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Research Article

In vitro Root Induction and *Ex vitro* Acclimatization of the Endangered Orchid *Eulophia nuda* Lindl. for Conservation and Large-Scale Propagation

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Abstract

Eulophia nuda Lindl. is a medicinally important terrestrial orchid facing rapid population decline due to overharvesting, habitat degradation, and inherently poor natural regeneration, underscoring the need for efficient propagation strategies. This study developed an optimized and reproducible protocol for *in vitro* root induction and *ex vitro* acclimatization of *E. nuda* plantlets. Regenerated shoots were cultured on half-strength Orchimax medium containing different concentrations of indole-3-butyric acid (IBA) to identify the most effective auxin regime for rooting. Among all treatments, 2.0 mg/L IBA proved optimal, promoting the earliest root initiation (11.10 days), the highest number of roots (4.59), and the greatest root length (4.12 cm) at 90 days after culturing in rooting media. Rooted plantlets were subsequently subjected to a two-stage acclimatization process, wherein primary hardening in sterile cocopeat achieved survival rates exceeding 95%. During secondary hardening, three substrate mixtures were evaluated, and the combination of red soil, sand, cocopeat, and vermicompost (2:1:1:1) resulted in the highest survival rates (86% at 30 days and 83% at 60 days). The integrated rooting and hardening system established in this study significantly enhances plantlet vigour and ex vitro establishment, offering a reliable and scalable protocol for large-scale propagation, conservation, and potential reintroduction of this economically and therapeutically valuable orchid species.

Keywords: Acclimatization; Conservation; Hardening; *In vitro* Rooting; Orchimax Medium

Abbreviations

CD:Critical Difference; DAC: Days After Culturing; *et al.*: And Others; Fig.: Figure; IBA: Indole 3 Butyric Acid; mg/L: Milligram Per Liter; S.Em: Standard Error of Mean

Introduction

The genus *Eulophia* R.Br. ex Lindl. comprises a diverse group of terrestrial orchids distributed across the palaeotropics, many of which possess significant ecological and medicinal value [1].

Among them, *Eulophia nuda* Lindl., commonly known as *manya*, *salam mishri,* or *goruma* is one of the most widely used medicinal orchids in traditional healthcare systems. The species is morphologically distinct, characterized by underground globose pseudobulbs, 2 to 3 oblong-lanceolate leaves, and an erect inflorescence bearing olive-green to brownish flowers with a purple labellum [2]. *E. nuda* is traditionally utilized for treating tumours, glandular swellings, bronchitis, blood disorders, skin infections, rheumatoid conditions, gastrointestinal disturbances, tuberculosis, and snake bites, and is also valued as a vermifuge, appetizer, and aphrodisiac [3]. Phytochemical investigations have revealed phenanthrene-based compounds with potent antioxidant, cytoprotective, and anticancer activities, supporting its medicinal reputation [4,5].

Despite its therapeutic importance, *E. nuda* faces severe threats in the wild. Its intrinsic reproductive constraints, including slow vegetative multiplication and extremely low natural seed germination due to the absence of endosperm, make population recovery difficult [6,7]. These biological limitations, coupled with extensive harvesting of tubers for medicinal use, have resulted in rapid depletion of natural populations, leading to its classification among the endangered orchids requiring urgent conservation attention [8]. As many orchid species are protected under CITES regulations, unsustainable removal from the wild poses a significant risk to long-term species survival [6].

A major research gap persists in the development of reliable propagation systems for *E. nuda*, especially concerning later developmental stages such as *in vitro* root induction and *ex vitro* acclimatization. Although advancements have been made in asymbiotic seed germination techniques since the pioneering work of Knudson in 1922 [9], standardized protocols specifically optimized for *E. nuda* are scarce. Efficient rooting and hardening are critical components for large-scale production, as weak root systems and high mortality during acclimatization remain major barriers in orchid micropropagation [10]. Without robust protocols, reintroduction programs and commercial cultivation remain severely limited. *In vitro* culture represents one of the most promising strategies for both conservation and sustainable utilization of *E. nuda*. Tissue culture overcomes the species' dependence on mycorrhizal fungi, accelerates developmental stages, and allows precise manipulation of plant growth regulators to enhance root formation [8]. Moreover, optimized hardening protocols ensure that regenerated plantlets develop sufficient physiological resilience to survive under natural conditions, an essential requirement for conservation programs and habitat restoration.

Considering the ongoing depletion of natural populations, the medicinal importance of the species, and the lack of consistent rooting and acclimatization protocols, the present investigation aims to establish an efficient *in vitro* rooting system and a reliable hardening method for *E. nuda*. This study contributes essential knowledge to support mass propagation, reduce dependence on wild harvesting, and promote long-term conservation of this valuable orchid.

Materials and Methods

Collection and authentication of plant material

Mature plants of *Eulophia nuda* Lindl. were collected from natural wild populations located near the Shri Ramalingeshwara Swamy Temple forest area, Halasi village, Belagavi district, Karnataka. The freshly collected plant material was immediately transported to the Plant Tissue Culture Laboratory, Department of Horticulture, University of Agricultural Sciences (UAS), GKVK, Bengaluru, to initiate conservation and *in vitro* culture experiments. Species identity was authenticated by Dr. A. N. Shringeshwar (Botanist), Mahatma Gandhi Botanical Garden, UAS, GKVK, Bengaluru. The authenticated accessions were maintained in the orchidarium under controlled environmental conditions with temperature (25 ± 2 °C) and relative humidity (70–80%) were regulated under a shade-net structure to ensure healthy growth and acclimatization. Mature flowers from established plants were hand-pollinated to obtain viable capsules. The harvested seeds were surface-sterilized and cultured on full-strength Orchimax medium for asymbiotic germination. The resulting seedlings developed into vigorous shoots, which served as explants for these experiments. The cultures were maintained under cool white fluorescent lamps providing a light intensity of 30–40 µmol m^{-2} s^{-1} (≈2000 lux) with a 16-hour photoperiod at 24 ± 2 °C.

Effect of Indole-3-Butyric Acid (IBA) on in vitro rooting of *E. nuda*

Regenerated shoots obtained from the shoot multiplication experiment were carefully excised and transferred to rooting media containing half-strength Orchimax solid medium supplemented with different concentrations of Indole-3-butyric acid (IBA). A control treatment without IBA was included for comparison, as shown in Table 1. Each medium contained 20 g/L sucrose, 6 g/L agar and 2 g/L activated charcoal. Data recorded were days taken for root initiation, number of roots, root length after 90 days of culturing in rooting media with 5 treatments and 10 replications.

Table 1: Rooting media composition and Indole-3-butyric acid (IBA) concentrations used for *in vitro* rooting of *E. nuda* shoots.

Treatments	Media	Concentrations
T_1	Half-strength Orchimax solid media	Control
T_2		IBA 0.5 mg/L
T_3		IBA 1.0 mg/L
T_4		IBA 1.5 mg/L
T_5		IBA 2.0 mg/L

Effect of different substrate mixtures on secondary hardening of *E. nuda*

After 90 days of rooting, well-developed plantlets were removed from the culture vessels. Adhering medium was removed by gently washing the roots with sterile distilled water. Plantlets were then dipped in a Bavistin (0.5 % for 3 min) solution to prevent fungal contamination during acclimatization.

Primary hardening

Plantlets were transferred aseptically into autoclaved polypropylene bags containing sterile cocopeat. The bags were sealed to create a high-humidity microenvironment and maintained in a growth chamber with controlled temperature and light. Over a period of one month, ventilation was gradually increased by making small perforations in the bag to help the plantlets acclimatize to ambient humidity and temperature.

Secondary hardening

After primary hardening, the plants were shifted to pots containing different substrate combinations for a secondary hardening trial (Table 2). Plant survival percentage was recorded at 30 and 60 days after transfer into Secondary hardening media, having 3 treatments and 10 replications

Table 2: Composition of various substrate mixtures used for secondary hardening of *in vitro* regenerated plants.

Treatments	Combinations
T_1	Red soil+ Sand + cocopeat (2:1:1)
T_2	Red soil+ Sand + vermicompost (2:1:1)
T_3	Red soil+ Sand + vermicompost + Cocopeat (2:1:1:1)

Statistical analysis

The experiment was carried out using a Completely Randomized Design (CRD) to assess the effects of various IBA concentration and substrate mixtures. Data were analyzed using OPSTAT software, and the significance of differences was determined using an F-test at the 1% level.

Results and Discussion

Effect of varied concentration of IBA on in vitro rooting in *E. nuda*

Rooting represents a critical phase in the *in vitro* propagation of terrestrial orchids, as it directly affects plantlet survival and successful acclimatization. A well-developed root system enhances water and nutrient absorption, provides better anchorage in the soil, and supports mycorrhizal associations essential for establishment in natural habitats. Inadequate rooting often leads to transplant shock and high mortality during hardening, highlighting the importance of auxin-mediated induction using IBA or NAA for effective transfer to *ex vitro* conditions [11]. Among auxins, indole-3-butyric acid (IBA) has been shown to effectively promote *in vitro* rooting in orchids [12].

The rooting response was strongly influenced by IBA concentration, with higher levels accelerating root initiation, likely due to enhanced auxin-mediated cell differentiation and elongation in root primordia. The 2.0 mg/L IBA treatment induced the earliest rooting at 11.10 days after culturing (DAC) in rooting media, followed by the 1.5 mg/L IBA treatment, which saw root emergence at 13.30 DAC, whereas the absence of IBA in the control delayed root formation to 38.20 DAC (Figure 1), highlighting the critical role of exogenous auxins in promoting *in vitro* root development.

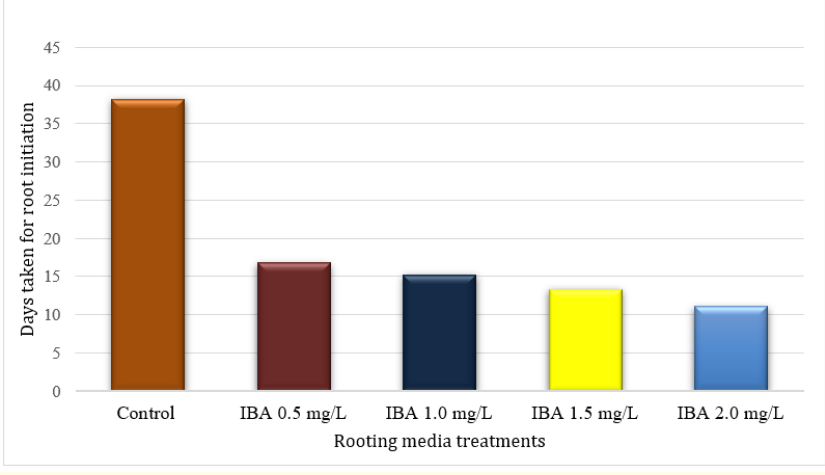

Figure 1: Effect of IBA on days taken for root initiation in *E. nuda*.

The data revealed a clear positive correlation between indole-3-butyric acid (IBA) concentration and root production (Table 3). The highest root number was recorded in the 2.0 mg/L IBA treatment, producing 4.59 roots at 90 days after culturing (DAC), followed by 1.5 mg/L IBA with 4.15 roots at 90 DAC. In contrast, the control lacking IBA exhibited a markedly lower root count of 1.05 at 90 DAC, possibly due to the absence of exogenous auxin, which is essential for initiating and stimulating root development *in vitro*. The maximum root length was observed in the 2.0 mg/L IBA treatment, reaching 4.12 cm at 90 DAC, followed by 1.5 mg/L IBA with 3.84 cm. The control group produced the shortest roots, measuring only 0.45 cm at 90 DAC (Figure 2 and Figure 3). The enhanced rooting observed at 2.0 mg/L IBA indicates that this concentration offers an optimal stimulus for activating root primordia while supporting structural development and elongation. IBA promotes pericycle cell division and differentiation of vascular tissues, leading to the rapid formation of thick, elongated, and physiologically robust roots. The well-developed root system obtained at 2.0 mg/L IBA indicates high functional quality, which is critical for improving acclimatization and post-transfer survival.

Table 3: Effect of different concentrations of Indole-3-Butyric Acid (IBA) on the number of roots of *E. nuda.*

Treatments	Number of roots
T_1- Control (no IBA)	1.05
T_2- IBA 0.5 mg/L	3.52
T_3- IBA 1.0 mg/L	3.65
T_4- IBA 1.5 mg/L	4.15
T_5- IBA 2.0 mg/L	4.59
F-Test	**
S. Em.±	0.08
CD at 1%	0.28

** Significant at p = 0.01.

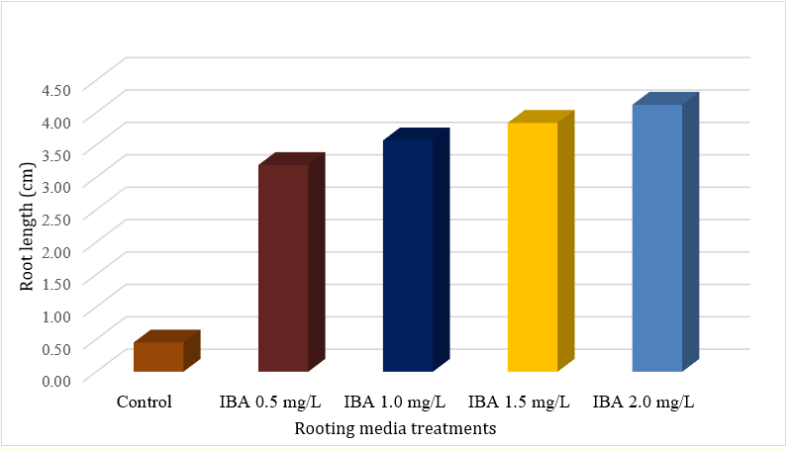

Figure 2: Effect of different concentrations of IBA on the root length (cm) of *E. nuda*.

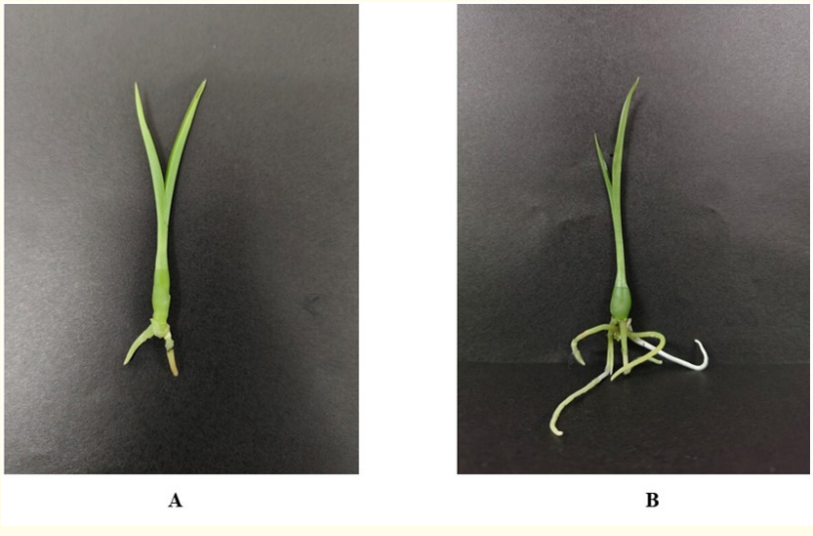

Figure 3: Root growth in *E. nuda* at 90 days after culturing in A) T1-Control and B) T5- Half strength orchimax media + 2.0 mg/L IBA.

IBA is considered one of the most stable and effective auxins for *in vitro* rooting due to its slower degradation and prolonged physiological activity compared to IAA or NAA. The superior rooting response of *E. nuda* at 2.0 mg/L IBA is consistent with observations in other orchid species. For instance, *Dendrobium thyrsiflorum* exhibited a strong rooting response at 2.0 mg/L IBA, producing an average of 4.93 roots [13]. In *Dendrobium nobile*, IBA significantly enhanced rooting efficiency compared to NAA, yielding 5.40 roots per shoot after eight weeks [14]. Similarly, in *Phalaenopsis circus*, IBA supplementation resulted in the highest root number (5.67) and root length (5.63 cm) [15]. Similar results have been reported in *Dendrobium heterocarpum* Wall. ex Lindl., *Dendrobium macrostachyum* Lindl., *Dendrobium ovatum* (L.) Kraenzl., *Pholidata imbricata* Lindl., and *Polystachya concreta* (Jacq.) Garay H.R. Sweet, where half-strength MS medium supplemented with 2 mg/L IBA exhibited the best overall performance, including early root initiation, maximum root number, and longer roots, compared to lower IBA concentrations [16]. The optimal concentration of indole-3-bu-

tyric acid (IBA) for effective root induction differs widely among genera, reflecting inherent variations in hormonal sensitivity, endogenous auxin content, and physiological responses unique to each taxonomic group. Therefore, propagation protocols need to be specifically optimized for each genus to achieve maximum rooting efficiency and quality.

Effect of different substrate mixtures on the survival rate of *in vitro* regenerated *E. nuda*

Hardening is a critical stage in acclimatizing *in vitro*–raised plants for successful establishment under *ex vitro* conditions. The gradual reduction of humidity and stepwise increase in light intensity facilitate the formation of a functional cuticle, the restoration of normal stomatal behaviour, and the development of a stronger root system. These physiological adjustments enable plantlets to transition from a protected, sugar-enriched *in vitro* environment to external conditions where they must independently photosynthesize, regulate water loss, and withstand environmental fluctuations [17]. During primary hardening under laboratory conditions, *E. nuda* exhibited over 95% survival rate.

The survival of *Eulophia nuda* plantlets during secondary hardening was strongly influenced by substrate composition. The mixture of red soil, sand, vermicompost, and cocopeat (2:1:1:1 v/v) resulted in the highest survival rates, with 86% at 30 days and 83% at 60 days after secondary hardening. This was followed by red soil, sand, and vermicompost (2:1:1 v/v), which recorded 78% and 75% survival, while red soil, sand, and cocopeat (2:1:1 v/v) exhibited the lowest survival of 70% and 68% at 30 and 60 days (Figure 4), respectively. These observations suggest that the inclusion of both vermicompost and cocopeat may have enhanced acclimatization and improved plantlet survival.

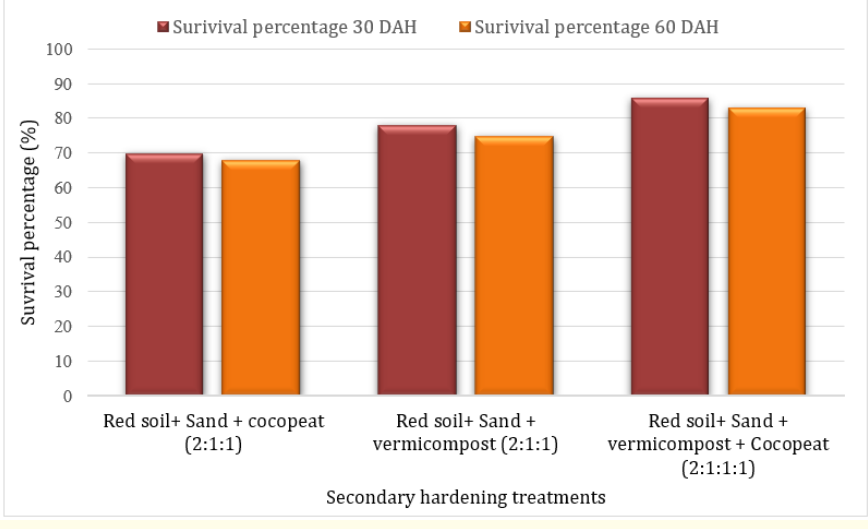

Figure 4: Survival percentage (%) of *E. nuda in vitro* plants on different substrate mixtures at 30, 60 days after secondary hardening.

The superior performance of the red soil: sand: cocopeat: vermicompost mixture may be attributed to the synergistic effects of its components. Red soil likely provided a stable matrix and essential nutrients, with its clay fraction possibly aiding water retention for the delicate plantlets. Sand may have facilitated adequate aeration and drainage, preventing waterlogging and root rot. Cocopeat could have contributed to balanced moisture retention and porosity, ensuring a steady water supply without suffocating the roots. Vermicompost may have supplied slow-release nutrients and beneficial microbes, supported sustained growth and potentially protecting the plantlets from pathogens. Collectively, this substrate facilitated a smoother transition from the sterile *in vitro* environment to the more challenging *ex vitro* conditions, resulting in higher survival rates.

Comparable survival was reported in *Eulophia nuda* plantlets, which exhibited 70% survival following gradual acclimatization under greenhouse conditions. Initially maintained in bottles containing Soilrite, the plantlets were subsequently transferred to earthen pots with a soil mixture of clay, sand, and vermicompost, where they displayed normal growth post-acclimatization [18]. Similarly, *Eulophia cullenii* seedlings showed 79% survival after transfer to community pots containing sand and farmyard manure [19].

Conclusion

This study successfully developed an efficient protocol for *in vitro* root induction and *ex vitro* acclimatization of *Eulophia nuda*. The optimal rooting response was achieved with 2.0 mg/L IBA, which significantly enhanced root initiation, root number, and root length. A substrate mixture comprising red soil, sand, cocopeat, and vermicompost proved most effective for secondary hardening, ensuring the highest plantlet survival. The combined rooting and acclimatization approach provides a robust platform for large-scale propagation and conservation of this endangered orchid. These findings offer valuable insights for sustainable utilization and restoration efforts aimed at protecting natural populations of *E. nuda*.

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