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RESEARCH ARTICLE

Asymbiotic In vitro Seed Germination and Seedling Development of *Eulophia nuda* Lindl., An Endangered Medicinal Orchid

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Abstract An optimized method was devised for asymbiotic seed germination, seedling development and field establishment of *Eulophia nuda* Lindl., an endangered orchid with tremendous therapeutic potentialities. Seeds of different ages were germinated on half and full strength MS (Murashige and Skoog), KC (Knudson) and BM1 (Van Waes and Debergh) media. Amongst various supplements,15 % coconut water (CW) and 500 mg/L casein hydrolysate proved beneficial for seed germination. BM1 fortified with CW (BM1 + CW) showed the highest germination (80.5 ± 2.1 %) after 60 days of inoculation, which was further improved to 91.3 ± 1.9 % upon incorporation of 1 mg/L each of α-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP) and eventually 50.5 ± 2.1 % cultures developed into seedlings. BM1 + CW nourished with 2.5 mg/L BAP and 1.5 mg/L Kin (kinetin) proved best for multiple shoot induction from seedlings. Shoot numbers further increased with each successive transfer onto the same medium. Sixty shoots per shoot clump were achieved after four cycles of 30 days each. Microshoots were rooted (81.3 ± 2.4 %) on MS containing 2.0 mg/L indole-3-butyric acid (IBA) and 200 mg/L activated charcoal. Rooted plantlets were gradually acclimatized to greenhouse (70 % survival) and they exhibited normal morphology and growth characteristics. Flow-cytometry based DNA content analysis revealed that the ploidy levels were maintained in in-vitropropagated plants.

Keywords Orchids · Asymbiotic seed germination · Ploidy · Flow cytometry · Scanning electron microscopy

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Introduction

Orchids represent a peak in plant evolution and uphold supremacy amongst the ornamentals due to their incredible diversity and fascinating long-lasting flowers besides their horticultural and medicinal values [1–4]. However, large proportion of orchid species in wild is under threat due to unscrupulous collection, habitat destruction, degradation, loss of pollinators [2, 5]. In nature, germination rate of orchids is extremely low due to suppressed endosperm and lack of nutrients and therefore requires highly specialized symbiotic fungal association to fulfill their nutritional requirements during germination and early development [3, 6]. Therefore, in situ conservation of dwindling populations of threatened orchid species is a difficult task which presses the need for viable alternatives for their live-stock maintenance, multiplication and conservation [4]. An efficient method for asymbiotic in vitro seed germination and propagation might thus prove to be crucial for mass multiplication, germplasm conservation and production of bioactive compounds of medicinally important orchid species under threat. In vitro seed germination on a suitable culture medium under controlled conditions has been positively tested in a number of commercial and threatened orchid taxa [6, 7].

Eulophia (Orchidaceae) is a large pan-tropical genus of 217 species [8], represented by 24 species in India [9]. *Eulophia nuda* Lindl.[Synonym: *Eulophia spectabilis* (Dennst.) Suresh] is a highly medicinal perennial orchid with underground tubers, found in central and south east Asian regions. In India, this plant is found in tropical Himalayas, from Nepal to Assam, and in Deccan from Konkan southwards. Its tubers are used against tumors, scrofulous glands of the neck, bronchitis, blood diseases, skin rash, rheumatoid arthritis, acidity, piles and stomach complaints, snake bite, tuberculosis, besides being used as a vermifuge, appetizer and an aphrodisiac agent [10–13]. A phenanthrene derivative 9,10-dihydro-2,5-dimethoxyphenanthrene-1,7-diol isolated by authors group from its tubers has shown noteworthy anti-proliferative activities against human cancer cells [14]. The tubers have also shown prominent antioxidant potential and DNA damage protecting activities [15]. Owing to its high therapeutic values, natural populations of *E. nuda* are under serious threat from over-exploitation. As a consequence this orchid has been reported as endangered [16] and seeks immediate steps towards its conservation.

Asymbiotic seed germination is considered as the most straight-forward method to produce large number of propagules [17]. Therefore, authors are reporting herein for the first time a reproducible, standardized method for in vitro asymbiotic seed germination and mass propagation of therapeutically important endangered orchid *E. nuda.* Various parameters including age of capsule, type of media and compositions, use of phytohormones and additives were optimized. The ploidy levels of in vitro regenerated plants were analyzed using flow cytometry based analysis by comparing their nuclear DNA content with the field-grown mother plants to confirm their true-to-typeness with the latter.

Material and Methods

Plant Collection, Capsule Preparation and Sterilization

Entire plants with capsules of *E.nuda* were collected from Western Ghat region of Belgaum, Karnataka, India (latitude 15°41.09′, longitude 74°25.02′ and 723 ma.s.l. altitude) during July 2012. The botanical identity was authenticated by the Botanical Survey of India, Pune (BSI/WC/Tech/2012/244).The mother plants were maintained in greenhouse and the capsules were hand-pollinated. Closed capsules with different maturity stages, including immature-green at 5 months after pollination (MAP), yellowish green at 6 MAP and mature-yellow at 7 MAP were used for germination. Initially, capsules were treated with 2 % NaOCl and 2–3 drops of Tween-20 (HiMedia, India) for 15–20 min and washed with distilled water. Capsules were then treated with 0.1 % bavistine (BASF, India) and 0.1 % streptomycin (HiMedia, India) for 15 min followed by 70 % ethanol for 60 s and finally disinfected with 0.1 % $HgCl_2$ (HiMedia, India) for 5 min in laminar air flow hood. After each treatment, the traces of chemicals were removed from capsules by rinsing three times with sterilized distilled water.

Asymbiotic Seed Germination and Seedling Development

Surface sterilized capsules of various stages were dissected transversally and longitudinally, into four sections in a sterile petri plate to scoop out the seeds using sterilized dissection needles. Total 13 asymbiotic seed germination media compositions as given in Table 1 were used for selecting optimal media for germination. Additionally, various plant growth regulators (PGRs) were also tried for germination, including indole-3-acetic acid (IAA), α-naphthaleneacetic acid (NAA), 6-benzylaminopurine (BAP) and kinetin (Kin) (all Sigma–Aldrich, Germany) with concentrations varying from 0.5 to 2.5 mg/L, either singly or in auxin-cytokinin combinations on BM1 + CW medium. Forty mL medium of each composition was dispensed per glass bottle (125 × 55 mm) and autoclaved for 20 min. The cultures were incubated at 25 ± 2 °C under 16-h photoperiod with 30–40 μm/m^2/s light intensity and 80–85 % relative humidity. All cultures were transferred to fresh media after every 30 days and responses were observed.

Seed germination process was also observed using scanning electron microscopy (SEM). The SEM was carried out under high vacuum conditions using 20 kV FEI-QUANTA-200 Scanning Electron Microscope equipped with luminescence fluorescence detector to examine seed germination stages including embryo swelling, seed coat breaking, PLB formation and shoot emergence.

Shoot Multiplication

In order to achieve prolific shoot multiplication, 30 days old asymbiotically germinated seedlings (5–10 mm) were transferred to BM1 + CW supplemented with varying concentrations (0.0–2.0 mg/L) of BAP and Kin either individually or in various combinations.

Rooting and Hardening

For rooting, elongated shoots of 5–6 cm with 2–3 leaves were detached from shoot clumps and transferred to various root induction media comprising MS, MS + A, BM1 and BM1 + CW, each containing 200 mg/L activated charcoal (AC, HiMedia, India) with or without 0.0–2.5 mg/L

Table 1 Different media compositions used for asymbiotic seed germination of *E. nuda*

Medium	Composition
½-MS	½-MS (half strength salts and vitamins), 30 g/L sucrose, 8 g/L agar, pH 5.8
MS	MS (full strength salts and vitamins), 30 g/L sucrose, 8 g/L agar, pH 5.8
MS + A	MS, additives (adenine sulfate, arginine, citric acid each 30 mg $l^{-1}$and 50 mg l^{-1}ascorbic acid), 30 g/L sucrose, 8 g/L agar, pH 5.8
MS + CH	MS, 500 mg/L casein hydrolysate, 30 g/L sucrose, 8 g/L agar, pH 5.8
MS + CW	MS, 15 % coconut water, 30 g/L sucrose, 8 g/L agar, pH 5.8
½-KC	½-KC (half strength salts and vitamins), 30 g/L sucrose, 8 g/L agar, pH 5.8
KC	KC (full strength salts and vitamins), 30 g/L sucrose, 8 g/L agar, pH 5.8
KC + A	KC, additives (adenine sulfate, arginine, citric acid each 30 mg $l^{-1}$and 50 mg l^{-1}ascorbic acid), 30 g/L sucrose, 8 g/L agar, pH 5.8
KC + CH	KC, 500 mg/L casein hydrolysate, 30 g/L sucrose, 8 g/L agar, pH 5.8
KC + CW	KC, 15 % coconut water, 30 g/L sucrose, 8 g/L agar, pH 5.8
½-BM1	½-BM1 (half strength salts and vitamins), 30 g/L sucrose, 8 g/L agar, pH 5.8
BM1	BM1 (full strength salts and vitamins), 30 g/L sucrose, 8 g/L agar, pH 5.8
BM1 + CW	BM1, 15 % coconut water, 30 g/L sucrose, 8 g/L agar, pH 5.8

MS Murashige and Skoog [18], *KC* Knudson [19], *BM1* BM1 terrestrial orchid medium (Van Waes and Debergh [20]. KC and BM1 media were purchased from HiMedia (India)

IBA. Rooted plantlets were removed from MS medium containing 2.0 mg/L IBA and washed in sterile distilled water to remove traces of agar and were then transferred to plastic pots filled with garden soil, vermiculture and sand (1:1:1). Plants were irrigated with ½-strength liquid MS medium. The pots were covered with polyethylene bags to maintain the relative humidity (70–80 %).These pots were maintained at 25 ± 2 °C under 16-h photoperiod with 30–40 μm/m^2/s light intensity for 3 weeks and were gradually shifted to greenhouse conditions in eighth week.

Flow Cytometry Based Ploidy Analysis

Wild and in vitro propagated plants were analyzed to check their ploidy levels by estimating plant nuclear genome sizes (C-values) using a flow cytometer (Attune® Acoustic Focusing Cytometer). Briefly, plant samples were chopped with a sharp razor blade in a petri plate and homogenized, the homogenate was then filtered to remove large debris, stained with propidium iodide (PI), and subjected to flow cytometric analysis. Preparation and staining of nuclei was performed by following the technique given by Galbraith et al. [21]. Flow histograms (PI fluorescence vs. test sample nuclei) were compared for in vitro regenerated and field-grown plants.The data were analyzed using Attune Cytometric Software version 2.0.

Statistical Analyses

Each treatment was repeated thrice with ten replicates. The results are presented as mean ± standard error of three experiments. Mean values were compared for significant differences using Duncan's multiple range test (DMRT) at $P \leq 0.05$. All the statistical analyses were carried out using MSTAT-C software package.

Results and Discussion

Seed Germination and Early Seedling development

Asymbiotic seed germination of orchids is greatly influenced by factors like seed age, nutrient media composition and addition of PGRs [7]. Terrestrial orchids are recalcitrant to germinate and may have a complicated dormancy pattern making them one of the most difficult groups of plants to grow from seeds [22]. In the present investigation, whole plant bearing capsules (Fig. 1a) were collected from wild conditions and maintained in greenhouse. The seeds were extracted from the capsules at varying maturity stages ranging from 5–7 MAP and used for in vitro asymbiotic germination. Among the seeds of different maturity stages, 6 MAP capsule seeds showed maximal germination rate (80.5 ± 2.1 %), after 60 days of inoculation followed by 7 MAP (50.9 ± 2.5 %) and 5 MAP (20.4 ± 0.7 %) on BM1 + CW. The seeds from capsules at 6 MAP (Fig. 1b, c) were therefore used in further experiments. Low germination rate in seeds of 5 MAP capsules might be attributed to the under developed embryo, which failed to absorb nutrients from the medium [23]. Similarly, probable seed coat formation and attenuating nutrient uptake might have lowered the germination in highly matured 7 MAP capsules. High germination rate of 6 MAP capsule seeds indicates the importance of proper embryo stage with thin

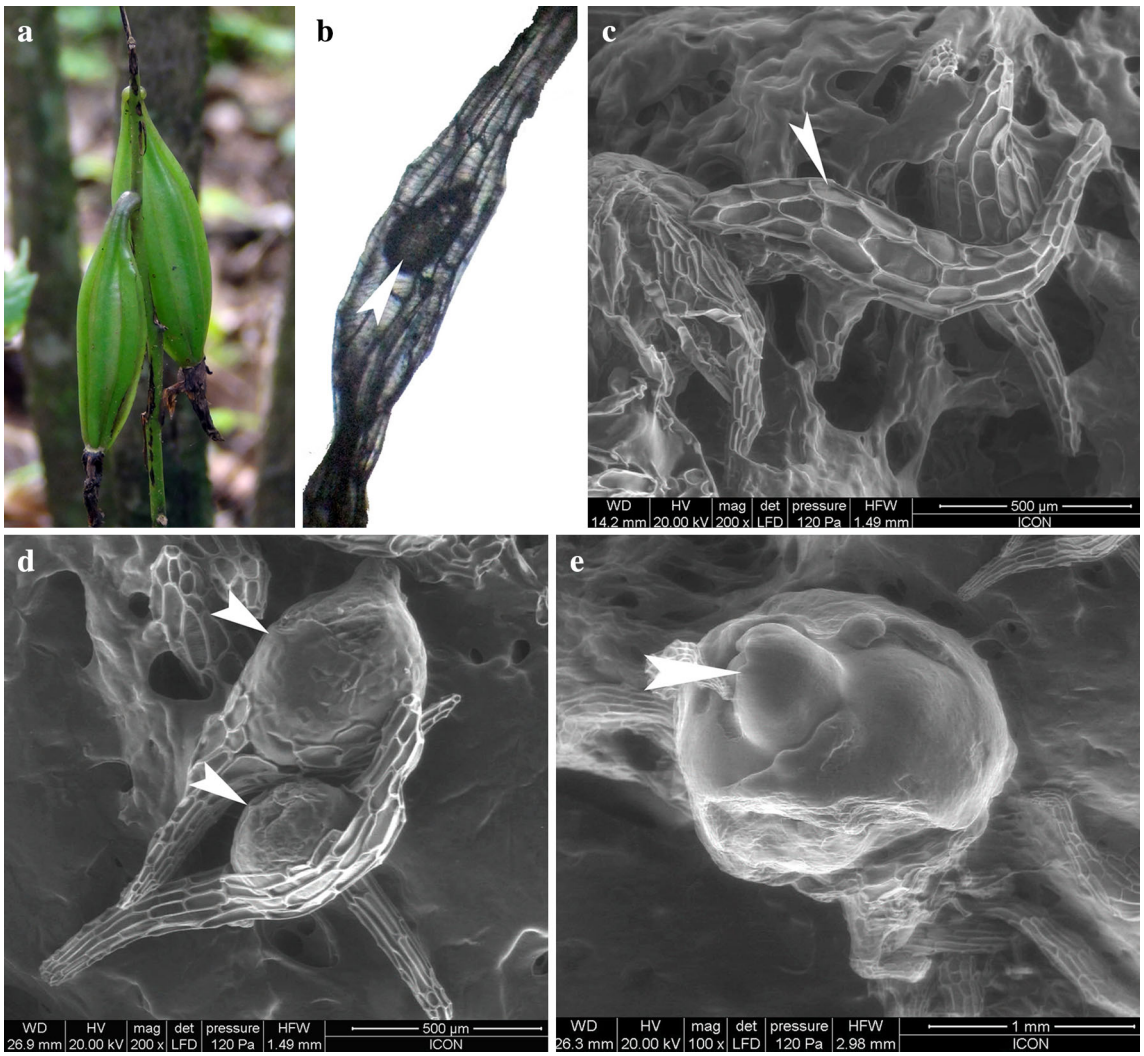

Fig. 1 Asymbiotic germination of *Eulophia nuda* seeds obtained from capsules. **a** Capsules of plants growing in wild conditions, **b** micrograph of seed with embryo (*arrow*) at pre-germination stage, **c** scanning electron micrographs of *E. nuda* seed (*arrow*), **d** scanning electron micrograph showing testa-breaking leading to protocorm formation (*arrows*), **e** shoot emergence from PLBs (*arrow*)

cuticle layer formation for easy nutrient uptake required for in vitro seed germination and seedling development of orchids [24]. Various reports are available where maximal germination have been achieved at various maturity stages, for instance *Paphiopedilum villosum* at 7 MAP [20] and *P.delenatii* at 5 MAP [24]. These results indicated that in vitro orchid seed germination is significantly affected by species-dependent specific maturity stage.

Further, no single medium is applicable universally for orchid cultures owing to their species specific nutrient regime [4]. Three basal media (MS, KC and BM1) selected on the basis of their effectiveness for orchid seed cultures reported previously, were evaluated to select best medium for *E. nuda* germination and seedling establishment. Out of these three media, BM1 showed significantly higher percentage of seed germination than its other two counterparts (Table 2). In addition, various additives and supplements including arginine, adenine sulfate, citric acid and ascorbic acid were tried in combination with MS and KC for optimal seed germination and seedling development. Since, BM1 is a commercially available widely used medium for in vitro orchid propagation (often termed as terrestrial orchid medium) and it essentially contains casein hydrolysate, folic acid, biotin and L-glutamine, additives were not used with this medium. The role of casein hydrolysate as source of organic nitrogen is well established, whereas adenine sulfate acts a source of organic nitrogen, hence the latter was not used as an additive with BM1. Antioxidants viz. ascorbic acid and citric acid were also not used with BM1 owing to the strong antioxidant properties of folic acid. Similarly, L-glutamine is an alternative to arginine and therefore was not used with BM1 unlike an additive to

KC and MS media. Use of additives in germination media did not show any beneficial effect on seed germination.

Interestingly coconut water and casein hydrolysate played a positive role in asymbiotic germination of *E. nuda* seeds. In a previous study, Kumar et al. [25] reported that casein hydrolysate not only promoted germination of *Rhynchostylis retusa* and *Cymbidium elegans* but also reduced the time required for germination. Since BM1 medium has folic acid, glutamine and casein hydrolysate as sources of organic nitrogen besides higher PO_4^{3-} concentration, all these might have been responsible for better germination frequency as compared to other media tried. Positive effects of phosphate on asymbiotic seed germination has been documented earlier [26] and present results are in accordance with these reports. However, contrary to these results, better seed germination has also been observed with inorganic form of nitrogen in a few *Dendrobium* species [27]. This reaffirms the fact that selection of optimal nutrient medium is most crucial for asymbiotic seed germination in orchids presumably because of the differences in the balance and supply of organic and inorganic nutrients [6, 22].

Table 2 Effect of capsule maturity stage, and type and compositions of culture media on seed germination of *E. nuda*

Media	Germination % of seeds		
	5 MAP	6 MAP	7 MAP
½-MS	0.0^{a}	0.0^{a}	0.0^{a}
MS	2.0 ± 0.1^{b}	5.1 ± 0.3^{b}	2.2 ± 0.2^{b}
MS + A	2.2 ± 0.2^{b}	5.0 ± 0.4^{b}	2.4 ± 0.3^{b}
MS + CH	2.1 ± 0.1^{b}	5.3 ± 0.3^{b}	5.5 ± 0.4^{c}
MS + CW	5.1 ± 0.3^{c}	10.4 ± 0.5^{c}	5.7 ± 0.4^{c}
½-KC	0.0^{a}	5.2 ± 0.4^{b}	2.0 ± 0.3^{b}
KC	2.1 ± 0.2^{b}	10.2 ± 0.6^{c}	5.2 ± 0.3^{c}
KC + A	2.2 ± 0.2^{b}	10.4 ± 0.5^{c}	5.5 ± 0.5^{c}
KC + CH	5.0 ± 0.4^{c}	15.6 ± 0.6^{d}	10.7 ± 0.8^{d}
KC + CW	10.4 ± 0.4^{d}	20.7 ± 0.8^{e}	15.3 ± 0.9^{e}
½-BM1	2.2 ± 0.3^{b}	20.5 ± 0.9^{e}	10.2 ± 0.8^{d}
BM1	10.3 ± 0.5^{d}	75.8 ± 1.5^{f}	25.4 ± 1.2^{f}
BM1 + CW	20.4 ± 0.7^{e}	80.5 ± 2.1^{g}	50.9 ± 2.5^{g}

Values represent mean ± standard error of three replicated experiments. Means in a column followed by different superscript letters were significantly different from each other according to DMRT at $P \leq 0.05$

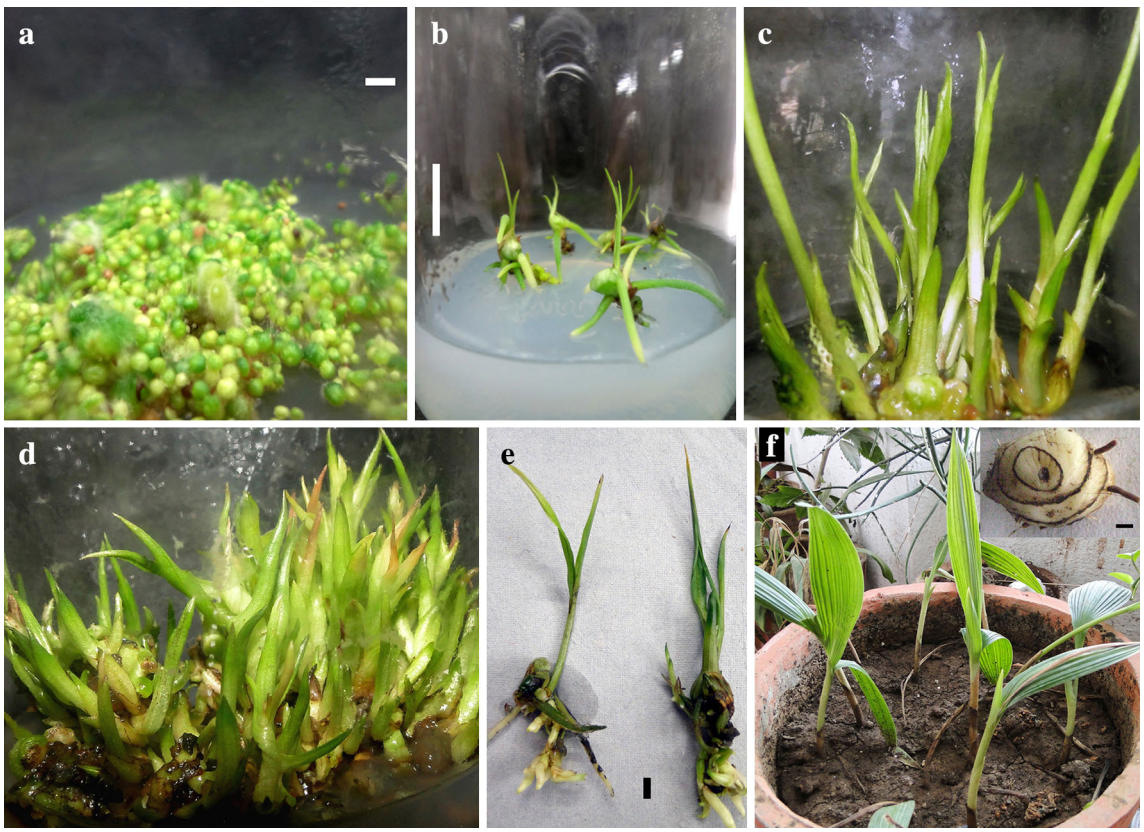

Fig. 2 Asymbiotic seed germination and plant developmental stages of *Eulophia nuda,* **a** Seed germination and PLB formation after 60 days of inoculation on BM1 + CW, **b** protocorm-derived seedling formation on BM1 + CW containing 1 mg/L each of NAA and BAP after120 days of seed inoculation, **c, d** stages of shoot multiplication on BM1 + CW containing 2.5 mg/L BAP and 1.5 mg/L Kin, 60 days after seedling inoculation (c) and further shoot proliferation after two subcultures of 30 days each (d)**, e** in vitro rooting of microshoots on MS medium supplemented with 200 mg/L AC and 2 mg/L IBA, **f** acclimatized plants of *E. nuda* in greenhouse, *inset*: tuber of *E. nuda*. Bar = 1 cm

Table 3 Effect of different growth regulators incorporated singly and in combinations in BM1 + CW medium on germination and early seedling development of *E. nuda*

PGRs				Germination percentage[$]	Percentage of cultures developed into seedlings*	Shoot length (mm)
NAA	IAA	BAP	Kin			
–	–	–	–	80.1 ± 1.8[a]	15.5 ± 0.9[a]	4.3 ± 0.13[a]
0.5	–	–	–	82.9 ± 3.4[d]	15.8 ± 0.7[a]	5.2 ± 0.21[b,c]
1.0	–	–	–	85.8 ± 2.7[e,f]	20.2 ± 1.1[b]	5.4 ± 0.20[c]
–	0.5	–	–	81.7 ± 2.2[c]	15.3 ± 0.9[a]	5.1 ± 0.19[b,c]
–	1.0	–	–	82.5 ± 1.9[d]	21.3 ± 0.9[c]	5.5 ± 0.31[c]
–	–	0.5	–	79.8 ± 2.4[b]	25.2 ± 1.1[d]	5.9 ± 0.22[d]
–	–	1.0	–	80.2 ± 2.5[b,c]	30.3 ± 1.2[e]	5.5 ± 0.30[c]
–	–	–	0.5	80.4 ± 2.6[b,c]	19.6 ± 0.9[b]	4.8 ± 0.18[b]
–	–	–	1.0	81.8 ± 3.1[c]	25.5 ± 0.8[d]	4.9 ± 0.21[b]
0.5	–	0.5	–	84.6 ± 2.7[e]	40.2 ± 1.4 [g]	6.1 ± 0.32[d]
0.5	–	1.0	–	85.2 ± 2.1[e,f]	41.6 ± 1.5 [h]	7.3 ± 0.33[f]
1.0	–	0.5	–	87.5 ± 1.8 [g]	39.9 ± 1.5 [g]	7.9 ± 0.24 [g]
1.0	–	1.0	–	91.3 ± 1.9 [h]	50.5 ± 2.1 [h]	12.2 ± 0.31 [h]
0.5	–	–	0.5	75.2 ± 2.3[a]	25.4 ± 0.8[d]	5.0 ± 0.22[b]
0.5	–	–	1.0	79.4 ± 2.6[b]	29.8 ± 1.0[e]	5.3 ± 0.27[c]
1.0	–	–	0.5	80.3 ± 2.8[b,c]	25.3 ± 0.9[d]	5.6 ± 0.29[c,d]
1.0	–	–	1.0	81.8 ± 2.1[c]	29.9 ± 0.9[e]	6.2 ± 0.21[d]
–	0.5	0.5	–	84.7 ± 2.2[e]	25.5 ± 1.1[d]	5.3 ± 0.17[c]
–	0.5	1.0	–	85.4 ± 2.3[e,f]	29.7 ± 0.9[e]	5.7 ± 0.19[c,d]
–	1.0	0.5	–	85.8 ± 3.5[e,f]	31.1 ± 1.2[e,f]	5.9 ± 0.15[d]
–	1.0	1.0	–	84.9 ± 3.2[e]	39.8 ± 1.2 [g]	6.1 ± 0.13[d]
–	0.5	–	0.5	78.8 ± 2.3[b]	25.6 ± 0.9[d]	5.1 ± 0.14[b,c]
–	0.5	–	1.0	81.6 ± 2.1[c]	29.9 ± 1.0[e]	6.2 ± 0.14[d]
–	1.0	–	0.5	80.5 ± 2.2[b,c]	30.2 ± 1.2[e]	6.6 ± 0.19[e]
–	1.0	–	1.0	79.6 ± 2.1[b]	30.9 ± 1.3[e,f]	7.2 ± 0.21[f]

[$] Germination percentage after 60 days of inoculation; * seedlings with a pair of leaves after 120 days of inoculation. Germination percentage was calculated at first stage (globular embryo), which was eventually followed by various developmental stages, namely seed-coat split, formation of protocorm followed by emergence of leaf primordia and lastly development of seedlings with pair of leaves.Values represent mean ± standard error of three replicated experiments. Means in a column followed by different superscript letters were significantly different from each other according to DMRT at $P \leq 0.05$

BM1 fortified with coconut water (BM1 + CW) was also proved to be the best for seedling development including protocorm formation followed by leaf primordium and ultimately the leaf development (detailed data not shown). Seed germination started with embryo swelling after 2 weeks followed by splitting of seed coat in next week. The oval shaped protocorm emerged in fourth week of inoculation from 6 MAP capsule on BM1 + CW medium (Fig. 1d and 2a) followed by shoot emergence (Fig. 1e). Leaves and roots were seen after 8 weeks which ultimately developed into plantlet.

Effect of Growth Regulators on Seedling Growth and Development

In order to formulate an optimal medium composition for abiotic seed germination, seedling development and shoot multiplication in *E. nuda*, various PGRs (auxins and cytokinins) were tested individually as well as in combinations. Both the auxins showed better seed germination than cytokinins when used singly (Table 2). However, protocorm formation occurred on IAA and remained green for 3 to 4 months but failed to develop further into seedlings. Similarly, protocorms developed on NAA became yellowish and eventually turned brown after 4 months of inoculation. These results suggested that auxins did not promote differentiation of germinated orchid seeds. Among two cytokinins tested individually, BAP showed highest seedling development response (30.3 ± 1.2 % with average shoot length of 5.5 ± 0.3 cm in comparison with Kin with 25.5 ± 0.8 % culture response and 4.9 ± 0.21 cm average shoot length).Current results are in agreement with those of previous assumptions that cytokinins encourage seed germination and subsequent differentiation [25]. However, out of all

Table 4 Effect of different concentrations of cytokinins used in combination on multiple shoot induction from in vitro germinated seedlings of *E. nuda* on BM1 + CW culture medium after 60 days of inoculation

Cytokinins (mg/L)		Percentage response	Number of shoots per seedling	Shoot length (cm)
BAP	Kin			
–	–	40.0 ± 1.2^{a}	1.0 ± 0.0^{a}	2.1 ± 0.1^{a}
0.5	0.5	60.2 ± 2.3^{c}	1.2 ± 0.1^{a}	3.4 ± 0.3^{b}
1.5	0.5	49.8 ± 1.7^{b}	1.4 ± 0.1^{a}	$3.7 \pm 0.4^{b,c}$
2.5	0.5	61.0 ± 2.6^{c}	$1.5 \pm 0.2^{a,b}$	3.9 ± 0.4^{c}
0.5	1.5	60.3 ± 3.1^{c}	2.0 ± 0.2^{b}	3.9 ± 0.2^{c}
1.5	1.5	60.7 ± 2.7^{c}	3.1 ± 0.2^{c}	4.3 ± 0.3^{d}
2.5	1.5	69.6 ± 2.8^{d}	4.2 ± 0.3^{d}	5.2 ± 0.3^{e}
0.5	2.5	49.9 ± 2.4^{b}	2.2 ± 0.1^{b}	3.2 ± 0.2^{b}
1.5	2.5	50.2 ± 2.3^{b}	3.2 ± 0.3^{c}	$4.1 \pm 0.2^{c,d}$
2.5	2.5	50.4 ± 1.9^{b}	4.1 ± 0.3^{d}	5.1 ± 0.4^{e}

Values represent mean ± standard error of three replicated experiments. Means in a column followed by different superscript letters were significantly different from each other according to DMRT at $P \leq 0.05$

PGR treatments tested either individually or in combination for seedling development, highest response was recorded on BM1 + CW containing a combination of auxin-cytokinin (NAA plus BAP, both 1 mg/L) where 50.5 ± 2.1 % cultures developed into plantlets with 12.2 ± 0.31 mm microshoots and a pair of leaves after 120 days of inoculation (Table 3, Fig. 2b). Such synergistic effect of NAA and BAP combination has been reported previously for PLB formation and seedling development in orchids [28, 29].

Shoot Multiplication

Combination of BAP and Kin with varying concentrations from 0.5 to 2.5 mg/L resulted in noticeable shoot multiplication and development on BM1 medium containing coconut water (Table 4). While increasing BAP concentration resulted in gradual escalation of shoot length, Kin displayed enhanced number of shoots with slight decrease in shoot length and this pattern was evidenced when these PGRs were used either singly or in their combinations. These results confirmed that cytokinin type, concentration and their combinations are vital for shoot multiplication. Numbers of shoots significantly increased with successive subcultures and after two subcultures of 30 days each, up to 15 shoots per explant were achieved on BM1 + CW containing a combination of 2.5 mg/L BAP and 1.5 mg/L Kin (Fig. 2c). Obtained shoots were divided into four clumps and transferred to the same media, which resulted into 55–60 shoots per clump after 2 subcultures of 30 days each (Fig. 2d). Similar results have been reported previously by Panwar et al. [16] for shoot multiplication of *E. nuda,* though, via cut-tuber explants. However, unlike present results, the authors used higher amount of BAP (10 mg/L) for bud breaking and culture initiation and obtained 16.2 ± 1.2 shoots per shoot clump after transferring them to 2 mg/L BAP plus 1 mg/L Kin. Since, subjecting the cultures to high levels of exogenous PGRs in culture media are known to exert genome instability or variations in ploidy levels; present results hold significance as greater number of shoots have been achieved on moderate levels of PGRs.

Table 5 Effect of various media compositions and IBA on in vitro rooting of shoots of *E. nuda* after 60 days of inoculation

Media	IBA (mg/L)	Number of days required for root initiation	Rooting percentage	Number of roots per shoot	Root length (cm)
MS + 200 mg/L AC	0.0	29	51.8 ± 2.3^{b}	2.3 ± 0.2^{b}	1.1 ± 0.1^{b}
	0.5	21	59.9 ± 1.8^{c}	3.4 ± 0.1^{c}	0.9 ± 0.1^{b}
	1.0	21	60.6 ± 1.9^{c}	3.7 ± 0.2^{c}	1.7 ± 0.1^{d}
	1.5	15	69.9 ± 2.1^{d}	4.3 ± 0.3^{d}	2.5 ± 0.2^{d}
	2.0	14	81.3 ± 2.4^{f}	5.7 ± 0.3^{f}	4.5 ± 0.2^{e}
	2.5	15	72.8 ± 2.5^{e}	5.1 ± 0.3^{e}	3.1 ± 0.2^{e}
BM1 + 200 mg/L AC	0.0	29	26.9 ± 2.1^{a}	1.1 ± 0.1^{a}	0.4 ± 0.1^{a}
	0.5	28	48.7 ± 1.7^{b}	1.2 ± 0.1^{a}	0.5 ± 0.1^{a}
	1.0	28	51.9 ± 1.9^{c}	1.4 ± 0.1^{a}	0.9 ± 0.1^{b}
	1.5	28	61.6 ± 2.1^{d}	2.1 ± 0.2^{b}	1.1 ± 0.2^{b}
	2.0	21	61.9 ± 1.8^{d}	2.5 ± 0.2^{b}	$1.3 \pm 0.1^{b,c}$
	2.5	21	61.8 ± 2.3^{d}	2.5 ± 0.3^{b}	$1.3 \pm 0.2^{b,c}$

Values represent mean ± standard error of three replicated experiments. Means in a column followed by different superscript letters were significantly different from each other according to DMRT at $P \leq 0.05$

In vitro Rooting and Hardening of Plantlets

Overall, MS medium showed better response than BM1, in terms of the time required for root initiation, percentage of culture response, number of roots per shoot and root length (Table 5). The time required for root initiation varied considerably from 14 to 29 days. Better rooting on MS medium might be attributed to the higher inorganic nitrogen content in MS than BM1, besides the carbon source (sugar) was also higher in MS (30 g/L) than BM1 (20 mg/L) which also might have played a role. Interestingly, zinc concentrations varied amongst BM1 and MS, which might have played an important role in root induction efficiency as even minor variations in zinc concentrations in medium can affect the rooting drastically, as described earlier by Castigalione et al. [30].

IBA was evidenced to be crucial for rooting process and rooting efficiency was significantly improved with increasing concentrations of IBA from 0 to 2.0 mg/L. MS media containing 200 mg/L AC and 2 mg/L IBA proved as the best medium composition for rooting, which resulted into root induction from *E. nuda* shoots after 14 days of inoculation with 81.3 ± 2.4 % rooting, with an average of 5.7 ± 0.3 roots and 4.5 ± 0.2 cm root length (Fig. 2e). These results are consistent with the reports of Asghar et al. [31], who showed 97.5 % rooting with an average 4.7 roots of 3.47 cm each at 2 mg/L IBA in *Dendrobium nobile*. The effectiveness of IBA in rooting has been well documented for various medicinal orchids including *Vanilla planifolia* [32] and *Satyrium nepalense* [33]. Activated charcoal was observed to be beneficial for rooting, possibly, owing to its capacity to stimulate rooting by maintaining the pH of medium, increasing the nitrogen uptake and adsorbing the phenolic substances released by the plantlets into the media [34].

Complete plantlets showed 70 % survival rate during their gradual acclimatization to greenhouse conditions after transfer of rooted plantlets to a mixture of garden soil, vermiculture and sand (1:1:1) and irrigation with half strength liquid MS medium (Fig. 2f). Hardened plants did not show detectable variation in morphological or growth characteristics as compared to the field-grown parent plants.

Flow Cytometry Based Ploidy Level Analysis

Acclimatized plants were subjected to ploidy level analysis using DNA flow cytometry, as in vitro propagated plants are often known for their genome instability or variations in ploidy levels. In vitro cultures are often regarded to promote genetic disturbances, which can result in somaclonal variations and therefore control of genome size is desirable [35]. The risk of genetic instability increases further with higher levels of exogenous PGRs used in culture media. The confirmation of genetic fidelity is of particular importance if plants are being used as a source of therapeutic compounds, as the presence, concentration and composition of these metabolites should remain unchanged in in-vitro propagated plants [35, 36]. Therefore, confirming the genetic stability in such plants holds utmost importance. Flow cytometry based nuclear DNA content and genomic size establishment is widely used for confirmation of true-to-typeness of in vitro propagated plants with their field-grown mother plants [35–38]. The results

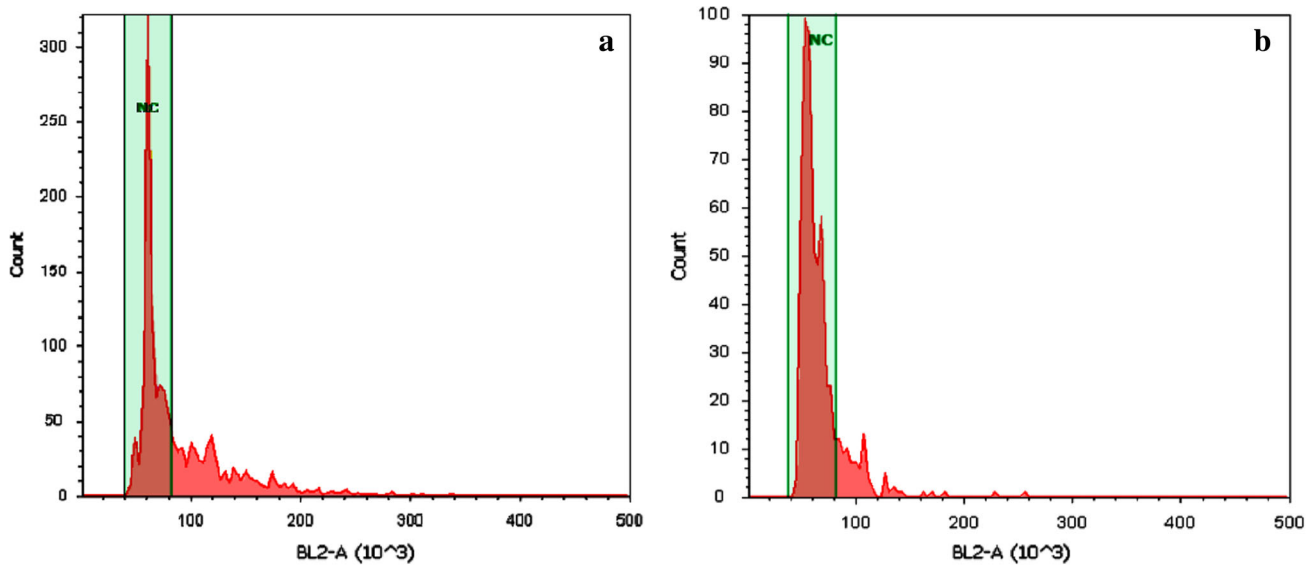

Fig. 3 Histograms of flow cytometric analysis of field-grown **(a)** and in vitro raised plants **(b)** of *E. nuda*

depicted in Fig. 3 a, b revealed that the seeds germinated and plants established through tissue culture maintained their genomic stability with their mother plants, as reveled by similar ploidy levels. The nuclear DNA content was maintained in in-vitro-propagated *E. nuda* plants, which might be attributed to the moderate levels of PGRs used in the present study as was also observed earlier by Thiem et al. [35] in micro-propagated plants of orchid, *Coelogyne cristata*. Likewise, genome size stability has been previously reported in other medicinal plants propagated in vitro including *Oenothera paradoxa*, *Inulaver bascifolia*, *Rubuscha maemorus*, *Solidago virgaurea, S. graminifolia* [37] and *Plantago asiatica* [35].These results reaffirm the usefulness of tissue culture based methods to produce plant material to obtain phytotherapeutics. To the authors knowledge, this is the first report on ploidy level analysis of *E. nuda.*

In conclusion, various parameters including appropriate seed age, culture medium compositions, use of phytohormones, additives and supplements were optimized and accordingly an efficient method has been described for optimal asymbiotic seed germination and subsequent large-scale plantlet regeneration of *E. nuda.* Present results may hold the key for mass multiplication and conservation of this highly important medicinal orchid, under severe threat from over-exploitation. In vitro raised plants were subjected for ploidy analysis to detect their DNA stability in comparison with their wild counterparts. It can be concluded that the in vitro seed raised plantlets maintained their genome stability and true-to-typeness with mother plants.

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