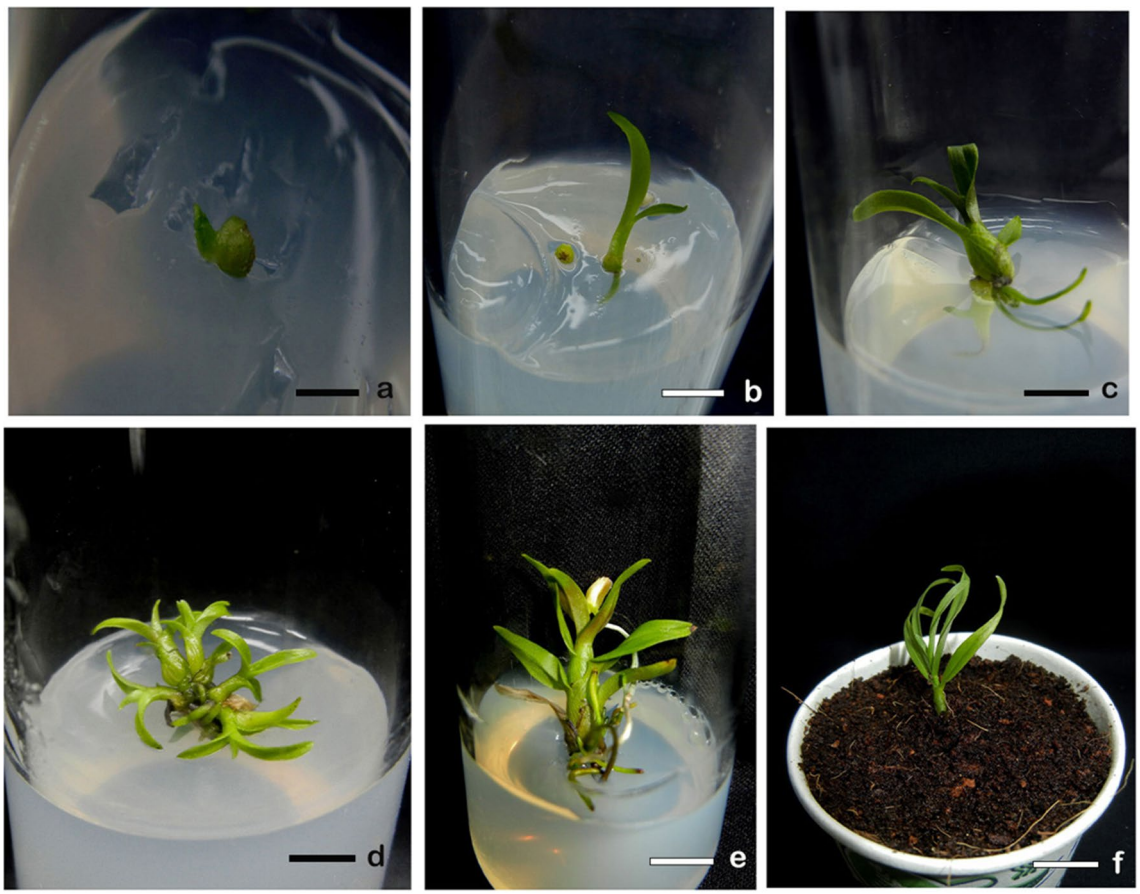

Fig. 5 Micropropagation of *Dendrobium heyneanum* Lindl. from protocorms – **a**) Protocorm (Stage IV); **b**) Seedling formation from protocorms; **c**) Shoot with developing pseudobulb and roots; **d**) Multiple shoot bud formation; **e**) Elongation of Pseudobulb and roots; **f**) Hardened plantlet

Conclusion

The study presents a comprehensive method for successful seed germination and micropropagation of *Dendrobium heyneanum* Lindl., addressing a critical aspect of its conservation by reducing dependency on the natural populations. By meticulously collecting mature pods from the field, minimal disruption to natural populations was ensured. The findings demonstrated that ½ macro-MS medium is highly effective for seed germination with subsequent optimization of cytokinin supplementation yielding diverse responses including callus formation, shooting, multiple bud growth, and flower bud initiation. Furthermore, the combination of cytokinin (KN) and auxins revealed optimal conditions for shoot elongation and rooting, enhancing the viability of propagated plants. Although the survival rate of hardened plants reached 52.73%, the study underscores the feasibility of raising *D. heyneanum* under controlled in vitro conditions. This developed protocol, with its demonstrated efficacy and adaptability, holds significant promise not only for the conservation of this taxon but also for the propagation of related species and other threatened epiphytic orchids. The future research could focus on implementing this methodology and broaden its applicability across diverse orchid taxa, thus contributing significantly to the conservation and sustainable management of these ecologically valuable species.

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Author's 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.

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Data availability Data is available upon reasonable request.

Declarations

The authors declare that they have no competing interests.

Ethical approval Not applicable.

Consent to participate Not applicable.

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