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Artificial pollination, seed germination, and micropropagation of *Coelogyne cristata* and *C. lawrenceana* by in vitro protocol for conservation of endangered orchids

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Abstract. *Hussien M, Koval VA, Ryabchenko AS, Makarov SS. 2025. Artificial pollination, seed germination, and micropropagation of* Coelogyne cristata *and* C. lawrenceana *by* in vitro *protocol for conservation of endangered orchids. Biodiversitas 26: 4384-4395.* *Coelogyne cristata* and *Coelogyne lawrenceana* are rare and endangered orchids valued for their ornamental and medicinal uses. These species are severely threatened by low natural propagation rates and declining wild populations, making effective conservation strategies urgently necessary. However, a complete in vitro propagation system has not yet been established, further limiting their conservation. This study reports the first successful and efficient in vitro propagation protocol for both species, integrating artificial pollination, asymbiotic seed germination, and clonal micropropagation using low concentrations of Plant Growth Regulators (PGRs) combined with potato homogenate, coconut water, and banana homogenate to achieve this. Artificial pollination under greenhouse conditions achieved fruit set rates of 65% in *C. cristata* and 45% in *C. lawrenceana,* respectively. Seeds germinated at high frequencies on half-strength Murashige and Skoog (½ MS) medium: 98% in *C. cristata* with 0.5 mg L^{-1} IAA and 92% in *C. lawrenceana* with 0.5 mg L^{-1} 6-BAP. Optimal protocorm development occurred with 0.5 mg L^{-1} meta-Topolin (mT) and banana homogenate for *C. cristata* and 0.5 mg L^{-1} 6-BAP with coconut water for *C. lawrenceana*. The highest shoot multiplication was obtained in *C. cristata* (3.93±0.47 shoots/explant) using 0.5 mg L^{-1} kinetin and potato homogenate, and in *C. lawrenceana* (6.28±0.15 shoots/explant) using 0.5 mg L^{-1} mT and coconut water, both significantly higher than controls ($p<0.05$). Root induction was most effective in *C. cristata* with 1.0 mg L^{-1} IBA or 0.5 mg L^{-1} IAA plus coconut water, while *C. lawrenceana* rooted best with 0.5 mg L^{-1} IAA. Acclimatized plants grown in a *Sphagnum* moss-charcoal-pine bark mixture (1:1:1) exhibited high survival rates: 87% for *C. cristata* and 82% for *C. lawrenceana*. This protocol offers a scalable solution for ex situ conservation and sustainable horticultural use of these threatened species.

Keywords: Asymbiotic germination, clonal propagation, *Coelogyne* spp., orchid conservation, protocorm

INTRODUCTION

The Orchidaceae family includes numerous species with significant ornamental and medicinal value. Among these, large-flowered species and hybrids of the genus *Coelogyne* are particularly prized for their aesthetic and pharmacological potential (Kolomeitseva et al. 2025). *Coelogyne* species are distributed from the Himalayas and Japan to Southeast Asia, New Caledonia, and Queensland (POWO 2025). Several *Coelogyne* species contain bioactive compounds (Teoh 2016), with *Coelogyne cristata* Lindl. and *C. fimbriata* Lindl. demonstrating antimicrobial activity (Wati et al. 2020), and *C. lawrenceana* exhibiting antimicrobial and antifungal properties (Buyun et al. 2017).

In addition to their medicinal properties, many *Coelogyne* species are widely cultivated as ornamental plants and cut flowers due to their striking floral displays (Subedi et al. 2011). Among the most notable ornamental species are *C. cristata* and *C. lawrenceana*, both characterized by large flowers (8-10 cm in diameter) and extended floral longevity. *C. cristata* produces pendulous racemes with snow-white, fragrant flowers and a trilobed lip ornamented with five yellow-crested keels. In contrast, *C. lawrenceana* develops erect inflorescences with pale-yellow flowers marked with ochre on the mid-lobe and three prominent lamellae on the lip.

In the face of ongoing climate change and increasing human impact, many orchids are at a higher risk of extinction. Epiphytic ornamental and medicinal species, including those in the genus *Coelogyne*, are especially vulnerable due to unsustainable harvesting from their natural habitats (Liu et al. 2010). *C. lawrenceana* is endemic to Vietnam and, like *C. cristata*, is currently listed in Appendix II of CITES (2023), emphasizing its ecological vulnerability and the need for ex situ conservation efforts.

Reproductive and biological constraints complicate such conservation efforts. Pollination in *Coelogyne* often encounters obstacles due to post-zygotic self-incompatibility mechanisms, such as ovule degeneration and early embryo abortion. These barriers frequently result in reduced seed set or the formation of parthenocarpic fruit under artificial autogamous and geitonogamous pollination. In contrast, xenogamous pollination is generally more effective in producing viable seeds (Koval 2023; Kolomeitseva et al. 2025).

Like many orchids, *Coelogyne* species produce dust-like seeds with limited nutrient reserves that require specific biotic and abiotic factors for germination. The most critical factor is embryo maturity (Lee and Yeung 2023). Germination also depends on establishing symbiotic relationships with mycorrhizal fungi, which facilitate carbon transfer to developing embryos (Rasmussen et al. 2015). Additionally, seed coat structure plays a key role, affecting dormancy, dispersal, and water absorption (Chen et al. 2022). In nature, seed germination is often limited due to the lack of essential nutrients, minerals, and water normally supplied through fungal symbiosis (Sisti et al. 2019). Given these reproductive and ecological constraints, artificial pollination combined with in vitro propagation has become a promising strategy. These methods are widely recognized for conserving and commercially producing rare orchid species. They also offer significant advantages over symbiotic germination and traditional vegetative propagation, which are often inefficient or difficult to scale. Several in vitro propagation protocols have been developed for *Coelogyne* species, including *C. nervosa*, *C. mossiae*, and *C. flaccida*, using MS, Knudson C, or Mitra media, often supplemented with coconut water or banana homogenate (Sebastianraj et al. 2006; Abraham et al. 2012; Kaur and Bhutani 2013).

For *C. cristata*, protocols have been developed for callus induction and Protocorm-Like Body (PLB) development using various explants, often involving high concentrations of PGRs such as 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine (6-BAP) (Naing et al. 2011). However, these methods skip the early stages of sexual reproduction and do not address artificial pollination or seed-based propagation. A thorough bibliographic review revealed a complete absence of published protocols for artificial pollination or in vitro propagation of *C. lawrenceana*, highlighting a significant gap in conservation strategies for this species. This lack of research is likely due not only to its endemic status and limited geographic range but also to its complex reproductive biology, which obstructs the production of viable seeds in both natural populations and cultivated collections (greenhouses). Additionally, a direct comparison between *C. cristata* and *C. lawrenceana* using the same propagation framework has yet to be systematically investigated. A reliable and scalable propagation system is therefore crucial not only for ex situ conservation but also for potential reintroduction into natural habitats or sustainable use in ornamental horticulture. Despite their horticultural and pharmacological importance, effective ex situ cultivation of both species remains limited by reproductive challenges and low survival rates during acclimatization. This study aims to fill these gaps by developing an efficient protocol for artificial pollination, asymbiotic seed germination, and clonal propagation of *C. cristata* and *C. lawrenceana* using selected PGRs combined with organic additives.

MATERIALS AND METHODS

Study area

The study was conducted at the Laboratory of Plant Biotechnology of the Main Botanical Garden, named after N.V. Tsitsin, Russian Academy of Sciences, using standard biotechnological techniques and established protocols for cultured plant tissue (Molkanova et al. 2018; Hussien et al. 2025a).

Plant materials

Two orchid species, *C. cristata* and *C. lawrenceana*, were used in this study. Specimens were obtained from the living collection of the Stock Greenhouse, Main Botanical Garden of the Russian Academy of Sciences (project No. 122042700002-6). The plants were cultivated under controlled greenhouse conditions, with each species maintained according to its specific temperature regime (Table 1).

Plants were grown in pots, mounted on blocks, or placed in large baskets using pine bark or a mixture of pine bark and *Sphagnum* moss as substrates. They were fertilized weekly with a liquid complex mineral fertilizer (NPK 3.4:3.8:4.0) containing magnesium, sulfur, and trace elements including Fe, B, Cu, Mn, Mo, and Zn. Flowering was monitored over three consecutive years to establish consistent blooming periods for each species.

Artificial pollination

Manual xenogamous pollination was performed by transferring pollen between flowers of the same species from different individuals. The maternal flower was prepared by removing the anther cap and pollinarium to expose the clinandrium. The pollinarium, or part of the pollinia, was carefully extracted from the paternal flower using sterilized steel tweezers and placed on the stigmatic surface beneath the rostellum. The rostellum was gently pressed against the stigma to secure the pollinarium. Pollinations were performed in the morning on four individual plants of each species, with a total of 100 flowers pollinated per species.

Table 1. Temperature regimes for the cultivation of *Coelogyne cristata* and *Coelogyne lawrenceana* in greenhouse conditions

Species	Inventory numbers, origin	Temperature regime	Temperature (°C)			
			Summer day	Summer night	Winter day	Winter night
Coelogyne cristata	2006.00718-2006.00722, Vietnam (nat)	Intermediate (cool day + cold night)	18-27°C	14-16°C	12-14°C	10-12°C
Coelogyne lawrenceana	1961.00678-1961.00681; 0000.01504, Vietnam (nat)	Warm (warm day + warm night)	24-29°C	18-20°C	20-22°C	18-20°C

Table 2. Application scheme of organic additives and cytokinins for the cultivation of *Coelogyne cristata* and *Coelogyne lawrenceana* protocorms

Organic additives	Cytokinins (0.5 mg L^{-1})
Potato homogenate (30 g L^{-1})	Without cytokinins (control)
	6-BAP
	Kinetin (Kin)
	meta-Topolin (mT)
Banana homogenate (50 g L^{-1})	Control
	6-BAP
	Kinetin (Kin)
	meta-Topolin (mT)
Coconut water (100 mL L^{-1})	Control
	6-BAP
	Kinetin (Kin)
	meta-Topolin (mT)

During pollination, greenhouse air temperature was 18-22°C for *C. cristata* in April and 24-26°C for *C. lawrenceana* in April, with relative humidity maintained at 70-80%, consistent with the summer daytime ranges in Table 1. Each fruit was tagged with its pollination date to allow timing by days after pollination, as morphological indicators of maturity are unreliable in *Coelogyne*. Harvest timing was therefore defined by the embryo-developmental stages described by Kolomeitseva et al. (2025); under these criteria, fruits of *C. cristata* were harvested six months after pollination (April → October) and fruits of *C. lawrenceana* eight months after pollination (April → December). These harvest times ensured embryo maturity while keeping fruits intact, which facilitated surface sterilization and minimized microbial contamination during in vitro culture. The fruit set rate was calculated as the percentage of fruits formed per 100 pollinated flowers. Seed maturity was verified microscopically to confirm the presence of a developed embryo before in vitro sowing, ensuring comparable developmental stages between the two species.

Asymbiotic seed germination

This stage aimed to induce successful germination and protocorm formation from the immature seeds of *C. cristata* and *C. lawrenceana*. Immature seeds were harvested from unopened, ripening fruits and used for in vitro germination. Fruits of *C. cristata* were collected six months after artificial pollination, and those of *C. lawrenceana* after eight months. Fruits were surface sterilized by immersion in a household sodium hypochlorite solution containing Tween 20 for 15 minutes, followed by a brief rinse in 70% ethanol (5 seconds), and final sterilization by flaming over an alcohol burner. The culture medium used in all experiments consisted of half-strength Murashige and Skoog (1962) (½ MS) supplemented with 20 g L^{-1} sucrose, 0.7 g L^{-1} activated charcoal, and 0.1 g L^{-1} myo-inositol. The medium and instruments were sterilized at 121°C and 15 psi for 20 minutes using a WAC-60 autoclave (Daihan Scientific, South Korea), and the pH was adjusted to 5.5-5.8 before autoclaving. Seeds were carefully sown onto the surface of the nutrient medium using a sterile scalpel, with three culture vessels per treatment established as replicates to ensure reproducibility. The following treatments were tested to optimize germination and protocorm development: (i) ½ MS without growth regulators (control), (ii) ½ MS + 0.5 mg L^{-1} 6-BAP, (iii) ½ MS + 0.5 mg L^{-1} Indole-3-Acetic Acid (IAA).

Cultures were maintained in a growth room at 24±2°C under a 16-hour light/8-hour dark, with an illumination intensity of approximately 60 μmol m^{-2} s^{-1} provided by LED floodlights. Germination was defined as the emergence of a green embryo with initial cell enlargement. Germination rates and protocorm development were recorded at 20, 30, and 45 days after sowing.

Development of protocorms and plantlet formation

Protocorms with leaf primordia were transferred to ½ MS medium supplemented with cytokinins and organic additives (Table 2) to promote further development and plantlet formation. The choice of this medium, supplemented with organic additives, was based on earlier reports demonstrating its effectiveness in supporting the regeneration of protocorms (An et al. 2021; Herawati et al. 2021; Safitri et al. 2024).

The protocorms were maintained for 70-90 days under the following growth conditions: 24±2°C, with a 16-hour light/8-hour dark photoperiod, and an illumination intensity of approximately 60 μmol m^{-2} s^{-1}. No intermediate subculture was performed during this period; protocorms were transferred only at the transition to the next developmental stage. At the end of the cultivation period, the following morphometric parameters were recorded: plantlet height (cm), rooting percentage, number of adventitious shoots, number of leaves, number of roots, and root length (cm). Root length was measured as the average length of all roots per plantlet using a digital caliper.

Rooting stage

Plantlets derived from the previous developmental stage were cultured on ½ MS medium supplemented with organic additives, including coconut water (100 mL L^{-1}) and banana homogenate (50 g L^{-1}), along with auxins—Indole-3-Butyric Acid (IBA) and Indole-3-Acetic Acid (IAA) at concentrations of 0.5 and 1.0 mg L^{-1}. Cultures were maintained for 70-90 days under the same growth room conditions: 24±2°C, with a 16-hour light/8-hour dark photoperiod and an illumination intensity of approximately 60 μmol m^{-2} s^{-1}. Plantlets remained on the rooting medium until the end of this period, after which measurements were taken. The following parameters were recorded: rooting percentage, plantlet height (cm), number of roots, number of leaves, and root length (cm).

Acclimatization stage

Plantlets with well-developed roots were transferred to greenhouse conditions for ex vitro acclimatization using three substrate compositions: (i) Peat, perlite, and pine bark (1:1:1), (ii) *Sphagnum* moss, charcoal, and pine bark (1:1:1), (iii) Pine bark and expanded clay pellets (2:1).

Before final transfer, plantlets were hardened off by gradually reducing humidity and placing them in a well-ventilated, shaded area for 6-8 hours.

Plantlets were cultivated under warm temperature conditions (23-25°C) with natural illumination corresponding to the latitude of Moscow (~1500 lux in winter, >23000 lux in summer) and a relative humidity of 70-80%. Supplemental artificial lighting (LED lamps) was used to maintain stable illumination levels during periods of low natural light intensity. Plants were grown in pots, and liquid complex mineral fertilizer was applied once a week. The survival rate was evaluated after 70 days. Plantlets were watered manually once per day, and an automatic fogging system was used to disperse fine water droplets every 30 minutes for 5 seconds.

Data analysis

All experiments were conducted using a Completely Randomized Design (CRD). Each treatment consisted of three replicates, with 10 explants per replicate (n = 30 per treatment). Statistical analyses were performed using One-Way and Two-Way Analysis of Variance (ANOVA), followed by Duncan's Multiple Range Test (DMRT) to compare treatment means at a significance level of $p<0.05$. Data are presented as means±Standard Deviations (SD). All analyses were conducted using CoStat 6.45 (CoHort Software, Monterey, CA, USA) and Microsoft Excel 2019.

RESULTS AND DISCUSSION

Artificial pollination

Under greenhouse conditions, however, both species exhibited shifts in flowering phenology. *Coelogyne lawrenceana* showed an extended blooming period from January to April and again in September, indicating an additional flowering cycle under warm, controlled conditions. *Coelogyne cristata* flowered from February to April (Figures 1.A and Figure 2.A).

To obtain seeds for in vitro germination, manual xenogamous pollination was conducted. Out of 100 pollinated flowers, *C. cristata* formed 65 fruits (65%) (Figure 1.B) and *C. lawrenceana* produced 45 fruits (45%) (Figure 2.B). These results indicate interspecific variation in reproductive success under controlled conditions.

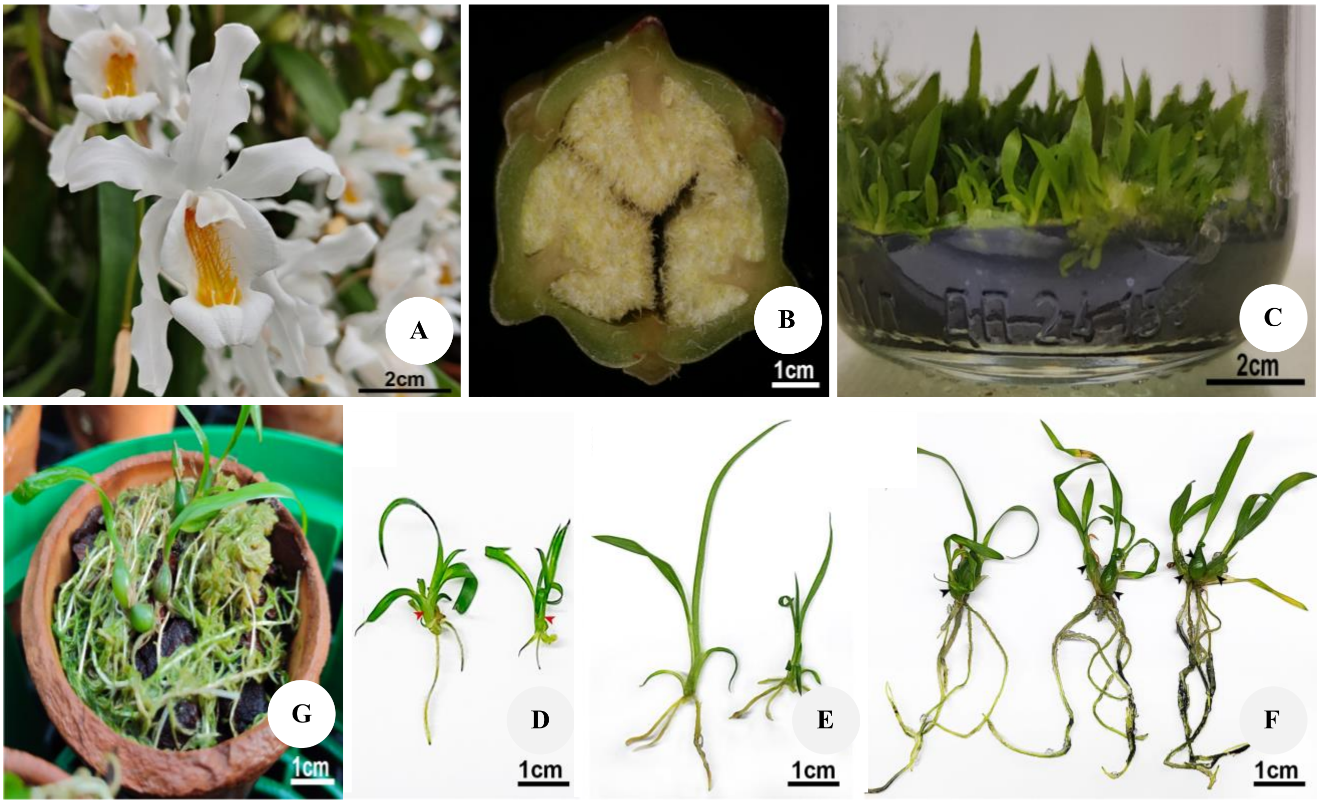

Figure 1. Stages of *Coelogyne cristata* clonal micropropagation: A. Flower of the donor plant, B. A six-month-old fruit containing seeds, C. Protocorm with the first emerging leaf on ½ MS medium supplemented with 0.5 mg/L IAA, D. Multiple shoots on ½ MS medium supplemented with potato homogenate and 0.5 mg/L kinetin, E. Regenerants with healthy leaves and roots on medium supplemented with banana homogenate and mT, F. Well-rooted plantlets ready for acclimatization, G. Hardened plantlets in earthen pots after 70 days. Note: Adventitious shoots (red arrowhead), pseudobulbs (black arrowhead)

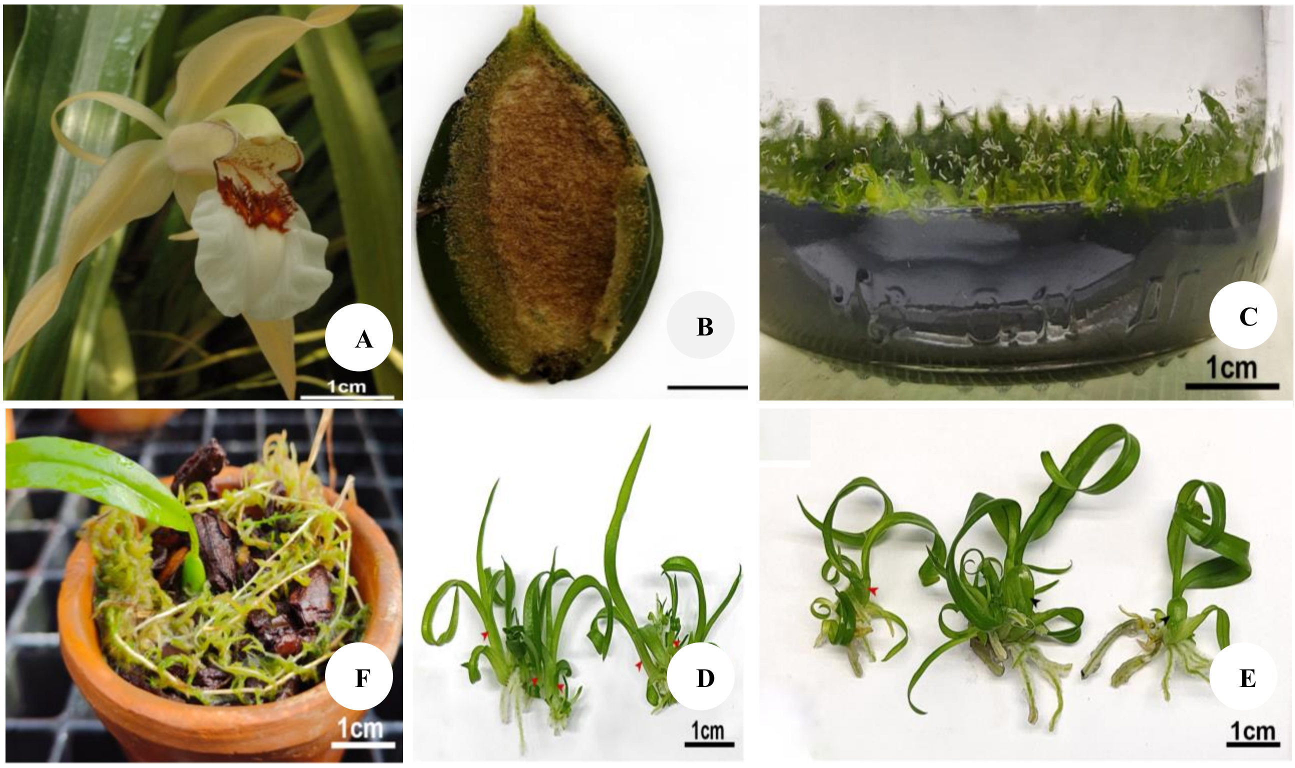

Figure 2. Stages of *Coelogyne lawrenceana* clonal micropropagation: A. Flower of the donor plant, B. An eight-month-old fruit containing seeds, C. Protocorm with the first emerging leaf on ½ MS medium supplemented with 0.5 mg/L 6-BAP, D. Multiple shoots and regenerants with healthy leaves and roots on medium supplemented with coconut water and 6-BAP, E. Well-rooted plantlets ready for acclimatization of *Coelogyne lawrenceana*, F. Hardened plantlets in earthen pots after 70 days. Note: Adventitious shoots (red arrowhead), pseudobulbs (black arrowhead)

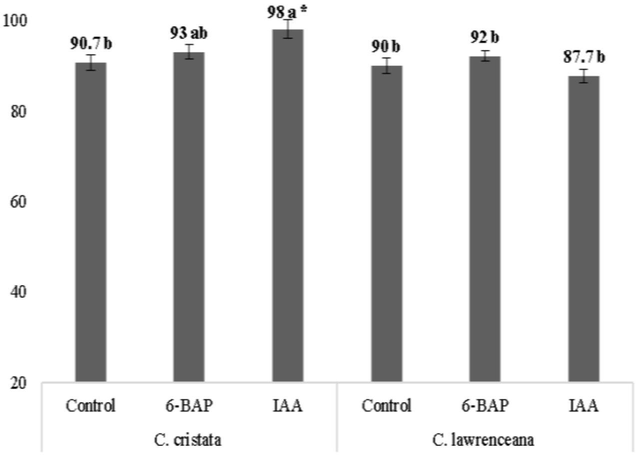

Figure 3. Effect of various PGRs on seed germination in *Coelogyne cristata* and *Coelogyne lawrenceana*. *Values are mean±SD of three replicates (n = 30), and the letters "a", "b", and "c" denote groups by Duncan's multiple range test at the 5% level

Asymbiotic seed germination

The immature fruits obtained were used as a seed source for asymbiotic germination experiments. After 20 days of cultivation, seeds of *C. cristata* showed dark green embryos on all tested nutrient media, indicating faster and more advanced embryo development compared to *C. lawrenceana*, which produced light green embryos only after 35 days ($p<0.010$). The highest germination rate for *C. cristata* (98%) was recorded on ½ MS medium supplemented with 0.5 mg L^{-1} IAA, followed by 93% on medium containing 0.5 mg L^{-1} 6-BAP, and 90.7% on the control medium without PGRs. In contrast, *C. lawrenceana* exhibited germination rates from 87.7% to 92% after 35 days, with no statistically significant differences between treatments ($p<0.286$) (Figure 3).

Despite the slower development, the consistently high germination rates in both species indicate that the embryos had reached sufficient maturity under asymbiotic conditions. Furthermore, the addition of PGRs to the nutrient medium significantly affected protocorm development ($p<0.013$). In *C. cristata*, rapid protocorm growth, along with early formation of primary roots and leaves, was observed after 40 days on medium supplemented with 0.5 mg L^{-1} IAA (Figure 1.C). These protocorms were suitable for transfer to fresh media for further development and the formation of plantlets. In contrast, *C. lawrenceana* showed slower protocorm development, with the highest percentage of protocorms forming primary leaves observed only after about 60 days on medium containing 0.5 mg L^{-1} 6-BAP (Figure 2.C).

Development of protocorms and plantlet formation

The developed protocorms of both species were transferred to ½ MS medium supplemented with various organic additives and cytokinins. The interaction between organic additives and PGRs showed significant differences in shoot formation ($p<0.001$). The highest number of adventitious shoots in *C. cristata* (3.39±0.47 units) was observed on medium containing 0.5 mg L^{-1} kin and 30 g L^{-1} potato homogenate (Figure 1.D). The lowest number of adventitious shoots (1.44±0.06 units) was recorded on medium supplemented with potato homogenate without cytokinins. Statistical analysis revealed that the interaction between the organic additive and the cytokinin type had a highly significant effect on all growth parameters ($p<0.001$). Plantlets of *C. cristata* grown on ½ MS medium supplemented with 0.5 mg L^{-1} mT and 50 g L^{-1} banana homogenate showed the highest number of leaves (3.28±0.03), the greatest plantlet height (2.76±0.05 cm), and the highest rooting rate (83%) (Figure 1.E). Additionally, the lowest plantlet growth rates were observed on the medium supplemented with potato homogenate, compared to those with coconut water or banana homogenate. Plantlets grown on medium supplemented with kinetin exhibited intermediate growth rates (Table 3).

For *C. lawrenceana*, protocorm development was significantly affected by various organic additives and cytokinins, especially in terms of the morphometric characteristics of the formed plantlets and adventitious shoot formation. Organic additives significantly impacted the number of leaves ($p<0.001$). Plantlets grown on medium supplemented with coconut water had the highest number of leaves compared to those on media with other organic additives. The interaction between organic additives and cytokinins also significantly influenced plantlet height ($p<0.001$). The greatest plantlet height was observed on medium supplemented with 0.5 mg L^{-1} 6-BAP and 100 mL L^{-1} coconut water, compared to other treatments (Figure 2.D). Notably, *C. lawrenceana* showed a greater ability for adventitious shoot formation than *C. cristata*, likely due to increased induction of formative cells in the primary protocorm. The highest number of adventitious shoots was recorded in regenerants cultured on medium with 0.5 mg L^{-1} mT and coconut water (Table 4).

Table 3. Morphometric parameters of *Coelogyne cristata* plantlets at 90 days after protocorm cultivation on media supplemented with various organic additives and cytokinins

Treatment	N. shoot (Unit)	N. leaves (Unit)	Plant height (cm)	Rooting %
P	1.44±0.06 f *	2.36±0.07 d	0.87±0.10 i	30.00±5.77 e
P 6-BAP	1.75±0.14 def	2.28±0.15 d	1.40±0.09 g	63.33±3.33 b
P Kin	3.39±0.14 a	2.75±0.14 bc	1.96±0.06 c	56.67±3.33 bc
P mT	1.56±0.06 ef	2.17±0.08 d	1.02±0.04 hi	16.67±1.67 f
CW	1.56±0.15 ef	1.83±0.17 e	1.13±0.07 h	30.00±5.77 e
CW 6-BAP	2.28±0.03 bcd	3.00±0.00 ab	2.26±0.01 b	46.67±3.33 cd
CW Kin	1.78±0.28 def	2.36±0.07 d	1.48±0.04 fg	43.33±3.33 d
CW mT	2.08±0.08 cde	2.28±0.03 d	1.58±0.05 efg	43.33±3.33 d
BH	2.75±0.14 b	2.44±0.15 cd	1.75±0.08 cde	60.00±5.77 b
BH 6-BAP	2.64±0.07 bc	2.39±0.14 d	1.69±0.09 def	43.33±3.33 d
BH Kin	2.44±0.47 bc	2.36±0.07 d	1.90±0.11 cd	36.67±3.33 de
BH mT	1.75±0.14 bcd	3.28±0.03 a	2.76±0.05 a	83.33±3.33 a

Note: P: Potato homogenate, CW: Coconut Water, BH: Banana Homogenate. Values are mean±SD of three replicates (n = 30), and the letters "a", "b", and "c" denote groups by Duncan's Multiple Range Test at the 5% level

Table 4. Morphometric parameters of *Coelogyne lawrenceana* plantlets at 90 days after protocorm cultivation on media supplemented with various organic additives and cytokinins

Treatment	N. shoot (Unit)	N. leaves (Unit)	Plant height (cm)	Rooting %
P	2.44±0.06 f *	2.36±0.22 bc	0.73±0.07 g	0.00 e
P 6-BAP	3.06±0.19 ef	2.58±0.22 bc	1.88±0.05 a	20.00±5.77 cd
P Kin	4.78±0.28 b	2.19±0.10 c	0.87±0.13 fg	0.00 e
P mT	3.44±0.15de	2.33±0.22 bc	1.04±0.12 ef	0.00 e
CW	3.53±0.12 de	2.28±0.03 bc	1.23±0.07 de	0.00 e
CW 6-BAP	4.36±0.36 bc	3.08±0.08 a	2.05±0.13 a	23.33±3.33 c
CW Kin	3.89±0.18 cd	2.64±0.14 abc	1.41±0.01 cd	13.33±3.33 d
CW mT	6.28±0.15 a	2.44±0.15 bc	1.53±0.03 bc	43.33±3.33 b
BH	3.06±0.31 ef	2.72±0.15 ab	1.99±0.11 a	53.33±0.00 a
BH 6-BAP	3.78±0.31 cde	2.33±0.17 bc	1.37±0.07 cd	23.33±3.33 c
BH Kin	3.53±0.24 de	2.25±0.07 bc	1.80±0.09 ab	36.67±3.33 b
BH mT	4.47±0.37 bc	2.36±0.07 bc	1.76±0.12 ab	60.00±3.33 a

Note: P: Potato homogenate, CW: Coconut Water, BH: Banana Homogenate. Values are mean±SD of three replicates (n = 30), and the letters "a", "b", and "c" denote groups by Duncan's Multiple Range Test at the 5% level

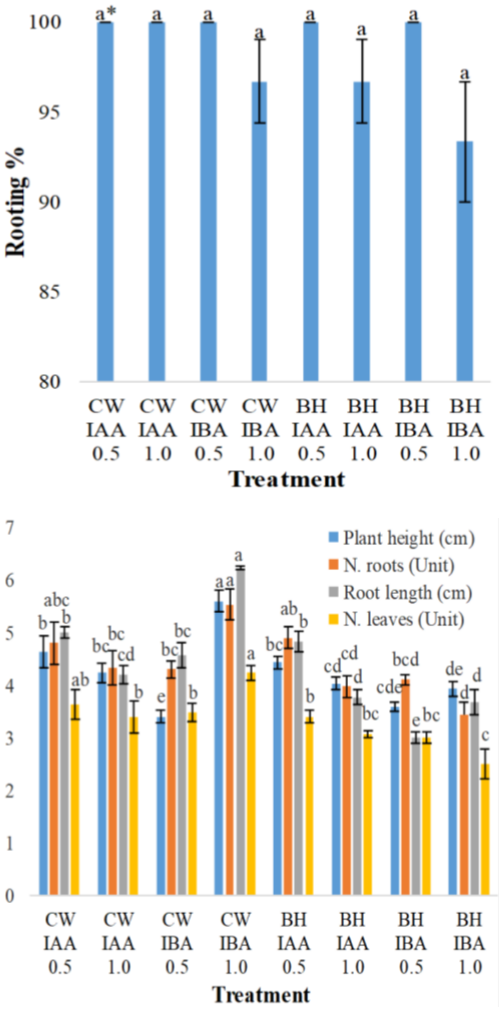

Figure 4. Rooting parameters of *Coelogyne cristata* plantlets at 180 days after cultivation on media supplemented with various organic additives and auxins. Note: CW: Coconut Water, BH: Banana Homogenate. Values are mean±SD of three replicates (n = 30), and the letters “a”, “b”, and “c” denote groups by Duncan’s multiple range test at the 5% level

Rooting stage

Coconut water and banana homogenate proved to be highly effective in increasing rooting percentages (Tables 3 and 4). Therefore, plantlets of both species were transferred to media supplemented with these organic additives and auxins at different concentrations to induce rooting. In *C. cristata*, high rooting percentages (93-100%) were recorded on all tested media, with no statistically significant differences noted regardless of the type of organic additive or auxin applied ($p<0.265$). However, the greatest plantlet height, the highest number of roots, and the longest root length were observed on medium supplemented with 100 mL L^{-1} coconut water and either 1.0 mg L^{-1} IBA or 0.5 mg L^{-1} IAA, compared to other treatments (Figure 1.F and Figure 4).

The interaction between auxin type and concentration significantly affected plantlet height ($p<0.001$). The greatest plantlet height was observed in the medium supplemented with 1.0 mg L^{-1} IBA and 100 mL L^{-1} coconut water. For root development, the type of organic additive significantly influenced both root number and root length ($p<0.002$). The highest values for both parameters were recorded in media containing coconut water. Among the auxins tested, IAA showed particularly promising results. Plantlets grown on a medium with 0.5 mg L^{-1} IAA, combined with either banana homogenate or coconut water, displayed a mean root number of 4.90±0.21 and a mean root length of 4.83±0.20 cm, ranking second among the treatments examined.

In *C. lawrenceana*, there was a general trend toward the formation of taller plantlets with greater vegetative mass, elongated pseudobulbs, and shorter roots compared to *C. cristata*. The type and concentration of organic additives and auxins used had a significant effect on the rooting percentage ($p<0.001$). Plantlets grown on medium supplemented with 0.5 mg L^{-1} IAA and 100 mL L^{-1} coconut water exhibited the highest rooting percentage (93%) among all treatments. Notably, the rooting percentage significantly declined when higher concentrations of auxins were applied, regardless of the type of organic additive used. For instance, the rooting percentage decreased to 60% when 1.0 mg L^{-1} IBA was used in combination with banana homogenate.

The number of roots and root length were also significantly influenced by the concentration of auxin ($p<0.002$ and $p<0.001$, respectively). The highest number of roots was recorded on media supplemented with either coconut water and 0.5 mg L^{-1} IAA or 0.5 mg L^{-1} IBA. Concurrently, the highest number of leaves (5.40±0.31) and the longest roots (2.07±0.09 cm) in *C. lawrenceana* were observed on medium supplemented with 0.5 mg L^{-1} IAA and 100 mL L^{-1} coconut water (Figure 2.E and Figure 5).

The proposed protocol allowed us to obtain plantlets with pseudobulbs, differentiated leaves, and a well-developed root system. These morphological characteristics indicated that the plantlets were sufficiently developed to proceed to the acclimatization stage.

Acclimatization stage

The plantlets were subsequently transferred to ex vitro conditions for acclimatization on substrates with varying compositions, as described in the Materials and Methods section. Only plantlets that appeared healthy and showed no signs of desiccation, chlorosis, or tissue necrosis were considered survived. The highest survival rates for both *C. cristata* (87%) and *C. lawrenceana* (82%) were recorded on a substrate consisting of *Sphagnum* moss, charcoal, and pine bark in a 1:1:1 ratio (Figure 1.G; Figure 2.F). These differences in survival rates among substrates were statistically significant ($p<0.001$). A lower survival rate (57%) was observed on the substrate containing peat, perlite, and pine bark (1:1:1), while the lowest survival (43%) was recorded on the combination of pine bark and expanded clay pellets (2:1).

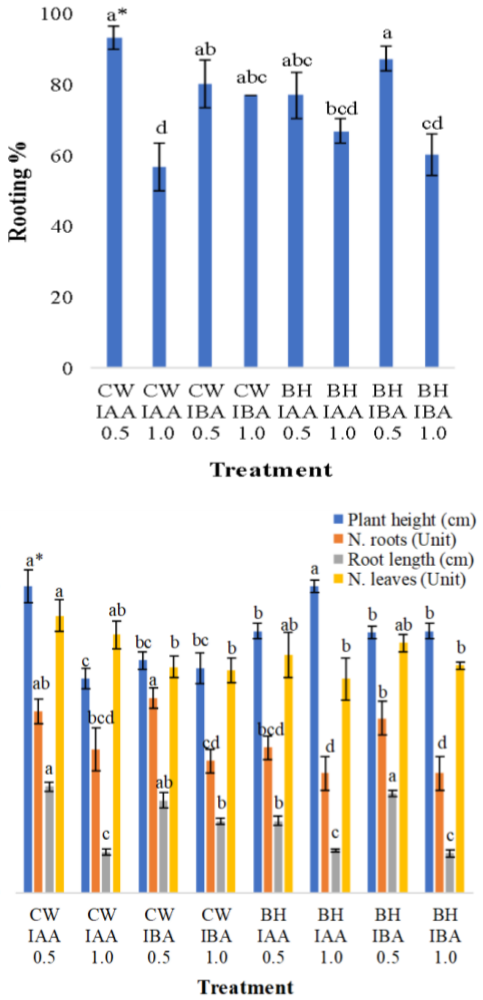

Figure 5. Rooting parameters of *Coelogyne lawrenceana* plantlets at 180 days after cultivation on media supplemented with various organic additives and auxins. CW: Coconut Water, BH: Banana Homogenate. Values are mean±SD of three replicates (n = 30), and the letters "a", "b", and "c" denote groups by Duncan's multiple range test at the 5% level

Discussion

In their natural habitats, *C. lawrenceana* flowers from December to March, while *C. cristata* typically blooms from April to August, with individual flowers lasting up to six weeks (Averyanov and Averyanova 2002). Under greenhouse conditions at Moscow's latitude (~56°N), both species exhibited notable shifts in flowering times: *C. lawrenceana* bloomed from January to April and again in September, while *C. cristata* flowered mainly from February to April. Variations in photoperiod, temperature, and humidity likely cause these shifts. They reflect adaptation to ex situ conditions but also demonstrate that species-specific phenological patterns are retained. *C. lawrenceana* maintained an earlier and more extended blooming period, including a second autumn peak, whereas *C. cristata* flowered within a more compact spring window. This phenological flexibility plays a key role in synchronizing manual pollination with the optimal reproductive period and in ensuring a consistent seed supply for subsequent in vitro propagation.

To generate viable seeds for asymbiotic germination, manual xenogamous pollination was carried out between genetically distinct individuals. This approach supports the maintenance of genetic variability and avoids the limitations of autogamy and geitonogamy, which can limit reproductive success despite successful pollen transfer. Xenogamous pollination, in contrast, is widely regarded as more effective in promoting successful fruit set and embryo viability (Barreda-Castillo et al. 2024). However, fruiting outcomes differed notably between the two species: *C. cristata* exhibited high fruit set, whereas *C. lawrenceana* produced significantly fewer fruits. This discrepancy may result from post-zygotic self-incompatibility mechanisms, such as ovule degeneration and early embryo abortion, which have been documented in large-flowered *Coelogyne* species even following xenogamous crosses (Kolomeitseva et al. 2025). These biological constraints must be considered when designing propagation protocols, particularly for conservation-targeted efforts. Ex situ conservation, especially through living collections in botanical gardens, plays a crucial role in protecting *Coelogyne* species from imminent extinction in the short to medium term (Zhao et al. 2021). Established micropropagation protocols provide effective strategies for both conservation and reintroduction, particularly for less studied species (Naing et al. 2011; De Stefano et al. 2022; Kaladharan et al. 2024).

In vitro propagation of orchids, such as *Phalaenopsis*, *Dendrobium*, and *Vanda*, using vegetative explants including inflorescence segments, pseudobulbs, or leaves, often faces a high risk of microbial contamination, with contamination rates exceeding 50% in some cases, leading to low survival rates (Teixeira et al. 2016; Baby et al. 2019). This increased risk is mainly due to the presence of endogenous endophytic bacteria and fungi within plant tissues, which are not easily removed through surface sterilization alone, as these microorganisms reside inside the tissues rather than solely on the surface (Alibrandi et al. 2020).

As an alternative, asymbiotic seed germination provides a reliable method to reduce contamination risk while supporting embryo development (Jolman et al. 2022). This study reports the successful development of efficient in vitro propagation protocols for two orchid species that are underrepresented in cultivation, *C. cristata* and *C. lawrenceana*. Both species responded well to the optimized culture conditions, showing high germination rates and quick progression from seed to acclimatized plantlets. In our experiments, *C. cristata* achieved 98% germination on ½ MS medium with IAA, while *C. lawrenceana* reached 92% on ½ MS medium with 6-BAP. Our findings build on previous research within the *Coelogyne* genus. For instance, *C. mossiae* achieved a 96.65% germination rate on MS medium (Kaladharan et al. 2024), while *C. asperata* reached 93.69% on VW medium (Alexander et al. 2021). The variation observed among species indicates that germination success depends not only on the choice of culture medium but also on species-specific physiological traits and hormonal needs.

Coelogyne cristata, a montane species adapted to cooler Himalayan environments, is relatively tolerant of lower temperatures. Such ecological adaptation may lead to embryos with higher physiological stability, which could partly explain the higher and more uniform germination observed in vitro. In contrast, *C. lawrenceana*, endemic to tropical Vietnam, is adapted to more stable thermal and moisture conditions, potentially contributing to its slower and less consistent germination response under controlled culture. This ecological divergence is also reflected in developmental timing: *C. cristata* protocorms develop within about 40 days, whereas *C. lawrenceana* takes nearly 60 days despite high germination rates. In *C. cristata*, IAA not only promotes high germination rates but also accelerates protocorm and shoot development. Conversely, although *C. lawrenceana* responds better to 6-BAP during germination, its subsequent development remains slower. These contrasting responses suggest that auxins and cytokinins may have species-dependent and sometimes opposing roles during the transition from germination to protocorm development. Auxins regulate germination through the TIR1/AFB-Aux/IAA-ARF signaling pathway, which modulates transcriptional repression of negative regulators such as ABI3 (Hussain et al. 2020). This mechanism is especially vital for seasonal species like *C. cristata*, where IAA-mediated responses help synchronize germination with favorable seasonal conditions, ensuring quick seedling establishment after dormancy. In contrast, *C. lawrenceana* displays a stronger response to cytokinin treatment. Cytokinins promote cell division, shoot initiation, and organ formation—processes that fit the biology of continuously growing or even-temperature species, where steady metabolic activity allows cytokinin-driven growth and strong early seedling development (Zhao et al. 2024). The balance between these pathways seems crucial and varies among *Coelogyne* species. Previous studies support this, showing that PGR responses significantly influence early development. For instance, in *C. fuscescens*, protocorms only developed after 10 weeks with 0.5 mg L^{-1} NAA (Koirala et al. 2013), while *C. fimbriata* showed delayed growth with auxin treatments (Anuprabha and Pathak 2019). In *C. flaccida*, the highest germination and protocorm formation rates were achieved with a combination of 0.5 mg L^{-1} 6-BAP and 0.5 mg L^{-1} NAA on MS medium after 22 weeks (Parmar and Pant 2015). Overall, these results emphasize that successful in vitro germination in *Coelogyne* depends on species-specific optimization of PGR combinations, not a universal protocol.

The choice of organic additives and cytokinins strongly influenced the efficiency of plantlet development and rooting in *Coelogyne* species. In *C. cristata*, plantlets cultured on medium supplemented with potato homogenate exhibited the highest number of shoots. This result aligns with previous findings for *C. pandurata*, where maximum shoot production was similarly achieved on medium containing potato homogenate (Hartati et al. 2017). This effect is likely attributed to the high content of carbohydrates, vitamins, and phytohormones in potatoes, which promote shoot proliferation (Sonin et al. 2024). Conversely, the lowest plantlet height and number of leaves in both *C. cristata* and *C. lawrenceana* were observed on media supplemented with potato homogenate. Similar findings have been reported in other epiphytic orchid species, such as *Cattleya gaskelliana*, where the addition of potato homogenate to the culture medium reduced growth performance (Hussien et al. 2024). This inhibitory effect is likely due to the high starch content in potato homogenate, which may act as a gelling agent in the medium, reducing nutrient diffusion and absorption, and limiting plantlet development (Lee et al. 2022). In contrast, the combined application of mT and banana homogenate was particularly effective in enhancing both plantlet development and rooting in *C. cristata*. Banana homogenate provides antioxidants, phenolic compounds, and essential nutrients that support morphogenesis while contributing to pH stabilization in the culture medium, an important factor for successful in vitro growth (Lee et al. 2022; Hussien et al. 2024). Furthermore, the addition of mT significantly improved shoot elongation and root induction. The efficiency observed in this study is consistent with earlier reports showing that mT promotes the formation of robust, healthy plantlets with high rooting success and acclimatization rates approaching 100% (Kucharska et al. 2020). Comparable effects of mT have been documented across a wide range of taxa, from woody species such as *Malus* to bulbous plants like *Zephyranthes*, as well as other orchids including *Phragmipedium*. In these cases, mT not only enhanced regeneration but also reduced morphological and physiological abnormalities (Abdalla and Dobránszki 2024; Hussien et al. 2025b; Verma et al. 2025). This enhanced performance of mT may be attributed to its unique metabolic pathway, as it does not lead to the accumulation of inhibitory N-glucosides at the plantlet base, a limitation commonly associated with other cytokinins (Strnad 2021). As a relatively novel cytokinin compared with 6-BAP, mT has received limited attention in *Coelogyne* research. The present findings not only confirm its strong potential for improving propagation efficiency in this genus but also provide a practical basis for developing more reliable and cost-effective micropropagation protocols.

In *C. lawrenceana*, vigorous plantlet formation was achieved on medium supplemented with coconut water and 6-BAP. This likely indicates a synergistic effect that enhances cell division at low cytokinin concentrations. Notably, the highest number of adventitious shoots was recorded in cultures supplemented with 0.5 mg L^{-1} mT and coconut water, further confirming the effectiveness of mT in the in vitro propagation of *Coelogyne* species. The use of mT has shown promising results in promoting shoot multiplication and regeneration in both terrestrial and epiphytic orchid species, including *Malaxis wallichii* and *Dendrobium chrysanthum* (Bose et al. 2017; Poeaim et al. 2022). Consequently, mT represents a promising alternative cytokinin for the propagation of priority orchid species, contributing significantly to ex situ conservation and long-term reintroduction programs. Its advantage is further reinforced by minimizing common limitations associated with other cytokinins, such as hyperhydricity and poor rooting (Nongdam et al. 2023).

At the rooting stage, interspecific differences in growth and rooting capacity remained evident. *C. cristata* plantlets exhibited a high rooting percentage, comparable to that of *C. lawrenceana*. Rooting was most successful on ½ MS medium supplemented with either 1.0 mg L^{-1} IBA or 0.5 mg L^{-1} IAA combined with coconut water. Similar positive effects of coconut water on rooting have been reported in other orchid species, including *Cymbidium aloifolium* and *Dendrobium moniliforme* (Rohmah and Taratima 2022; Hwang et al. 2024). This is likely due to the presence of carbohydrates, such as mannose and sorbitol, along with vitamins and naturally occurring plant hormones like zeatin and kinetin, in coconut water (Aishwarya et al. 2022). Additionally, the inclusion of auxins in the nutrient medium regulates internal physiological processes and mobilizes biochemical compounds that stimulate root initiation and elongation (Kurepa et al. 2023). Interestingly, *C. lawrenceana* demonstrated a higher rooting capacity at a lower IAA concentration, suggesting greater sensitivity to exogenous auxins. Comparable findings were reported by Hussien et al. (2025c), who observed that high concentrations of IBA, particularly when combined with banana homogenate, inhibited root formation in various orchid species, including *C. gaskelliana*. Overall, the high rooting success achieved in *C. cristata* and *C. lawrenceana* confirms the suitability of the developed protocol for efficient large-scale propagation.

During the acclimatization stage, both species adapted successfully to a substrate composed of *Sphagnum* moss, charcoal, and pine bark (1:1:1). However, the overall acclimatization rate remained relatively low compared to the success achieved in vitro. This limited survival may be attributed to several factors, primarily related to the epiphytic nature of *Coelogyne* species. Under natural conditions, these orchids rely on symbiotic associations with mycorrhizal fungi, which enhance water and nutrient uptake. The absence of the necessary symbiotic fungi in artificial substrates likely reduced resource acquisition efficiency, resulting in physiological stress and lower survival rates (Castillo-Pérez et al. 2021; Kaladharan et al. 2024). Additionally, factors such as inadequate aeration, suboptimal moisture retention, and insufficient physiological adaptation of in vitro-derived plantlets to ex vitro conditions may have further contributed to reduced acclimatization success. Such adaptation limitations are often associated with structural anomalies in leaves and roots, including immature stomata, underdeveloped cuticle layers, and poorly differentiated vascular tissues, which reduce the ability of plantlets to cope with external stresses (Mani et al. 2022). Mycorrhizal inoculation can significantly enhance acclimatization success; for instance, *Guarianthe skinneri* exhibited a higher survival rate when inoculated with endophytic fungi such as *Nigrospora* sp. and *Coprinellus* sp., highlighting the critical role of fungal associations during ex vitro transfer (Aucencia and Anne 2024). Moreover, growth retardants have been shown to improve abiotic stress tolerance by modulating hormonal pathways that regulate stomatal function, delay leaf senescence, and activate stress signalling (Thakur et al. 2025). Therefore, combining symbiotic inoculation with the application of growth retardants may represent a promising strategy to enhance survival rates during the acclimatization of *Coelogyne* species and to optimize overall propagation protocols.

In conclusion, this study successfully developed an efficient and reproducible in vitro protocol for artificial pollination, seed germination, and clonal micropropagation of *C. cristata* and *C. lawrenceana*. Controlled pollination resulted in fruit set rates of 65% in *C. cristata* and 45% in *C. lawrenceana*. Asymbiotic germination was most effective on ½ MS medium supplemented with 0.5 mg L^{-1} IAA for *C. cristata* and 0.5 mg L^{-1} 6-BAP for *C. lawrenceana*, achieving germination rates of 98% and 92%, respectively. The addition of organic additives significantly improved protocorm and plantlet development: 0.5 mg L^{-1} mT with banana homogenate for *C. cristata*, and 0.5 mg L^{-1} 6-BAP with coconut water for *C. lawrenceana*. Rooting was most successful in *C. cristata* on ½ MS medium containing 1.0 mg L^{-1} IBA or 0.5 mg L^{-1} IAA with coconut water, whereas *C. lawrenceana* responded best to 0.5 mg L^{-1} IAA. The acclimatization phase showed high survival rates 87% for *C. cristata* and 82% for *C. lawrenceana,* when transferred to a substrate mix of *Sphagnum* moss, charcoal, and pine bark. This protocol provides a practical and scalable framework for the ex situ conservation of these threatened species, demonstrating strong potential for application in botanical gardens, orchid nurseries, and conservation programs. Its high success rate from seed to acclimatized plantlet not only enables large-scale propagation but also contributes to reducing pressure on wild populations.

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