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IN VITRO PROPAGATION OF *COELOGYNE FLACCIDA* LINDL.: AN ORCHID HAVING HORTICULTURAL AND THERAPEUTIC POTENTIAL

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Abstract

Coelogyne flaccida Lindl. is a rare pseudobulbous epiphyte having considerable therapeutic as well as medicinal potential. The present investigation describes a protocol for mass propagation of this conservation reliant orchid. About 95% and 78% germination was recorded on half strength MS basal medium and Knudson C medium fortified with 3% sucrose, 0.8% agar, 10% coconut water, and 0.3% activated charcoal within 4 and 8 wks of culture respectively. Protocorm multiplication was induced on half strength MS basal medium fortified with varying concentrations (0.5-2.0 mgL^{-1}) of TDZ, BAP, and KN along with 0.5 mgL^{-1} NAA and 0.5 mgL^{-1} IBA. A notable increment in shoot number was noticed when protocorm derived explants were exposed to varying levels of additives like adenine sulphate and banana homogenate. Rooting (100%) was obtained on half strength MS basal medium fortified with varying concentrations of IBA (0.5-2.0 mgL^{-1}). After 3 months of initial acclimatization, the seedlings/plantlets exhibited appreciable growth and a healthy survival rate (85%).

Introduction

COELOGYNE LINDL. (Orchidaceae) is a large asiatic genus comprising of 598 species is distributed throughout SouthEast Asia, Sumatra, and the Himalayas (POWO, 2024). *Coelogyne flaccida* Lindl., commonly known as *loose Coelogyne*, is a cool growing forest epiphyte restricted to the subtropical regions of the Himalayas (900-2300 m amsl) (Clayton, 2002). The species blooms from spring to early summer and produces large, heavily scented flowers having considerable horticultural value.

Besides being horticulturally significant, the species has medicinal importance as well. Biologically active compounds like flaccidin, oxaloflaccidin (Phenanthrene derivatives), and stilbinoids like isoflaccidin and isooxoflaccidin have been isolated from this species (Majumdar and Maity, 1989, 1991). Pharmacological studies conducted previously have shown that the species possess antibacterial activity and the paste of its pseudobulbs is utilized for treating headaches. The juice obtained from pseudobulbs is also administered orally for treating indigestion (Pyakurel and Gurung, 2008). Due to its high ornamental value as cut flower and its profound therapeutic importance, natural populations of *C. flaccida* have steadily declined primarily due to over collection of this beautiful species from wild habitats.

Hence, in order to conserve the ever dwindling germplasm and for future commercialization of this conservation reliant medicinally important species, quality planting material on a large scale is an absolute necessity, which in turn requires the development of an easy, rapid, and economically viable propagation method. Some attempts have been made earlier to propagate and conserve a few medicinally and commercially important orchid species using *in vitro* micropropagation techniques (Bhowmik and Rahman, 2022, 2023; Dhillon and Pathak, 2023; Kirti *et al.,* 2023; Lalduhsanga *et al.,* 2021; Mutum *et al.,* 2022; Pathak *et al.,* 2023; Sunita *et al.,* 2021; Thakur and Pathak, 2021; Tripura *et al.,* 2022; Vasundhra *et al.,* 2021). Application of biotechnological methods can offer us an ideal solution for the development of conservation strategies. Keeping all these points into consideration, the present investigation was carried out for the development of a rapid, reliable, and cost effective protocol for *in vitro* asymbiotic seed germination, mass propagation and successful *ex vitro* establishment of *C. flaccida* plantlets.

Material and Methods

Plant Material

Mature *Coelogyne flaccida* plants with immature green capsules were collected from the wild (lower catchment areas of Neora Valley National Park, district: Kalimpong, West Bengal; Elevation: ± 1100m in the month of June). The specimens were identified using standard taxonomic literature and by comparison with herbarium specimens available at the Central National Herbarium (CAL), Botanical Survey of India (Howrah, West Bengal). A voucher specimen was deposited in the Departmental Herbarium of Barasat Government College, West Bengal, India.

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Surface Disinfection of Immature Green Capsules

Immature green capsules were washed thoroughly under running tap water followed by a brief rinse in 2% Teepol solution (for 10 min). This was followed by incubation in 1% (w/v) Carbendazim [2-(methoxycarbamoyl)-benzimidazole] for 20 min. The capsules were finally disinfected with aqueous $HgCl_2$ (0.1% w/v) for 10-12 min in laminar air flow cabinet and eventually rinsed with sterile distilled water (3-4 times). Surface sterilized capsules were longitudinally split into two halves and the dry seeds were scooped out and these were eventually inoculated on semisolid media for aseptic germination.

Media Composition and Culture Conditions

For aseptic germination, the surface sterilized seeds were inoculated on Knudson C (KC) (Knudson, 1951) medium and full as well as half strength Murashige and Skoog's (MS) medium (Murashige and Skoog, 1962). All nutrient media used for germination were fortified with 3% sucrose, 0.8% agar (bacteriological grade), 10% (v/v) coconut water (CW), and 0.3% (w/v) activated charcoal. For induction of multiple shoots, about 12 wks old meristematic mounds (obtained from the conglomeration of protocorms) were inoculated on half strength MS basal medium fortified with varying concentrations (0.5-2.0 mgL^{-1}) of Thidiazuron (TDZ), Benzylaminopurine (BAP), and Kinetin (KN) along with 0.5 mgL^{-1} Naphthalene acetic acid (NAA) and 0.5 mgL^{-1} Indole-3 butyric acid (IBA). In order to accelerate the shoot multiplication rate, protocorm derived meristematic mounds (about 15 wks old) were inoculated on half strength MS basal medium supplemented with the best hormonal combination obtained during the shoot multiplication experiment (a combination of 2 mgL^{-1} TDZ and 0.5 mgL^{-1} NAA was used) along with varying levels of two complex additives [coconut water (5-15%) and banana homogenate (BH) (100-300 mgL^{-1})] and sulphonated form of purine (adenine sulphate/Ads) (100-300 mgL^{-1}).

For induction of roots, half strength MS medium supplemented with varying concentrations (0.5-2.0 mgL^{-1}) of three different auxins *i.e.*, IAA, IBA, and NAA were used. All media used for shoot multiplication and rooting were fortified with 0.8% (w/v) bacteriological grade agar. The pH of the various media used was set to 5.7 by using 1(N) NaOH and 1(N) HCl as required prior to autoclaving at 121°C for 17 min. All chemicals and reagents used were of analytical grade and were obtained from Sigma-Aldrich (USA), E. Merck (Germany), Himedia (Mumbai, India), and Qualigens Fine Chemicals (Mumbai, India).

All cultures were maintained at 22±2°C under a 16/8 hr of photoperiod provided by cool white fluorescent lamps (Phillips, India) and sub culturing was performed regularly after 4/8 wks interval. Photographs were taken using a Zeiss stemi 508 stereo zoom microscope and captured images were processed using ProgRes Capture software package.

Hardening and Ex Vitro Transfer of the Seedlings/ Plantlets

About 24 wks old seedlings/plantlets with well-developed roots were removed carefully from the rooting medium in the post monsoon season (October) and transferred to small earthen pots containing a mixture of sterile charcoal bricks, cocopeat, and Pine wood chips (in the ratio 1:1:1). The pots were covered with plastic in order to maintain the high humidity level. The potted seedlings/plantlets were sprinkled with sterile water thrice a week. The seedlings were maintained in this condition in the greenhouse (27±2°C) for about 12 wks (under 65% RH and 12/12 photoperiod) after which the plastic covers were removed and the seedlings were eventually transferred to 4 inch earthen pots filled with charcoal bricks, coconut husk, and vermiculite (in the ratio 1:1:1); the roots were covered with a layer of moss in order to retain the moisture level. These pots were maintained under partial shade and irrigated with tap water as and when required.

Statistical Analysis

The data was recorded after every 30 days interval for *in vitro* germination. Data related to shoot multiplication were recorded after 4 and 8 wks respectively. Data related to *in vitro* rooting of micro shoots were recorded after 4 wks of culture. All experiments were repeated thrice with 5 explants in each treatment. Each experimental set was in triplicate. All data were subjected to one-way analysis of Variance (ANOVA) using SPSS version 22.0 for Windows (Jinn, 2011). Means were separated using Duncans multiple range test at 5% probability level (Duncan, 1955).

Results and Discussion

During the present study, immature seeds procured from green capsules were used for experimentation (Fig. 1a). The dust like fusiform seeds (120 μm in length and 45 μm in breadth) present within the capsules underwent appreciable swelling and exhibited signs of germination after 3 wks of culturing. Half strength MS medium fortified with 3% sucrose, 0.8% agar, 10% (v/v) coconut water, and 0.3% (w/v) activated charcoal proved to be the best in terms of both onset and frequency of germination (Table 1). Seeds successfully germinated

with 95% frequency on half strength MS basal medium after 4 wks, while the seeds inoculated on the KC medium took an additional 4 wks to germinate. The germination percentage of 78.3% was recorded on the KC medium. No sign of germination was observed on full strength MS basal medium. About 99% and 87% of the swelled embryos developed into globular protocorms in case of half strength MS and KC media, respectively (Table 1).

Initial signs of leaf formation in the protocorms were noticed after about 66 days of inoculation and signs of root emergence were observed after 78 days of inoculation on half strength MS medium. On the other hand, in KC medium, first leaf formation was noticed after 74 days of inoculation and emergence of first root was observed after 90 days of inoculation.

Orchid seeds are known to successfully germinate and develop into seedlings better in nutrient media having low-salt and mineral concentration (Zeng *et al.,* 2015). The role of coconut water in promoting *in vitro* germination of orchid seeds has also been reported previously in *Coelogyne stricta* and *Dendrobium densiflorum* (Pant *et al.,* 2022; Thapa *et al.,* 2020). Symbiotic germination of orchid seeds is a complex phenomenon and has not yet been fully understood. *In vitro* asymbiotic germination on the other hand can provide us a suitable alternative for circumventing constrains encountered during *in vivo* germination. *In vitro* germination of *C. flaccida* seeds have been previously reported by De and Sil (2015) using Orchimax medium supplemented with 1 mgL^{-1} NAA and 15% coconut water. Hormone free Mitra *et al*. (1976) medium has also been utilized by Kaur and Bhutani (2013) for asymbiotic germination of *C. flaccida* seeds. In both these reports, the time taken for germination and subsequent development were much longer (>5 wks). In the present investigation, however, germination took place within 4 wks on half strength MS medium supplemented with 10% CW. The results obtained in the present study also report a much higher germination percentage (> 90%). Development of globular protocorms (~ 1.5 mm in diameter) with distinct absorbing hair at basal ends (Table1; Fig. 1b) took place in the same germination medium (both in half MS as well as in KC medium). This is in accordance with the results obtained previously in *Coelogyne perverula* (Nelson and Rusdi, 2018). The protocorms obtained in half strength MS medium showed rapid multiplication (> 60%) after 8 wks and subsequently a substantial percentage of globular protocorms (with growth of absorbing hair at the basal ends) exhibited a tendency of clumping together to form green, compact, nodular clumps (~ 2-3 mm in diameter) after almost 10 wks of culture (Fig. 1c). These protocorm derived coalesced clumps had 2-3 nodular meristematic mounds on their apical portions (Fig.1d). These meristematic mounds served as organogenic centres in future experiments. A perusal of literature describing micropropagation protocols for different *Coelogyne* species clearly reveals the utility of various explants (*i.e.*, protocorms, foliar explants, pseudobulbs, shoot meristems, seeds, and stem nodal buds) for culture initiation and subsequent regeneration (Abraham *et al.,* 2012; Deb, 2010; De and Sil, 2015; Kaur and Bhutani, 2013; Sil and De, 2015; Singh and Kumaria, 2019; Thapa *et al.,* 2020). The present investigation, however describes the utility of green compact pseudobulb clusters with prominent meristematic mounds (procured from *in vitro* raised cultures) for induction of multiple shoots.

The green meristematic mounds yielded prominent shoot buds when inoculated on half strength MS medium supplemented with varying concentrations of TDZ, BAP, and KN (0.5, 1.0, and 2.0 mgL^{-1}) in separate combinations with 0.5 mgL^{-1} NAA and IBA (Table 2). Hormone free basal medium was unable to evoke any response in the inoculated explants. Out of the three cytokinins used in combination with two different auxins, TDZ proved to be more effective in comparison to both BAP and KN. A concentration of 2 mgL^{-1} TDZ used in combination with 0.5 mgL^{-1} NAA produced nearly 15 and 26 shoots from a single meristematic mound after 4 and 8 wks of culture, respectively (Table 2; Fig. 1f-

Table 1. Effect of different media components on *in vitro* germination and seedling development of *Coelogyne flaccida*.

Nutritional combination	Germination (%)	Wks taken for germination	Wks taken for protocorm	Protocorm formation (%)	Days taken for the 1st leaf formation	Days taken for the 1st leaf formation
½ MS +3% S + 10% CW+0.3% AC	95.00	4 wks	8 wks	99	66	78
KC + 3% S + 10% CW+0.3% AC	78.30	8 wks	12 wks	87	74	90
MS + 3% S + 10% CW+0.3% AC	0 ± 0 a	N.R	N. R	0 ± 0 a	N.R	N. R

Results are Mean ± S.E of 3 replicates; means followed by same letters in each column are not significantly different at 5% probability level using Duncan's multiple range test (DMRT); N.R-no response

g). A combination of 1 mgL^{-1} BAP in combination with 0.5 mgL^{-1} NAA on the other hand, yielded as many as 13 and 25 shoots after 4 and 8 wks of culture (Fig. 1e). Varying levels of KN when combined with 0.5 mgL^{-1} NAA, however, resulted in a lower turnover of shoots (Table 2). When combined with 0.5 mgL^{-1} IBA, varying levels of all three cytokinins yielded a much lower number of shoots. Out of the three cytokinins used, a combination of 2 mgL^{-1} TDZ and 0.5 mgL^{-1} IBA produced 3.26 and 9.30 shoots after 4 and 8 wks of culture, followed by the combination of 1 mgL^{-1} BAP and 0.5 mgL^{-1} IBA (2.26 and 7.32 shoots). On the other hand, a combination of 2 mgL^{-1} KN and 0.5 mgL^{-1} IBA yielded only 1.76 and 6.10 additional shoots from a single meristematic mound after 4 and 8 wks, respectively (Table 2). No sign of root induction could be noticed in any of the hormonal treatments inspite of auxin supplementation. The regenerated shoots in case of all the treatments appeared healthy and exhibited no sign of hyperdydricity.

Ever since the discovery of its stability and cytokinin like activity (even at extremely low concentrations), the substituted phenyl urea compound TDZ has been effectively utilized to induce adventitious shoots, roots, somatic embryos, and calli in numerous plants including orchids. TDZ has been successfully utilized for the induction of multiple shoots in *Coelogyne cristata; Rhynchostylis retusa*, and *Vanda coerulea* (Naing *et al.,* 2010; Seeni and Latha, 2000; Thomas and Michael, 2007. PLB induction followed by shoot multiplication in *Cymbidium sinense* (Chang and Chang, 2000), induction of direct somatic embryos in *Oncidium* (Chen and Chang, 2001), and embryogenic callus induction in *Dendrobium ovatum* (Pyati, 2022) was also been reported.

A remarkable enhancement in shoot number as well as shoot length was noticed when the best shoot multiplication medium (1/2 strength MS + 2 mgL^{-1} TDZ + 0.5 mgL^{-1} NAA) was fortified with varying levels of the complex additive BH (100-300 mgL^{-1}) and the sulphonated form of a nucleotide base [Adenine sulphate (Ads) (100-300 mgL^{-1})]. Out of the three additives used, Adenine sulphate proved to be the most effective one at a concentration of 300 mgL^{-1}; Ads accelerated the shoot multiplication rate remarkably (Table 3) by yielding nearly 23 and 43 additional shoots

Table 2. Effect of growth regulators on shoot multiplication and elongation from protocorm derived meristematic clumps of *Coelogyne flaccida*.

PGR's (mgL^{-1})					Number of shoots obtained after 4 wks	Number of shoots obtained after 8 wks	Mean shoot length in cm (after 8 wks)
TDZ	BAP	KN	NAA	IBA			
0	0	0	0	0	0 ± 0 [a]	0 ± 0 [a]	0 ± 0 [a]
0.5	0	0	0.5	0	12.20 ± 0.3912 [i]	23.72 ± 0.3412 [j]	1.22 ± 0.2728 [de]
1	0	0	0.5	0	13.68 ± 0.3200 [j]	25.20 ± 0.3033 [kl]	2.10 ± 0.2828 [fg]
2	0	0	0.5	0	14.78 ± 0.3680 [k]	26.30 ± 0.3050 [m]	2.70 ± 0.2608 [g]
0	0.5	0	0.5	0	10.26 ± 0.3320 [g]	23.44 ± 0.3265 [j]	1.48 ± 0.3412 [ef]
0	1	0	0.5	0	13.46 ± 0.3415 [j]	25.78 ± 0.3397 [l]	2.46 ± 0.3265 [g]
0	2	0	0.5	0	11.30 ± 0.3406 [h]	24.68 ± 0.3200 [k]	2.12 ± 0.2557 [fg]
0	0	0.5	0.5	0	8.46± 0.3696 [f]	11.60 ± 0.2702 [h]	0.62 ± 0.1985 [abcd]
0	0	1	0.5	0	8.84± 0.3544 [f]	12.18 ± 0.2691 [hi]	0.80 ± 0.2429 [bcde]
0	0	2	0.5	0	9.18± 0.3412 [f]	12.90 ± 0.2345 [i]	1.08 ± 0.2417 [cde]
0.5	0	0	0	0.5	1.86± 0.3628 [bcd]	7.10 ± 0.2933 [e]	0.60 ± 0.1924 [abcd]
1	0	0	0	0.5	2.30± 0.2983 [d]	8.38 ± 0.3184 [f]	0.70 ± 0.1703 [abcd]
2	0	0	0	0.5	3.26 ± 0.3043 [e]	9.30 ± 0.3256 [g]	0.80 ± 0.2280 [bcde]
0	0.5	0	0	0.5	1.30± 0.2627 [bc]	5.30 ± 0.3332 [c]	0.50 ± 0.1414 [abcd]
0	1	0	0	0.5	2.26 ± 0.2337 [cd]	7.32 ± 0.3484 [e]	0.70 ± 0.2214 [abcd]
0	2	0	0	0.5	1.92± 0.2691 [bcd]	6.84 ± 0.3544 [de]	0.58 ± 0.1772 [abcd]
0	0	0.5	0	0.5	1.20± 0.2121 [b]	4.18 ± 0.2634 [b]	0.18 ± 0.0374 [ab]
0	0	1	0	0.5	1.50± 0.1414 [bcd]	5.38 ± 0.2905 [c]	0.28 ± 0.0583 [ab]
0	0	2	0	0.5	1.76 ± 0.1990 [bcd]	6.10 ± 0.2915 [cd]	0.48 ± 0.1281 [abc]

Results are Mean ± S.E of 3 replicates; means followed by same letters in each column are not significantly different at 5% probability level using Duncan's multiple range test (DMRT).

Table 3. Effect of different additives on shoot multiplication in *Coelogyne flaccida.*

CW (%)	Additives Ads (mgL^{-1})	BH (mgL^{-1})	Number of shoots after 4 wks	Number of shoots (after 8 wks)	Mean shoots length in cm (after 8 wks)
5	0	0	7.14 ± 0.2909 [a]	12.6 ± 0.2408 [a]	0.4 ± 0.0894 [a]
10	0	0	8.2 ± 0.3536 [b]	17.88 ± 0.3397 [c]	0.72 ± 0.1855 [ab]
15	0	0	9.48 ± 0.3412 [c]	19.8 ± 0.3536 [d]	0.92 ± 0.1772 [abc]
0	100	0	13.58 ± 0.2557 [d]	27.7 ± 0.3114 [f]	1.54 ± 0.2581 [cd]
0	200	0	15.32 ± 0.3397 [e]	33.58 ± 0.2764 [g]	1.8 ± 0.2 [d]
0	300	0	22.8 ± 0.3332 [g]	42.78 ± 0.3611 [h]	2.54 ± 0.2482 [e]
0	0	100	8.2 ± 0.3256 [b]	16.9 ± 0.3755 [b]	0.74 ± 0.172 [ab]
0	0	200	13.32 ± 0.2835 [d]	23.32 ± 0.3121 [e]	0.96 ± 0.1778 [abc]
0	0	300	16.46 ± 0.3544 [f]	33.22 ± 0.3397 [g]	1.28 ± 0.2764 [bcd]

Results are Mean ± S.E of 3 replicates; means followed by same letters in each column are not significantly different at 5% probability level using Duncan's multiple range test (DMRT)

from a single meristematic mound previously obtained from coalesced protocorms after 4 and 8 wks of culture respectively (Fig. 1i-j). Varying levels of BH also enhanced the shoot number appreciably (Table 3). Application of 300 mgL^{-1} BH in the shoot multiplication media resulted in 16 and 33 shoots after 4 and 8 wks of culture, respectively (Fig. 1h). Varying levels of coconut water (5-15 %) however, was not as effective as Ads and BH and resulted in a much lower turnover of shoot after 4 and 8 wks of culture. As far as the shoot length is concerned, the superiority of Ads over the other two additives was also evident from the results depicted in Table 3. It is also noteworthy to mention here that in all treatments containing varying levels of Ads, rooting took place simultaneously along with multiple shoots within 8 wks of culture. Each shoot cluster possessed at least 5-6 healthy roots with considerable length (Fig.1k). These rooted clusters can be directly transferred to *ex vitro* conditions for hardening and acclimatization, which in turn may shorten the duration of our protocol by circumventing the 4 wks long rooting experiments. Spontaneous rooting was, however, not observed in any other experimental sets containing additives like BH or CW.

Adenine sulphate (known commonly as 6-aminopurine hemisulphate salt) is a purine which serves as a precursor in the cytokinin biosynthetic pathway (Khan

Table 4. Effect of different auxins on *in vitro* root induction, in *Coelogyne flaccida.*

Auxins used (mgL^{-1})			Mean number of roots after		Root length
IAA	IBA	NAA	4 wks	8 wks	(in cm)
0	0	0	0 ± 0 [a]	0 ± 0 [a]	0 ± 0 [a]
0.5	0	0	2.50 ± 0.2309 [c]	4.53 ± 0.2028 [c]	5.24 ± 0.0723 [e]
1	0	0	3.30 ± 0.2082 [cd]	5.50 ± 0.1155 [d]	5.71 ± 0.0551 [f]
2	0	0	3.70 ± 0.1732 [de]	6.27 ± 0.2603 [e]	6.12 ± 0.0681 [g]
0	0.5	0	4.40 ± 0.3055 [ef]	6.30 ± 0.2517 [e]	6.95 ± 0.0713 [h]
0	1	0	4.70 ± 0.1528 [fg]	6.90 ± 0.1732 [f]	7.09 ± 0.0608 [h]
0	2	0	5.40 ± 0.2887 [g]	7.27 ± 0.1453 [f]	8.14 ± 0.0677 [i]
0	0	0.5	1.40 ± 0.1000 [b]	3.23 ± 0.1202 [b]	3.08 ± 0.0578 [b]
0	0	1	2.40 ± 0.4041 [c]	3.67 ± 0.1764 [b]	4.57 ± 0.0669 [c]
0	0	2	2.50 ± 0.5508 [c]	4.37 ± 0.2186 [c]	5.03 ± 0.1026 [d]

Results are Mean ± S.E of 3 replicates; means followed by same letters in each column are not significantly different at 5% probability level using Duncan's multiple range test (DMRT)

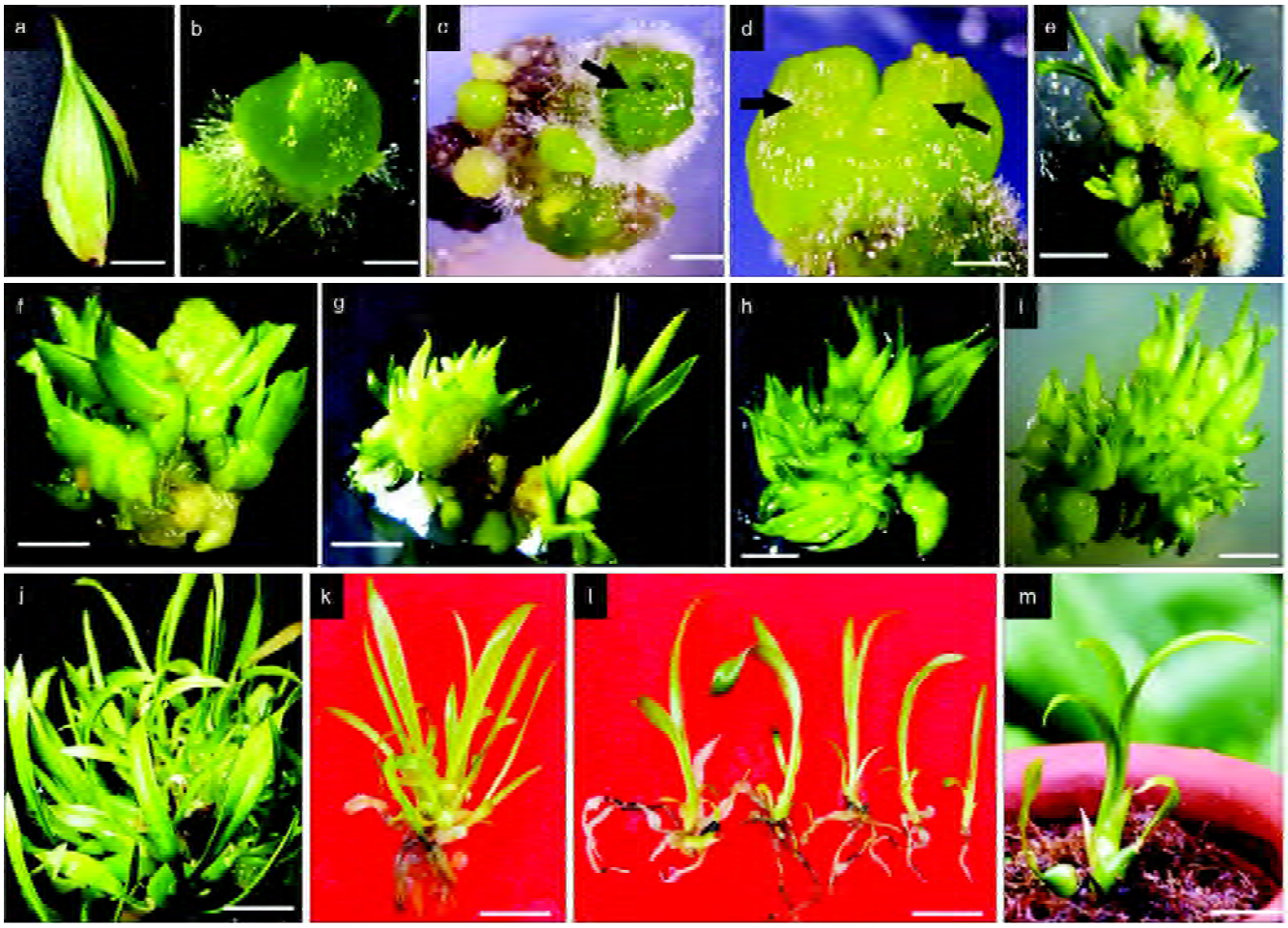

Fig. 1a-m. *In vitro* asymbiotic seed germination and seedling development in *Coelogyne flaccida* a; Green capsule (Scale bar: 1 cm); b, Globular protocorms (obtained after 8 wks) with growth of absorbing hair at basal ends (Scale bar: 10 mm); c, Mass of developing protocorms coalesced together to form a green, compact cluster (after 10 wks) (Scale bar: 10 mm); d, Protocorm clumps with distinct nodular meristematic masses (after 10 wks) (Scale bar: 10 mm); e, A cluster of shoots obtained from a single clump of protocorms (after 8 wks of culture) on half strength MS fortified with 1 mgL^{-1} BAP and 0.5 mgL^{-1} NAA. (Scale bar: 1cm); f, A cluster of shoots obtained from a single clump (after 4 wks of culture) on half strength MS fortified with 2 mgL^{-1} TDZ and 0.5 mgL^{-1} NAA. (Scale bar: 1cm); g, A cluster of shoots obtained from a single clump (after 8 wks of culture) on half strength MS fortified with 2 mgL^{-1} TDZ and 0.5 mgL^{-1} NAA. (Scale bar: 1cm); h, Profuse shoot multiplication from a single coalesced protocorm clump (after 8 wks) on half strength MS+ mgL^{-1} TDZ & 0.5 mgL^{-1} NAA fortified with 300 mgL^{-1} Banana Homogenate (Scale bar: 1cm); i, Profuse shoot multiplication from a single coalesced protocorm clump (after 4 wks) on half strength MS+ mgL^{-1} TDZ & 0.5 mgL^{-1} NAA fortified with 300 mgL^{-1} Adenine sulphate (Scale bar: 1cm); j, Elongated shoot clusters after profuse multiplication (after 8 wks) on half strength MS+ mgL^{-1} TDZ & 0.5 mgL^{-1} NAA fortified with 300 mgL^{-1} Adenine sulphate (Scale bar: 1cm); k, A single spontaneously rooted shoot cluster obtained after 8 wks when cultured on half strength MS+ mgL^{-1} TDZ & 0.5 mgL^{-1} NAA fortified with 300 mgL^{-1} Adenine sulphate (Scale bar: 1cm); l, Rooted shoots prior to *ex-vitro* transfer (after 8 wks of culture on half strength MS+ 2 mgL^{-1} IBA) (Scale bar: 1cm); m, A single tissue culture raised regenerant (after 4 months of *ex-vitro* transfer) successfully established on a potting mixture comprising charcoal bricks, coconut husk and vermiculite with a toping of moss (Scale bar: 1cm).

et al., 2014). Ever since its growth regulatory activity has been discovered, Ads has been effectively used in tissue culture studies in combination with other cytokinins. Although the promotional effects of Ads in multiple shoot induction, regeneration, and cell growth stimulation has been previously elucidated in several woody as well as herbaceous plants like *Bacopa monnieri* (Ramesh *et al*., 2006), *Cichorium intybus* (Nandagopal and Ranjithakumari, 2006), *Clitoria ternatea* (Rency *et al*., 2018), *Holarrhena antidysenterica* (Raha and Roy, 2001), *Melia azadirachta* (Husain and Anis, 2004), *Pterocarpus marsupium* (Husain *et al.,* 2008), literature describing the effectiveness of Ads in orchid tissue culture is scanty. Extensive perusal of available literature reveals the existence of a solitary report elucidating the promotary effect of Ads on *in vitro* seed germination of the endangered Korean terrestrial orchid *Calanthe sieboldii* (Park *et al.,* 2000). In the present investigation, however, the effectiveness of Ads in profuse shoot multiplication has been clearly demonstrated. This notable acceleration is shoot multiplication rate may be attributed to the elicitor or enhancer like action of Ads used in combination with

exogenous growth regulators (Gantait and Mandal, 2010; Khan *et al.,* 2014).

Rooting took place in all the experimental sets supplemented with varying levels (0.5- 2 mgL^{-1}) of three different auxins. The rooting response, however, varied with type of auxins used. Hormone free medium on the other hand could not evoke any response (Table 4). Out of the three auxins used, IBA exhibited the best rooting response (100%) in all the three concentrations tested. A concentration of 2 mgL^{-1} IBA yielded maximum number of roots (5.40 and 7.27) with appreciable length after 4 and 8 wks of culture, respectively (Fig. 1l). This was followed by IAA in terms of the number of roots produced, (79% rooting response was obtained) and a concentration of 2 mgL^{-1} IAA produced a substantial number of healthy roots (3.70 and 6.27 after 4 and 8 wks, respectively) having appreciable length (Table 4). Varying concentrations of NAA on the other hand produced less roots (58% explants responded) in comparison to both IAA and IBA (Table 4). The roots produced after NAA treatment appeared stunted and also exhibited a tendency towards callus formation. Lower concentrations of IAA and NAA could evoke feeble response in terms of the number of roots produced. The length of the roots obtained was, however, noteworthy (Table 4).

As a result of its low toxicity, high stability against catabolism, ability to resist inactivation by conjugation with growth inhibitors and inherent capacity to enhance the endogenous auxin content, IBA has been extensively used for inducing *in vitro* roots in case of several plants including orchids (Van Staden *et al.,* 2008). Successful root induction using different concentrations of IBA has been reported earlier in case of several monopodial as well as sympodial orchids (*i.e. Acampe praemorsa*, *Coelogyne stricta, Dendrobium chryserum, D. nobile*, *Satyrium nepalense*, and *Spathoglottis plicata*) by various authors (Asghar *et al.,* 2011; Maharajan *et al.,* 2020; Manokari *et al*., 2021; Nayak *et al.,* 1997; Singh *et al*., 2021; Thapa *et al*., 2020).

Well rooted seedlings/plantlets when exposed to greenhouse conditions exhibited appreciable leaf growth. Nearly 85% of the regenerants survived after 3 months of acclimatization and were eventually transferred to *ex vitro* conditions during the winter months (January-February). The seedlings successfully survived the winter months and exhibited healthy root and pseudobulb development at the onset of spring, a few seedlings also produced new leaves (Fig. 1m). *In vitro* grown plantlets often fail to develop good quality roots which eventually lead to their poor survival under *ex vitro* environment. In the present investigation, all seedlings/plantlets exhibited appreciable leaf and root growth and were also morphologically identical with their donor plants and no anomaly was noticed. A combination of charcoal bricks, coco peat and pine wood chips was beneficial for the initial stages of acclimatization, however, replacement of the previously used substrate was done in the latter stages, with a combination of charcoal bricks, coconut husk and vermiculite which proved highly effective and resulted in a high survival rate (>80%) of the seedlings.

Tissue culture is an indispensable tool for clonal propagation of conservation reliant wild orchid species as well as horticulturally important hybrids having immense commercial value. The results obtained in the present investigation may be effectively utilized for rapid clonal propagation of this conservation reliant medicinally important *Coelogyne* species.

Conclusion

The present communication reports the development of a rapid, reliable, and cost effective micropropagation protocol for the rare pseudobulbous epiphyte, *Coelogyne flaccida,* using protocorm derived meristematic clumps. Asymbiotic germination and successful *ex vitro* establishment of the seedlings/plantlets may be useful not only for re-establishment of *C. flaccida* plants in their natural habitats, but also for commercial cultivation of this horticulturally important species in future breeding programmes.

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References

Abraham, S., J. Augustine, and T. D. Thomas. 2012. Asymbiotic seed germination and *in vitro* conservation of *Coelogyne nervosa* A. Rich. An endemic orchid to Western Ghats. *Physiol. Mol. Biol. Plants*, **18**: 245-51.

Asghar, S., T. Ahmad, I. Ahmad Hafiz, and M. Yaseen. 2011. *In vitro* propagation of orchid (*Dendrobium nobile*) var. Emma White. *Afr. J. Biotechnol.,* **10**: 3097-103.

Bhowmik, T. K. and M. M. Rahman. 2022. Seed germination, protocorm multiplication, and seedling development in *Dendrobium formosum* Roxb. ex Lindl. of Bangladesh- A study *in vitro*. *J. Orchid Soc. India*, **36**: 1-7.

Bhowmik, T. K. and M. M. Rahman. 2023. *In vitro* seed germination, seedling and SPSs development in *Habenaria digitata* Lindl.

on different growth additives and PGRs supplemented MS medium. *J. Orchid Soc. India*, **37**: 59-67.

Chang, C. and W. C. Chang. 2000. Effect of thidiazuron on bud development of *Cymbidium sinense* Willd *in vitro*. *Plant Growth Regul.,* **30**: 171-75.

Chen, J. T. and W. C. Chang. 2001. Effects of auxins and cytokinins on direct somatic embryogenesis from leaf explants of *Oncidium* 'Gower Ramsey'. *Plant Growth Regul.*, **34**: 229-32.

Clayton, D. 2002. *The Genus Coelogyne: A Synopsis*. Royal Botanic Gardens, Kew, U.K.

De, K. K. and S. Sil. 2015. Protocorm-like bodies and plant regeneration from foliar explants of *Coelogyne flaccida,* a horticulturally and medicinally important endangered orchid of Eastern Himalaya. *Lankesteriana*, **15**(2): 151-58.

Deb, C. R. 2010. Regenerative competence of foliar explants of *Coelogyne suaveolens* and *Taenia latifolia*: Two threatened orchids of NorthEast India. *Appl. Biol. Res.*, **12**(1): 1-9.

Dhillon, M. K. and Promila Pathak. 2023. Asymbiotic seed germination in a medicinally important and near threatened terrestrial orchid, *Crepidium acuminatum* (D.Don) Szlach. from NorthWestern Himalayas: A study *in vitro. J. Orchid Soc. India,* **37**: 49-57.

Duncan, D. B. 1955. New multiple range test. *Biometrics*, **11**(1): 1-42.

Gantait, S. and N. Mandal. 2010. Tissue culture of *Anthurium andreanum*: A significant review and future prospective. *Int. J. Bot.*, **6**: 207-19.

Husain, M. K. and M. Anis. 2004. *In vitro* proliferation of shoots of *Melia azedarach* L. from mature trees. *In: Biotechnology for a Better Future* (eds. L. D'Souza, M. Anuradha, S. Nivas, S. Hegde, and K. Rajendera) pp. 294-301. SAC, Mangalore, India.

Husain, M. K., M. Anis, and A. Shahzad. 2008. *In vitro* propagation of a multipurpose leguminous tree (*Pterocarpus marsupium* Roxb.) using nodal explants. *Acta Physiol. Plant.*, **30**: 353-59.

Jinn, J. H. 2011. *SPSS for windows (version 20).* IBM Corporation., Armonk, New York, U.S.A.

Kaur, S. and K. K. Bhutani. 2013. *In vitro* conservation and asymbiotic propagation of *Coelogyne flaccida* (Lindl.): A threatened orchid. *Plant Biosyst.,* **148**(5): 935-44.

Khan, M. K., P. Misra, T. Sharma, P. K. Shukla, and P. W. Ramteke. 2014. Effect of adenine sulphate on *in vitro* mass propagation of *Stevia rebaudiana* Bertoni. *J. Med. Plant Res.,* **8**(13): 543-49.

Kirti, Promila Pathak, and K. C. Mahant. 2023. Asymbiotic seed germination and seedling development in a commercially important and endemic orchid of Western Ghats, *Aerides crispa* Lindl.- A study *in vitro. J. Orchid Soc. India,* **37**: 141-49.

Knudson, L. 1951. Nutrient solutions for orchids. *Bot. Gaz.*, **112**(4): 528-32.

Lalduhsanga, R. Jayanthi, B. N. Sathyanarayana, K. S. Nirmala, and Vena S. Anil. 2021. A comparative study of different nutrient media on the *in vitro* asymbiotic seed germination of two threatened wild orchids. *J. Orchid Soc. India*, **35**: 109-13.

Maharjan, S., L. S. Thakuri, B. B. Thapa, S. Pradhan, K. K. Pant, G. P. Joshi, and B. Pant. 2020. *In vitro* propagation of the endangered orchid *Dendrobium chryseum* Rolfe from protocorms culture. *Nepal J. Sci. Technol.,* **19**(1): 39-47.

Majumder, P. L. and D. C. Maiti. 1989. Flaccidinin and oxoflaccidin, two phenanthrene derivatives of the orchid *Coelogyne flaccida*. *Phytochem.*, **28**(3): 887-90.

Majumder, P. L. and D. C. Maiti. 1991. Isoflaccidinin and isooxoflaccidin, stilbenoids from *Coelogyne flaccida*. *Phytochem.*, **30**(3): 971-74.

Manokari, M., S. Priyadharshini, and M. S. Shekhawat. 2021. Direct somatic embryogenesis using leaf explants and short term storage of synseeds in *Spathoglottis plicata* Blume. *Plant Cell Tissue Organ Cult.*, **145**: 321-31.

Mitra, G. C., R. N. Prasad, and A. R. Chowdhury. 1976. Inorganic salts and differentiation of protocorms in seed callus of an orchid and correlated changes in its free amino acids content. *Indian J. Exp. Biol.*, **14**: 350-51.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant.*, **15**(3): 473-97.

Mutum, R. D., N. M. Chanu, T. N. Khanganba, and B. Thongam. 2022. Propagation and conservation of selected orchids of Manipur. *J. Orchid Soc. India*, **36**: 95-101.

Naing, A. H., K. T. Myint, Y. J. Hwang, I. S. Park, J. D. Chung, and K. B. Lim. 2010. Micropropagation and conservation of the wild medicinal orchid, *Coelogyne cristata*. *Hortic. Environ. Biot.*, **51**(2): 109-14.

Nandgopal, S. and B. D. Ranjitha. 2006. Adenine sulphate induced high frequency shoot organogenesis in callus and *in vitro* flowering of *Cinchorium intybus* L. cv. Focus- A potent medicinal plant. *Acta Agric. Slov.,* **87**: 415-25.

Nayak, N. R., S. Patnaik, and S. P. Rath. 1997. Direct shoot regeneration from foliar explants of an epiphytic orchid, *Acampe praemorsa* (Roxb.) Blatter and McCann. *Plant Cell Rep.*, **16**: 583-86.

Nelson, H. V. and N. A. Rusdi. 2018. Effect of basal media, plant growth regulators and complex additives on asymbiotic seed germination and shoot induction of *Coelogyne pulverula*. Agriculture. *In: Proc. International Conference of Sustainable Agriculture.* pp 181-190. Universiti Malay Sabah, Malaysia.

Pant, B., K. Chand, M. R. Paudel, P. R. Joshi, B. B. Thapa, S. Y. Park, S. Shakya, L. S. Thakuri, S. Rajbahak, and A. K. Sah. 2022. Micropropagation, antioxidant and anticancer activity of pineapple orchid: *Dendrobium densiflorum* Lindl. *J. Plant Biochem. Biotechnol.,* **31**: 399-409.

Park, S. Y., H. N. Murthy, and K. Y. Paek. 2000. *In-vitro* seed germination of *Calanthe sieboldii*, an endangered orchid species. *J. Plant Biol.*, **43**: 158-61.

Pathak, Promila, Anamika Kumari, Brent D. Chandler, and Lawrence W. Zettler. 2023. *In vitro* propagation and phytochemical analysis of *Vanda cristata* Wall. ex Lindl.: An endangered medicinal orchid of biopharmaceutical importance. *S. Afr. J. Bot.*, **153**: 109-23.

POWO. 2024. *Plants of the World Online*. Facilitated by the Royal Botanic Gardens, Kew. Published on the Internet. http://www.plantsoftheworldonline.org.

Pyakurel, D. and K. Gurung. 2008. Uses of orchids. *In: Enumeration of Orchids and Estimation of Current Stock of Traded Orchids in Rolpa District*. A report submitted to district forest office, Rolpa, Nepal.

Pyati, A. N. 2022. *In vitro* propagation of orchid (*Dendrobium ovatum* (L.) Kraenzl.) through somatic embryogenesis. *Plant Tissue Cult. Biotechnol.*, **32**: 53-66.

Raha, S. and S. C. Roy. 2001. *In vitro* plant regeneration in *Holarrhena antidysenterica* Wall., through high-frequency axillary shoot proliferation. *In Vitro Cell. Develop. Biol. Plant*, **37**: 232-36.

Ramesh, M., R. M. Saravanakumar, and S. Karutha Pandian. 2006. Benzyl amino purine and adenine sulphate induced multiple shoot and root induction from nodal explants of brahmi, *Bacopa monnieri* (Linn.) Penn. *Nat. Prod. Rad.*, **5**: 44-51.

Rency, A. S., S. Pandian, and M. Ramesh. 2018. Influence of adenine sulphate on multiple shoot induction in *Clitoria ternatea* L. and analysis of phyto-compounds in *in vitro* grown plants. *Biocatal. Agric. Biotechnol.*, **16**: 181-91.

Seeni, S. and P. G. Latha. 2000. *In vitro* multiplication and eco rehabilitation of the endangered Blue Vanda. *Plant Cell Tissue Organ Cult.*, **61**: 1-8.

Sil, S. and K. K. De. 2015. *In vitro* mass propagation of an endangered, ornamentally and medicinally important orchid, *Coelogyne flaccida* Lindl. through shoot tip culture. *J. Biotech.*, **5**(3): 54-60.

Singh, N. and S. Kumaria. 2019. *Ex situ* multiplication of *Coelogyne ovalis* Lindl.: Nutrient optimization for asymbiotic seed germination and mass scale propagation of genetically stable plantlets. *Int. J. Life Sci. Res.*, **7**: 503-12.

Singh, D. K., B. A. Mir, S. Babbar, and S. B. Babbar. 2021. Cost-effective *in vitro* multiplication and phenolic profile of an important medicinal orchid, *Satyrium nepalense* D.Don. *J. Biol. Act. Prod. Nat..*, **11**(2): 162-82.

Sunita, Promila Pathak, and K. C. Mahant. 2021. Green pod culture of an endangered and medicinally important orchid, *Vanda cristata* Wall. ex Lindl. from Himachal Pradesh. *J. Orchid Soc. India*, **35**: 25-33.

Thakur, Babita and Promila Pathak. 2021. Application of organic additives for the enhancement of seed germination and seedling development in an endangered and medicinal orchid, *Rhynchostylis retusa* (L.) Blume through asymbiotic culture. *J. Orchid Soc. India*, **35**: 99-107.

Thapa, B. B., L. S. Thakuri, P. R. Joshi, K. Chand, S. Rajbahak, A. K. Sah, and B. Pant. 2020. *Ex-situ* conservation and cytotoxic activity assessment of native medicinal orchid: *Coelogyne stricta*. *J. Plant Biotechnol.*, **47**(4): 330-36.

Thomas, T. D. and A. Michael. 2007. High-frequency plantlet regeneration and multiple shoot induction from cultured immature seeds of *Rhynchostylis retusa* Blume., an exquisite orchid. *Plant Biotech Rep.*, **1**: 243-49.

Tripura, A., M. A. Sumi, T. K. Bhowmik, and M. M. Rahman. 2022. *In vitro* seed germination and phytochemical screening of an epiphytic medicinal orchid, *Pholidota imbricata* W. J. Hook. of Bangladesh. *J. Orchid Soc. India,* **36**: 137-45.

Van Staden, J., E. Zazimalova, and E.F. George. 2008. Plant Growth Regulators II: Cytokinins, their Analogues and Antagonists. *In: Plant propagation by tissue culture* (eds. E. F. George, M. A. Hall, and G. J. Deklerk) pp. 206-17. Springer.

Vasundhra, Promila Pathak, and Anuprabha. 2021. *In vitro* asymbiotic seed germination and regeneration competence of leaf explants in *Satyrium nepalense* D.Don, a medicinally important, and an endangered terrestrial orchid of Kasauli Hills, Himachal Pradesh (NorthWestern Himalayas). *J. Orchid Soc. India*, **35**: 73-82.

Zeng, S., W. Huang, K. Wu, J. Zhang, J. A. T. da Silva, and J. Duan. 2015. *In vitro* propagation of *Paphiopedilum* orchids. *Crit. Rev. Biotechnol.,* **36**(3): 521-34. DOI: 10.3109/07388551.2014.993585.