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RESEARCH ARTICLE

In vitro propagation and asymbiotic seed germination for the conservation of the endangered orchid *Eulophia andamanensis* Rchb.f.

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Abstract

Eulophia andamanensis Rchb.f., an endangered terrestrial orchid endemic to the Andaman and Nicobar Islands, is severely threatened by habitat loss, over-collection and climate change. The present study developed an optimized *in vitro* propagation protocol encompassing seed germination, protocorm-like body (PLB) formation, shoot multiplication, root induction and acclimatization to support *ex situ* conservation and potential reintroduction. Immature seeds cultured on various media showed the best response in full-strength Orchimax medium, with early germination observed at 60.30 days after culturing (DAC), maximum seed germination (92.20 %) at 90 DAC and early PLB formation at 74.90 DAC. Later, PLBs were transferred to full-strength Orchimax medium supplemented with 1.0 mg L^{-1} 6-benzylaminopurine (BAP) and 0.5 mg L^{-1} α-naphthalene acetic acid (NAA) initiated shoots within 10.20 DAC, producing 4.73 shoots per explant with an average shoot length of 4.67 cm and 4.05 leaves after 90 DAC in the shooting medium. Rooting was optimal in Orchimax half-strength medium containing 2.0 mg L^{-1} indole-3-butyric acid (IBA), which induced root initiation at 10.30 DAC, with 4.77 roots per shoot and an average root length of 4.40 cm at 90 DAC in rooting media. Hardened plantlets grown in a red soil: sand: vermicompost: cocopeat (2:1:1:1) mixture exhibited an 85% survival rate after 60 days of secondary hardening in the greenhouse. This efficient and reproducible protocol provides a reliable platform for the large-scale propagation, genetic conservation and restoration of *E. andamanensis* in its natural habitats.

Keywords: acclimatization; conservation; endangered; *in vitro* propagation; seed germination

Introduction

The Orchidaceae is the second largest family of flowering plants, comprising approximately 900 genera and 28,000 to 32,000 species (1). Orchids hold significant ecological, cultural and economic value and are renowned for their extraordinary floral diversity, striking aesthetic appeal and important roles in horticulture and ethnomedicine (2). Among terrestrial orchids, the genus *Eulophia* R.Br. ex Lindl. holds a prominent place. First described by John Lindley in 1821, its name derives from the Greek *eu* ("well") and *lophos* ("plume"), referring to the distinctive crested ridges on the labellum of many species (3). Today, *Eulophia* comprises about 230 species, of which 203 are formally recognized, predominantly in Palaeotropical regions. Africa serves as its primary diversity centre, with a small contingent of six species extending into the Neotropics; most species are terrestrial, though some adopt epiphytic or lithophytic habits (4).

A notable member of this genus is *Eulophia andamanensis* Rchb. f., a terrestrial orchid of deciduous forest endemic to Southeast Asia. It develops above-ground pseudobulbs and linear leaves (10 to 26 × 1.5 to 2 cm) that abscise at flowering and produces an erect inflorescence (20 to 50 cm) bearing green, red-veined flowers with a deeply cleft trilobed lip. Flowering occurs from February through May (5). Despite its ornamental allure and documented antiseptic use of dried pseudobulbs in Thai traditional medicine (6), *E. andamanensis* is threatened by habitat loss, overcollection and inherently low natural germination rate. Humans have long used orchids as sources of food, medicine, adhesives and fragrances. Their longevity, high yield and transportability support their dominance in the global floriculture trade. They also feature prominently in traditional medicinal systems worldwide, exhibiting antimicrobial, antioxidant, anti-inflammatory, anticancer, antidiabetic and wound-healing activities (7, 8). Within *Eulophia*, tubers have been used traditionally to treat scrofulous gland swellings (9), bronchitis and rheumatoid arthritis (10) and to act as vermifuges, tonics and aphrodisiacs (11), further underscoring the genus's therapeutic potential. Conservation of *E. andamanensis* and related species remains challenging due to poor seed germination, low seedling survival and obligatory mycorrhizal associations, making conventional *in situ* approaches inadequate. Consequently, *ex situ* strategies, particularly plant tissue culture, have become essential for germplasm preservation and mass multiplication (12). In natural habitats, orchid seeds rely on mycorrhizal fungi for the acquisition of essential nutrients, as the

dust-like seeds lack endosperm and depend entirely on the fungal partner for carbon and mineral nutrition during early germination and protocorm development. Asymbiotic seed culture, which provides sterile, nutrient-rich media to support protocorm-like bodies (PLBs) initiation and seedling development without fungal partners, has emerged as a vital tool (13). Standard basal media, such as Murashige and Skoog (MS) and Lindemann, are widely used but show variable success among orchid genera and species (14). Proprietary formulations such as Orchimax medium, enriched with organic nitrogen sources and activated charcoal, have demonstrated improved seed germination rates and PLBs formation in various terrestrial orchids (15). Successful micropropagation relies on optimizing the composition of culture media (16). Despite the widespread use of tissue culture in many orchid species, *E. andamanensis* remains poorly studied and no standardized *in vitro* propagation protocol is currently available for this species. This gap limits both conservation and large-scale propagation efforts. Therefore, the present study aims to (i) assess different basal media for asymbiotic seed germination and PLBs formation, (ii) evaluate the effect of plant growth regulators (PGRs) on shoot initiation and proliferation from PLBs and (iii) determine the optimal concentration of indole-3-butyric acid (IBA) for efficient *in vitro* rooting in *E. andamanensis*.

Materials and Methods

Plant material and explant source

The *E. andamanensis* plants were sourced from the Mahatma Gandhi Botanical Garden, University of Agricultural Sciences, Gandhi Krishi Vigyana Kendra, Bengaluru, in November 2023 and maintained in the orchidarium (13° 4′ 47.05″ N, 77° 34′ 38.54″ E) of the Plant Tissue Culture Laboratory, Department of Horticulture, University of Agricultural Sciences, Gandhi Krishi Vigyana Kendra.

Seed sterilization and culture initiation

Hand-pollinated seed capsules were collected 150 to 180 days after pollination to ensure optimal embryo development. Immature capsules were first immersed in 200 mL of distilled water supplemented with 0.01 % (v/v) Tween-20, with continuous agitation for 30 min. They were then rinsed three times in sterile distilled water (5 min each) to remove surfactant residues. Capsules were then treated with 0.2 % Bavistin for 20 min under continuous agitation, followed by three sterile water rinses (each for 5 min). Surface sterilization was continued by soaking the capsules in 0.1 % mercuric chloride ($HgCl_2$) for 3 min, after which the explants were washed 5 times in sterile distilled water (2 min each). Excess moisture was blotted onto sterile filter paper and the capsules were finally surface sterilized by dipping in 70 % ethanol and flaming for 5 sec.

Culture condition

Cultures were incubated under cool white fluorescent lamps with a light intensity of 30-40 µmol m^{-2} s^{-1} (approximately 2000 lux) with a 16-hr photoperiod at 24 ± 2 °C.

Seed germination and protocorm-like bodies formation on different media

Six culture media formulations were assessed (Table 1), comprising full and half-strength Murashige and Skoog (MS) medium, Lindemann medium and Orchimax medium. Each medium was solidified with 6 g L^{-1} agar and adjusted to pH 5.7. Sucrose was incorporated at 30 g L^{-1} into MS medium and at 20 g L^{-1} into Lindemann and Orchimax media formulations. The Orchimax media was further supplemented with tryptone, MES (2- (N-morpholino) ethanesulfonic acid) buffer and activated charcoal. Detailed compositions and concentrations are listed in supplementary Tables 1 - 3. Under aseptic conditions in a laminar airflow chamber, approximately 0.3 mg of *E. andamanensis* seeds (equivalent to 150 -200 seeds) were sown into each 250 mL culture vessel containing 40 mL of media, with ten replicates per treatment. Key parameters measured in the study included the days to seed germination, the days to PLB formation, and the seed germination percentage (%). Specifically, seed germination was considered as the time taken for the visible emergence of embryos. At the same time, PLB formation was recorded from culture initiation until compact, globular, creamy white to pale green structures with spherical protrusions appeared. The germination percentage was recorded at 90 days after culturing (DAC), following ISTA guidelines, using the formula (17).

Table 1. Culture media treatments for *in vitro* seed germination and PLBs formation in *E. andamanensis*

Treatments	Solid media	Media strength
T_1	Murashige and Skoog	Full-strength
T_2		Half-strength
T_3	Lindemann	Full-strength
T_4		Half-strength
T_5	Orchimax	Full-strength
T_6		Half-strength

$$\text{Germination percentage (\%)} = \frac{\text{No. of seeds germinated}}{\text{Total no. of seeds inoculated}} \times 100 \quad \text{(Eqn. 1)}$$

Shoot induction and multiplication from PLBs using different PGRs

As Orchimax, full-strength medium, yielded the best results, it was selected for subsequent experiments. Protocorm-like bodies measuring 4 to 5 mm in diameter from *E. andamanensis*, obtained from a previous experiment, were utilized as explants, with one PLB per bottle and ten replicates per treatment. The effect of various concentrations of 6-benzylaminopurine (BAP), Kinetin (KIN), α-naphthalene acetic acid (NAA), each ranging from 0.5 to 1.5 mg L^{-1}, applied alone and in combinations on the growth and proliferation of PLBs was carried out (Table 2). Observations recorded included days to shoot initiation, number of shoots, shoot length (cm), and number of leaves at 90 DAC in the shooting media.

Rooting of regenerated shoots with IBA treatments

After 90 DAC, regenerated shoots were carefully excised from the shooting medium and transferred to rooting media for further trials. Rooting was initiated using Orchimax half-strength medium supplemented with varying concentrations of IBA, along with a control treatment lacking IBA. Each treatment consisted of ten replicates, with one shoot cultured per bottle (Table 3). Observations of days to root initiation, number of roots per shoot and root length (cm) were recorded at 90 DAC in rooting media. After 90 days of rooting, well-rooted plantlets were subjected to *in vitro* primary hardening for one month in sealed, autoclaved polypropylene bags containing sterilized cocopeat. Later, they were transferred to pots containing a substrate mixture of red soil, sand, vermicompost and cocopeat (2:1:1:1) and maintained under

Table 2. Composition of culture media and PGR treatments used for shoot regeneration from PLBs of *E. andamanensis*

Treatments	Concentration (mg L^{-1})	
T_1		**Control**
T_2		0.5
T_3	BAP	1.0
T_4		1.5
T_5		0.5
T_6	NAA	1.0
T_7		1.5
T_8		0.5
T_9	KIN	1.0
T_{10}		1.5
T_{11}		0.5+0.5
T_{12}		0.5+1.0
T_{13}		0.5+1.5
T_{14}		1.0+0.5
T_{15}	BAP+NAA	1.0+1.0
T_{16}		1.0+1.5
T_{17}		1.5+0.5
T_{18}		1.5+1.0
T_{19}		1.5+1.5
T_{20}		0.5+0.5
T_{21}		0.5+1.0
T_{22}		0.5+1.5
T_{23}		1.0+0.5
T_{24}	KIN+NAA	1.0+1.0
T_{25}		1.0+1.5
T_{26}		1.5+0.5
T_{27}		1.5+1.0
T_{28}		1.5+1.5

BAP- Benzylaminopurine, KIN- Kinetin and NAA- Naphthalene acetic acid.

Table 3. Composition of rooting media and IBA concentrations used for *in vitro* rooting of *E. andamanensis* shoots

Treatments	Solid media	Concentrations
T_1		Control
T_2		IBA 0.5 mg L^{-1}
T_3	Orchimax half strength	IBA 1.0 mg L^{-1}
T_4		IBA 1.5 mg L^{-1}
T_5		IBA 2.0 mg L^{-1}

greenhouse conditions for two months with regular watering and care.

Statistical analysis

The experiment was conducted using a completely randomized design (CRD) to evaluate the effects of different media and PGRs. Data were analyzed using OPSTAT software and the *F*-test was used to test significance at a 1 % level.

Results and Discussion

Effect of different basal media on seed germination and PLBs formation

The composition and strength of the culture media significantly influenced seed germination and subsequent PLBs development in *E. andamanensis*. Among the tested media, Orchimax full-strength solid medium (T_5) exhibited the earliest germination (60.30 DAC) and the highest germination rate (93.96 %) after 90 DAC. In contrast, Lindeman full-strength solid medium (T_3) exhibited delayed germination (79.70 DAC) with the lowest germination rate (72.70 %), indicating that Orchimax full-strength medium was the most effective for seed germination (Table 4). Statistically significant variations were observed in the days taken for PLB formation among the different media. Orchimax full-strength medium recorded the earliest PLB formation (74.90 DAC), while Lindemann full-strength medium showed the slowest PLB formation (93.50 DAC), as shown in Fig. 1.

The superior performance of Orchimax medium can be

Table 4. Influence of culture media on days taken for seed germination and germination percentage in *E. andamanensis*

Treatments	Days taken for seed germination	Germination percentage (%) after 90 days of culturing
T_1- MS full-strength solid media	71.80	81.20
T_2- MS half-strength solid media	68.20	85.64
T_3- Lindeman full-strength solid media	79.70	72.70
T_4- Lindeman half-strength solid media	75.20	76.88
T_5- Orchimax full-strength solid media	60.30	93.96
T_6- Orchimax half-strength solid media	64.40	90.10
F-test	**	**
SEm ±	0.83	0.86
CD at 1 %	3.12	3.23

** Significant at *p*= 0.01.

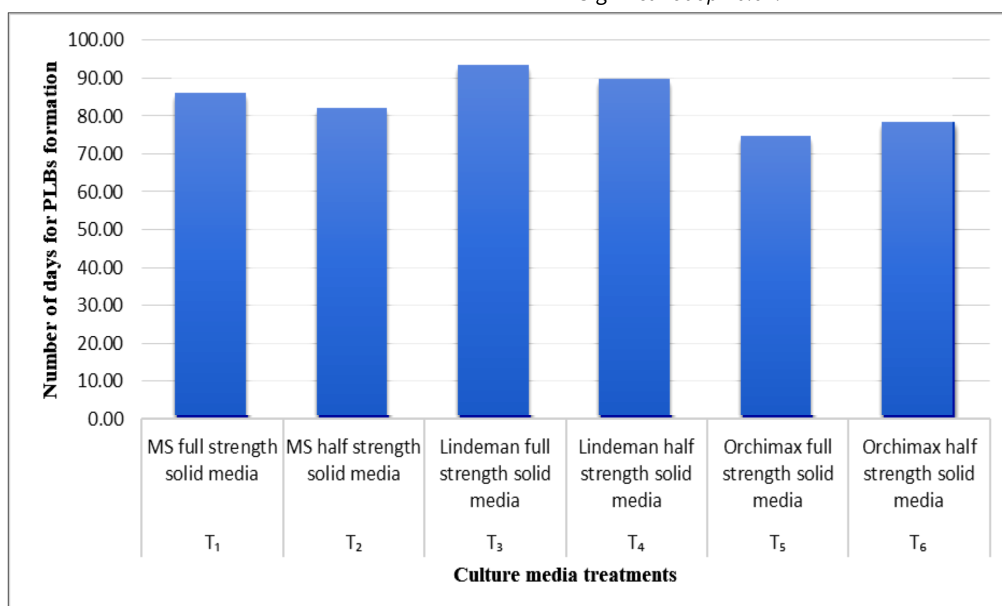

Fig. 1. Effect of varied culture media on the days taken for PLBs formation from seeds of *E. andamanensis.*

attributed to its optimally balanced macronutrient composition and enriched organic additives, including activated charcoal and essential vitamins, which collectively enhance cellular division, morphogenic competence and early differentiation. These findings are corroborated by biochemical evidence indicating that the improved nutrient bioavailability, the adsorptive and antioxidative properties of activated charcoal and vitamin-mediated stimulation of metabolic pathways significantly enhance PLBs' vigor and developmental progression in orchids (18). In contrast, the delayed response observed on Lindemann medium could result from ionic or salt imbalances that impede embryo metabolism. Comparable trends have been reported in *Sedirea japonica* (19), *Orchis coriophora* (20), *Eulophia nuda* (21) and *Eulophia graminea* (22), underscoring the critical role of optimized nutrient formulations in supporting asymbiotic germination and protocorm development. Similarly, *Eulophia alta* exhibited maximum germination and PLB formation (87.90 %) on Orchimax medium when cultured from immature seeds (23). The present findings reaffirm that Orchimax full-strength medium provides optimal physicochemical conditions for rapid and uniform germination, making it a reliable choice for large-scale propagation and conservation of *E. andamanensis*.

Effect of different PGRs on shoot induction and multiplication

The response of *E. andamanensis* to different PGRs treatments showed pronounced variation in shoot initiation and morphogenic development from PLB. Among the treatments, the combination of BAP (1.0 mg L^{-1}) and NAA (0.5 mg L^{-1}) (T_{14}) was the most effective, producing the earliest shoot initiation (10.20 DAC), highest shoot number (4.73 per explant), maximum shoot length (4.67 cm) and greatest leaf number (4.05 per explant) at 90 DAC in shooting media. At the same time, the control showed delayed initiation (35.20 DAC) with lower number of shoots (0.98) and minimum shoot length of 0.48 cm, indicating a poor morphogenic response overall as shown in Table 5 and Fig. 2. The superior performance of BAP (1.0 mg L^{-1}) and NAA (0.5 mg L^{-1}) treatment can be attributed to the synergistic interaction between cytokinin and auxin, which promotes both meristematic cell division and cellular elongation. Cytokinins, such as BAP, stimulate shoot meristem activation and rapid bud differentiation, whereas auxins, like NAA, facilitate cellular expansion and organized tissue development, resulting in a balanced morphogenic response. The optimized BAP: NAA ratio (2:1) provided an ideal hormonal environment, preventing excessive callusing and stunted shoot formation commonly seen under cytokinin-rich conditions. This hormonal equilibrium facilitated coordinated shoot and leaf development, resulting in healthy, elongated and morphologically stable plantlets, which indicates its suitability for large-scale micropropagation of *E. andamanensis*.

Shoot elongation was greatest under the combined treatment, reflecting the synergistic interaction of cytokinin and auxin in regulating cell division, expansion and tissue differentiation, as previously demonstrated in *Hyssopus officinalis* (24). Elevated cytokinin levels alone can suppress elongation through ethylene accumulation, underscoring the importance of maintaining an optimal cytokinin-to-auxin ratio (25). Similar synergistic effects of BAP and NAA have been reported across several orchid species, where combined applications promoted multiple shoot formation and enhanced elongation compared to individual hormone treatments, including *Aerides multiflora* (13), *Bulbophyllum crassipes* (26), *Cattleya* spp. (27), *Rhynchostylis retusa* (28) and *Dendrobium* spp. (29). In the present study, the combination of 1.0 mg L^{-1} BAP and 0.5 mg L^{-1} NAA yielded the most favourable response in *E. andamanensis*, resulting in early and synchronized shoot

Table 5. Effect of BAP, KIN and NAA on days taken for shoot initiation and shoot length from PLBs of *E. andamanensis*

Treatments (mg L^{-1})	Days taken for shoot initiation	Shoot length (cm) after 90 days of culturing in shooting media
T_1-Control	35.20	0.48
T_2- BAP 0.5	12.80	4.01
T_3- BAP 1.0	11.40	4.47
T_4- BAP 1.5	12.60	4.20
T_5- NAA 0.5	14.80	2.88
T_6- NAA 1.0	15.20	2.65
T_7- NAA 1.5	15.80	2.40
T_8- KIN 0.5	14.20	3.89
T_9- KIN 1.0	12.80	4.01
T_{10}- KIN 1.5	12.60	4.25
T_{11}- BAP 0.5 + NAA 0.5	12.60	4.14
T_{12}- BAP 0.5 + NAA 1.0	12.80	3.91
T_{13}- BAP 0.5 + NAA 1.5	13.20	3.73
T_{14}- BAP 1.0 + NAA 0.5	10.20	4.67
T_{15}- BAP 1.0 + NAA 1.0	11.80	4.38
T_{16}- BAP 1.0 + NAA 1.5	12.80	4.15
T_{17}- BAP 1.5 + NAA 0.5	12.40	4.33
T_{18}- BAP 1.5 + NAA 1.0	12.60	4.13
T_{19}- BAP 1.5 + NAA 1.5	12.80	3.93
T_{20}- KIN 0.5 + NAA 0.5	13.40	4.08
T_{21}- KIN 0.5 + NAA 1.0	13.80	3.81
T_{22}- KIN 0.5 + NAA 1.5	14.20	3.76
T_{23}- KIN 1.0 + NAA 0.5	12.80	4.20
T_{24}- KIN 1.0 + NAA 1.0	13.40	3.96
T_{25}- KIN 1.0 + NAA 1.5	13.80	3.75
T_{26}- KIN 1.5 + NAA 0.5	12.60	4.25
T_{27}- KIN 1.5 + NAA 1.0	12.80	4.08
T_{28}- KIN 1.5 + NAA 1.5	13.20	3.72
F-test	**	**
SEm ±	0.29	0.04
CD at 1 %	1.08	0.14

** Significant at p= 0.01.

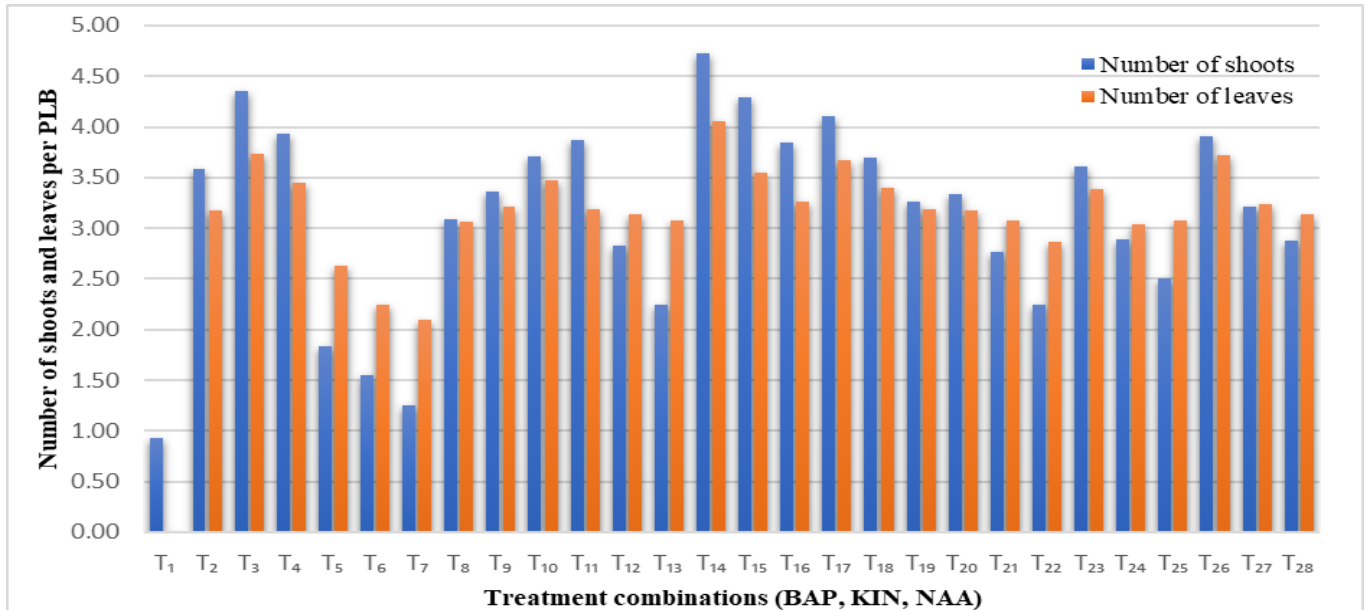

Fig. 2. Influence of BAP, KIN and NAA alone and in combination on the number of shoots and the number of leaves produced from PLBs of *E. andamanensis* at 90 DAC in the shooting media. T_1 – Control, T_2 – BAP 0.5, T_3 - BAP 1.0, T_4 - BAP 1.5, T_5 – NAA 0.5, T_6 – NAA 1.0, T_7 – NAA 1.5, T_8 - KIN 0.5, T_9 - KIN 1.0, T_{10} - KIN 1.5, T_{11} – BAP 0.5 + NAA 0.5, T_{12} - BAP 0.5 + NAA 1.0, T_{13} - BAP 0.5 + NAA 1.5, T_{14} - BAP 1.0 + NAA 0.5, T_{15} - BAP 1.0 + NAA 1.0, T_{16} - BAP 1.0 + NAA 1.5, T_{17} - BAP 1.5 + NAA 0.5, T_{18} - BAP 1.5 + NAA 1.0, T_{19} - BAP 1.5 + NAA 1.5, T_{20} – KIN 0.5 + NAA 0.5, T_{21} - KIN 0.5 + NAA 1.0, T_{22} - KIN 0.5 + NAA 1.5, T_{23} - KIN 1.0 + NAA 0.5, T_{24} - KIN 1.0 + NAA 1.0, T_{25} - KIN 1.0 + NAA 1.5, T_{26} - KIN 1.5 + NAA 0.5, T_{27} - KIN 1.5 + NAA 1.0, T_{28} - KIN 1.5 + NAA 1.5.

initiation, rapid multiplication and enhanced leaf development. These findings underscore the pivotal role of a balanced cytokinin: auxin interaction in optimizing *in vitro* morphogenesis and ensuring the development of vigorous, true-to-type plantlets.

Effect of IBA on rooting

Rooting response in *E. andamanensis* was significantly influenced by the concentration of IBA in the Orchimax half-strength culture medium. All IBA-supplemented treatments induced root formation, whereas the control showed delayed and weak rooting. Root initiation occurred progressively earlier with increasing IBA concentration, indicating a positive correlation between auxin level and rhizogenesis. The earliest root emergence was observed at 10.30 DAC in 2.0 mg L^{-1} IBA, followed by 12.70 DAC in 1.5 mg L^{-1} IBA, while the control exhibited root initiation only after 35.50 DAC in the rooting medium.

The treatment containing 2.0 mg L^{-1}IBA proved to be the most effective, producing the highest mean number of roots (4.77 per shoot) and the greatest root length (4.40 cm) at 90 DAC. The 1.5 mg L^{-1} IBA treatment resulted in slightly fewer and shorter roots (4.30 roots and 3.95 cm, respectively). In contrast, the control produced only 1.25 roots with an average length of 0.53 cm (Table 6, Fig. 3 & 4). These results indicate that *E. andamanensis* responds optimally to moderately high concentrations of IBA, which promote early root initiation, increased root number and vigorous elongation. The superior rooting at 2.0 mg L^{-1}IBA suggests that this concentration provides a balanced stimulus for root primordia activation while maintaining structural integrity and elongation potential. The auxin IBA is known to enhance pericycle cell division and vascular strand differentiation, resulting in the rapid emergence of thick, elongated and physiologically strong roots. Lower concentrations generally lead to delayed or fewer roots, whereas higher levels may cause basal swelling or callusing, reducing elongation efficiency (30). The well-developed root system obtained at 2.0 mg L^{-1} IBA indicates high functional quality, which is critical for improving acclimatization and post-transfer survival.

One of the most stable and efficient auxins for *in vitro* rooting is IBA, owing to its slower degradation rate and sustained physiological activity compared to IAA or NAA. The superior response of *E. andamanensis* a 2.0 mg L^{-1} IBA agrees with observations in several orchid taxa where IBA outperformed other auxins. In *Dendrobium nobile*, IBA significantly improved rooting efficiency compared to NAA, producing 5.40 roots per shoot after eight weeks (31). Likewise, *Phalaenopsis circus* showed the highest root number (5.67) and length (5.63 cm) when cultured with IBA (32). *Dendrobium thyrsiflorum* exhibited a strong rooting response at 2.0 mg L^{-1} IBA with an average of 4.93 roots (33).

Table 6. Influence of IBA concentrations on days taken for root initiation and number of roots per shoot in *E. andamanensis*

Treatments (mg L^{-1})	Days taken for root initiation	Number of roots after 90 days of culturing in rooting media
T_1- Control	35.50	1.25
T_2- IBA 0.5	16.10	3.56
T_3- IBA 1.0	14.90	3.75
T_4- IBA 1.5	12.70	4.30
T_5- IBA 2.0	10.30	4.77
F-test	**	**
SEm ±	0.34	0.10
CD at 1 %	1.30	0.39

** Significant at p= 0.01.

Comparable results were reported in *Dendrobium heterocarpum*, *D. macrostachyum*, *D. ovatum*, *Pholidota imbricata* and *Polystachya concreta*, where MS half-strength medium with 2.0 mg L^{-1} IBA promoted early and elongated root formation (34). Similarly, 2.4 mg L^{-1} IBA enhanced rooting efficiency in *Cattleya* orchids (35). Species-specific auxin optimization remains crucial for promoting vigorous root development and ensuring successful acclimatization in orchid micropropagation systems. After two months in the greenhouse, plantlets grown in a substrate mixture of red soil, sand, vermicompost and cocopeat (2:1:1:1) exhibited an 85 % survival rate, indicating effective acclimatization and successful establishment under *ex vitro* conditions. The high survival rate can be attributed to the well-aerated and nutrient-rich substrate composition, optimal moisture retention provided by cocopeat,

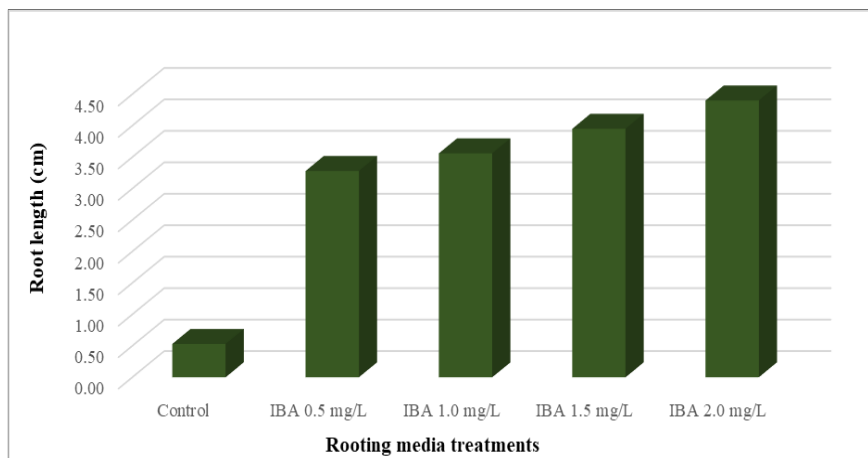

Fig. 3. Effect of IBA concentration on root length (cm) per shoot in *E. andamanensis* at 90 DAC in rooting media.

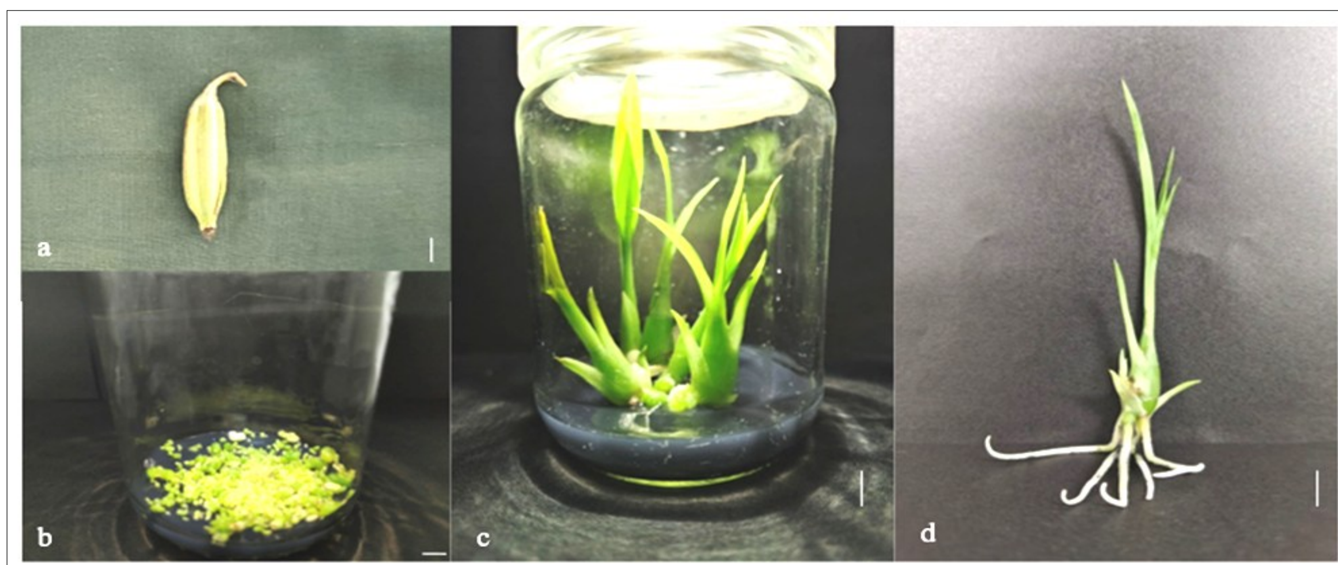

Fig. 4. Asymbiotic seed germination and *in vitro* developmental stages of *E. andamanensis,*(a) Immature seed pod, (b) Seed germination and PLBs formation at 90 DAC on Orchimax full-strength medium, (c) Shoot proliferation at 90 DAC on Orchimax full-strength medium supplemented with BAP (1.0 mg L^{-1}) and NAA (0.5 mg L^{-1}), (d) *In vitro* rooting of regenerated shoots at 90 DAC on Orchimax half-strength medium containing IBA (2.0 mg L^{-1}). Bar = 1 cm.

improved root aeration from the addition of sand and the controlled greenhouse environment that minimized stress and promoted healthy growth.

Conclusion

This study established an efficient, reliable and reproducible *in vitro* regeneration protocol for *E. andamanensis* optimized asymbiotic seed germination, PLB proliferation, shoot multiplication, rooting and hardening were achieved using Orchimax medium. Orchimax full-strength medium promoted rapid germination and PLB formation, while BAP (1.0 mg L^{-1}) and NAA (0.5 mg L^{-1}) enhanced shoot morphogenesis. Rooting was optimal with 2.0 mg L^{-1} IBA, yielding early root initiation and an 85 % survival rate during acclimatization. The standardized protocol enables large-scale propagation and *ex situ* conservation of this rare orchid. Future research should explore cost-effective organic media and native mycorrhizal integration for sustainable conservation.

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Authors' contributions

BGS conducted the lab experiments, collected the primary data, performed the statistical analysis and drafted the original manuscript. PVM reviewed and edited the manuscript and provided resources for the experiment. CS and NN contributed to conceptualization, experimental planning and editing of the original manuscript. NKS designed and conceptualized the experiments. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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