

Induction of Protocorm-like Bodies (PLBs) and Plant Regeneration in *Cymbidium chloranthum* (Orchidaceae).

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Abstract. *Cymbidium chloranthum* Lindl. is an indigenous orchid native to Sabah and several tropical countries in Asia. The significant horticultural value of orchids has led to many wild orchid species being threatened with extinction due to overcollection and habitat destruction. In this study, an efficient protocol for the in vitro regeneration and mass propagation of this orchid was developed. Protocorms of *C. chloranthum* were cultured on Murashige and Skoog (MS) basal medium containing plant growth regulators (PGRs) such as meta-Topolin (mT) and 6-benzylaminopurine (BAP) at concentrations of 0.5 or 1.0 mg/L, and coconut water (CW) at 5 or 10% (v/v). The cultures were incubated at 25±2°C under 12h of photoperiod. The MS basal medium devoid of any PGRs served as a control. After 90 days of culture, the combination of 0.5 mg/L mT and 0.5 mg/L BAP significantly promoted 2.97±0.53 shoots and 3.83±1.07 roots, respectively. The same treatment also promoted protocorms multiplication with an average production of 2.24±0.55 PLBs. During the acclimatization phase, 66% of the regenerated plants survived. The fully acclimatized plants were reintroduced into their natural habitat in Tenom Orchid Centre. The current approach offers a sustainable way to meet commercial demand while conserving the remarkable species in its natural habitat.

1 Introduction

Orchidaceae is one of the most varied and extensive families of flowering plants with over 28,500 species. The diverse shape, growth form, life cycle, and habitat of the Orchidaceae family members create a variety of physiological characteristics that attract human interest. In Malaysia, approximately 978 species of orchids are reported in Peninsular Malaysia, and around 3,000 more species are found in Sabah and Sarawak [1].

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Most orchid species are facing extinction in the wild and losing their native habitats worldwide due to human overexploitation, deforestation, and conversion of land for agricultural purposes, with some species on the verge of becoming endangered [2]. *Cymbidium chloranthum* is one of the declining natural populations of terrestrial orchid species, native to Sabah. This orchid is commonly known as the green flowered *Cymbidium* and is classified as a rare species that holds horticulture importance value. *Cymbidium chloranthum* is distributed in the lowland forests of Borneo, Sumatra, Kalimantan, and Java [3]. In Sabah, this orchid has only been discovered in the Crocker Range foothills of the Crocker Range Park boundary, growing at elevations of 200 to 1,000 meters [3]. The flowering season of *C. chloranthum* was not fixed in any month, but it can be found blooming from June to November [4]. The high market value of many Sabah wild orchids has led to their increasing popularity and commercial growth, resulting in unsustainable harvesting and frequent illegal collecting. Other factors, including deforestation, agricultural expansion and climate change are also contributing to the declining population of *C. chloranthum*, which has led to this species being listed on the International for Conservation of Nature (IUCN) Red List of Threatened Species in 2011 [5], and included in CITES Appendix II to regulate trade prevent practices incompatible with the species' survival (<https://cites.org/eng>). Conservation of orchid plants has become a major concern, because of the risk of losing a rich genetic resource. In natural environments, the multiplication rate of orchids through sexual reproduction is relatively low due to the reliance on specific mycorrhizal fungi in the soil for seed germination and growth. The in vitro culture technique has proved to be the most significant method for commercial propagation and the conservation of rare orchid species. This approach has become an important and informative method for reproducing and increasing the availability of many *Cymbidium* orchids [6-10]. The plant regeneration of orchids involved the formation of secondary protocorms known as protocorm-like bodies (PLBs) from the explant. It has been suggested that the formation of PLBs is a favoured method for orchid propagation, as a substantial mass of PLBs can be obtained and produced within a short period when cultured in appropriate nutrient media, potentially leading to the mass production of plantlets [11]. The incorporation of plant growth regulators (PGRs) such as 6-benzylaminopurine (BAP) in culture media has substantially promoted PLBs induction in *Dendrobium barbatulum* [12], *Oncidium* hybrid [13], and *Bulbophyllum auricomum* [14]. Meanwhile, the effect of meta-Topolin (mT) on orchid regeneration is still underexplored, and, so far, has been tested on certain species, including *Coelogyne ovalis* [15], *Dendrobium chrysanthum* [16], and *Vanilla planifolia* [17]. Additionally, coconut water was previously reported to be beneficial for plant regeneration in *Cymbidium aloifolium* [18]. Therefore, the current study was the first to determine the effectiveness of BAP, mT, and coconut water in promoting protocorm proliferation and plant regeneration in *C. chloranthum*.

2 Materials and Methods

2.1. Effects of PGRs and coconut water on protocorm development

Seeds of *C. chloranthum* were previously germinated on Murashige and Skoog (MS), 1962 medium [19]. The green and healthy protocorms at stage 3 (Fig. 1) were then selected and used as explants in this study. Protocorms were transferred into petri dishes containing MS basal media supplemented with various types of PGRs such as meta-Topoline (mT), 6-benzylaminopurine (BAP) and coconut water (CW) at different concentrations. Basal medium devoid of growth regulators served as the control. The pH of the media was adjusted to 5.6-5.8 with 0.1N of HCl or NaOH before autoclaving at 121°C, 103.4 kPa for 20 min. All cultures were incubated for 90 days at 25 ± 2°C under 12 hours of photoperiod. Subculturing into the fresh medium was conducted monthly.

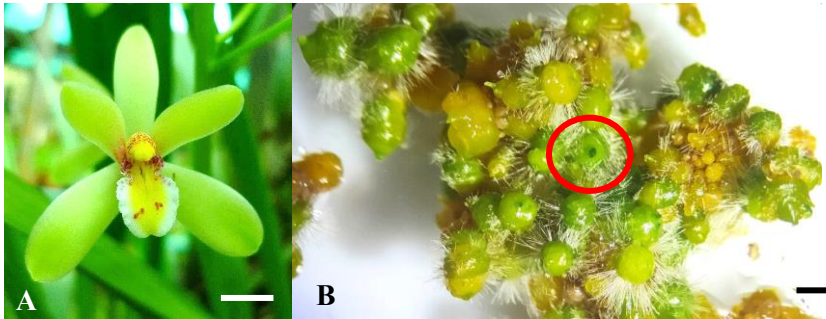


Fig. 1. (A) Flower of *C. chloranthum*; (B) Stage 3 protocorms of *C. chloranthum* cultured on MS medium. Bar: (A) 1 cm; (B) 1.0 mm

2.2. Further development of seedlings and plantlets

To observe the further development of the seedlings and plantlets regenerated from PLBs, these explants were transferred to MS basal media containing 0.5 mg/L mT supplemented with or without 0.5 mg/L of BAP. Each treatment consists of three individual seedlings or three plantlets. MS medium without any PGRs served as control. The medium pH was adjusted to 5.6-5.8 with 0.1N of HCl or NaOH before autoclaving at 121°C, pressure of 105kPa for 20 minutes. All cultures were incubated for 90 days at 25±2°C and exposure to 12 hours of photoperiod. Subculturing was conducted every 30 days of culture.

2.3. Acclimatization of regenerated plants

Regenerated plants with leaves and roots were removed from the culture jars and washed thoroughly under a running water tap to remove residual medium gel. The seedlings were transferred to a small plastic pot filled with charcoal, sphagnum moss and coconut husks at a ratio of 1:1:1 [20]. Acclimatization was performed in a plant nursery with 70% shading. The pots were initially covered with a polythene sheet for two to three weeks to maintain high humidity.

2.4. Experimental design and statistical analysis

The experiment utilized a completely randomized design (CRD). Data were analyzed using IBM SPSS Statistics version 29.0. A one-way analysis of variance (ANOVA) was performed to assess the effects of growth regulators on plant regeneration. Each treatment included 10 replications, with each replicate consisting of five protocorms. The means were compared using Duncan's multiple range test at a significance level of $p < 0.05$.

3 Results and Discussion

3.1. Protocorm developmental stages

Table 1 describes the developmental stages of *C. chloranthum* from seed to seedling. In this study, the stage 3 protocorm of *C. chloranthum* was used as an explant (Fig. 2A). After 15 days of culture, the formation of absorbing hair (rhizoid) was observed from the basal protocorms (Fig. 2B). It was then followed by the formation of leaf primordium at the tip of protocorm after 30 DAC (Fig. 2C). The formation of leaf and root was observed after 60

DAC (Fig. 2D). Complete seedling of *C. chloranthum* with two leaves and roots was achieved within 90 DAC (Fig. 2E).

Table 1. *Cymbidium chloranthum* seed-to-seedling developmental stages.

Stage	Description
1	Mature seed without embryo
2	Swollen embryo that breaks out from testa
3	Protocorm
4	Protocorm with rhizoid and formation of leaf primordium
5	Appearance of first leaf and root
6	Complete seedling with elongated leaves and roots

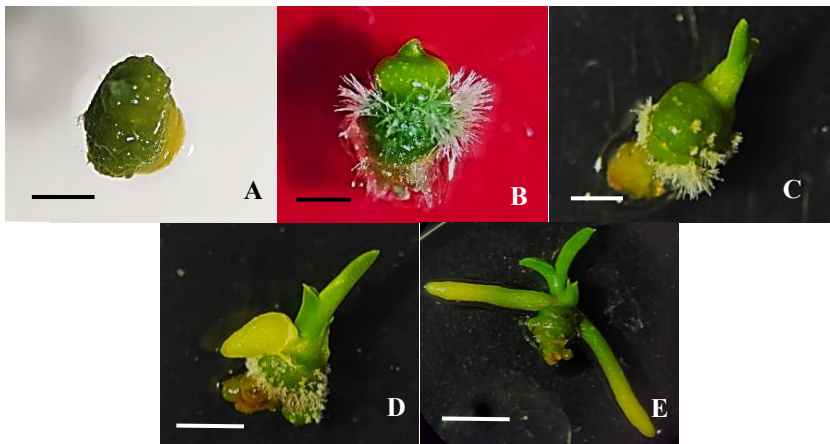


Fig. 2. Protocorm developmental stages of *C. chloranthum*. (A) Stage 3: protocorm; (B) Protocorm with rhizoid; (C) Stage 4: Protocorm with rhizoid and formation of leaf primordium; (D) Stage 5: Appearance of first leaf and root; (E) Stage 6: Complete seedling with elongated leaves and roots. (Bar (A) 1 mm; (B) 2 mm; (C-D) 3 mm; (E) 4 mm).

3.2 Effect of PGRs and coconut water on protocorm proliferation

Protocorms of *C. chloranthum* cultured on MS basal medium supplemented with PGRs and CW gave various responses depending on the treatment. The percentage of protocorm proliferation ranged from 14% to 92% (Table 2). The addition of 5% CW, 0.5 mg/L mT, 1 mg/L mT, 0.5 mg/L BAP, 1.0 mg/L BAP, and the combination of 0.5 mg/L mT + 0.5 mg/L BAP significantly promoted protocorm proliferation compared to the control medium and treatment with 10% CW, which recorded at 14% and 42% of proliferation, respectively. The proliferation of protocorms occurred when the secondary protocorms named protocorm-like bodies (PLBs), were induced directly from the protocorm explant without callus formation. The combination treatment of 0.5 mg/L mT + 0.5 mg/L BAP has effectively promoted the formation of 2.24 ± 0.55 new PLBs per protocorm. It was also observed that the secondary protocorms (PLBs) were developed with shoot and root at 2.34 ± 0.54 and 2.97 ± 1.59 , respectively (Fig 3F). Previously, MS basal media was reported to be effective in enhancing the growth and development of *C. devonianum* Paxt [21]. Studies on the synergistic effect of mT and BAP in orchid micropropagation are still limited. However, Praminik [22] reported that this combination effectively promoted secondary somatic embryo formation in *Phalaenopsis* 'AMP 17'. In another study, mT produced a better response than BAP in inducing shoot and root of *Dendrobium chrysanthum* Wall ex. Lindl [16]. Meanwhile, the

combination of BAP and NAA enhanced the formation of secondary protocorms (PLBs) from the primary protocorm of *C. mastersii* [11]. The current finding revealed that the combination of BAP and mT promoted protocorm proliferation and plantlet development of *C. chloranthum*, even with the absence of auxin in the treatment.

Treatment with BAP or mT alone was also effective in promoting protocorm proliferation. However, root formation was decreased when higher BAP or mT at 1.0 mg/L was applied (Table 2). The current finding indicates that *C. chloranthum* protocorms preferred lower-concentrated cytokinin treatments to encourage root production. On the contrary, the increased concentration of CW at 10% (v/v) inhibited root formation and resulted in necrosis affecting $52.00\pm 43.4\%$ of the explants. Coconut water has been widely utilized in the orchid in vitro propagation industry and was reported beneficial in culturing many orchid species including *Dendrobium aqueum* Lindley [23], *Vanda dearei* [24], and *Phalaenopsis amboinensis* J.J.Sm [25]. Coconut water contains essential nutrients for plant cell development such as organic acids, vitamins, amino, organic ions, carbohydrates, and enzymes [24]. However, the addition of CW in this study was unfavourable to enhance the protocorm proliferation of *C. chloranthum*. A report by Tawaro [26] suggested that an improper balance of nutrients or lack of required growth-stimulating components may promote the yellowing, browning or necrosis of protocorms without any further growth during the culture of *C. finlaysonianum* protocorms.

3.3 Further development of seedlings and regenerated plantlets

To assess the further development of seedlings and regenerated plantlets from PLBs, these explants were transferred into a new basal medium containing 0.5 mg/L mT, with or without 0.5 mg/L BAP. Results in Table 3 show that all seedlings and plantlets survived 80 -100% on all treatments with a minimum production of two leaves. The combination of 0.5 mg/L BAP + 0.5 mg/L mT significantly promoted a higher number of roots compared to other treatments, with an average production of 5.80 ± 3.12 and 5.83 ± 1.98 roots from seedlings and plantlets, respectively. At this stage, all treatments still promoted PLBs, exhibiting a range of two to three PLBs per responsive explant. Previously, Guo [27] described the PLBs explants as developed structures that originated from the protocorms and had a different path of growth mechanism as they underwent differentiation and specialization, resulting in slower growth rate compared to seedling developmental growth paths. However, the current study proved that the plantlets of *C. chloranthum* can regenerate and perform proliferation similarly to the seedling. The current findings agreed with Ramesh and Renganathan [10] that the PLBs of *C. elegans* undergo organogenesis to induce the plant organs (leaf and root) and later transform into complete plantlets due to the presence of meristematic cells that continue to differentiate to form leaves and roots.

Table 2. Effects of PGRs and coconut water on protocorm development of *C. chloranthum* in treatment using MS basal medium under 12h photoperiod at 25±2°C after 90 DAC.

Treatment	Protocorm Survival Rate (%±SD)	Protocorm Shoot (%±SD)	Shoot per explant (Mean±SD)	Protocorm with Root (%±SD)	Root per explant (Mean±SD)	Protocorm Proliferation (%±SD)	Number of Secondary Protocorm (Mean±SD)	Protocorm Mortality (%±SD)
Control	92.00±13.98 ^a	100.00±0.00 ^a	1.20±0.29 ^{cd}	69.50±28.52 ^a	1.76±0.75 ^b	14.50±13.83 ^c	1.20±1.23 ^{bc}	8.00±13.98 ^b
5% CW (v/v)	94.00±9.66 ^a	89.50±17.39 ^a	1.52±0.34 ^{bcd}	8.00±13.98 ^d	0.45±0.76 ^{cd}	70.50±24.09 ^a	1.61±0.61 ^{abc}	6.00±9.66 ^b
10% CW (v/v)	48.00±43.41 ^b	51.50±46.07 ^b	1.06±0.98 ^d	0.00±0.00 ^d	0.00±0.00 ^d	42.33±41.75 ^b	1.11±1.11 ^c	52.00±43.41 ^a
1.0 mg/L mT	72.00±28.60 ^a	82.50±33.44 ^a	1.62±0.69 ^{bcd}	35.83±32.87 ^{bc}	1.77±1.34 ^b	68.17±28.58 ^a	1.68±0.79 ^{abc}	28.00±28.60 ^b
0.5 mg/L mT	82.00±33.27 ^a	88.00±31.55 ^a	2.14±1.22 ^{ab}	51.33±29.32 ^{ab}	2.21±1.50 ^{ab}	80.00±31.27 ^a	1.97±0.95 ^{ab}	16.00±32.39 ^b
1.0 mg/L BAP	86.00±13.50 ^a	83.67±15.25 ^a	1.85±0.49 ^{abc}	11.83±20.91 ^{cd}	0.33±0.54 ^{cd}	85.17±13.27 ^a	1.78±0.67 ^{abc}	14.00±13.50 ^b
0.5 mg/L BAP	92.00±19.32 ^a	96.00±8.43 ^a	1.68±0.58 ^{abcd}	38.50±39.16 ^b	1.26±1.05 ^{bc}	81.50±11.56 ^a	2.03±0.56 ^{ab}	8.00±19.32 ^b
0.5 mg/L mT + 0.5 mg/L BAP	82.00±27.41 ^a	94.00±9.66 ^a	2.34±0.54 ^a	51.83±31.39 ^{ab}	2.97±1.59 ^a	92.00±10.33 ^a	2.24±0.55 ^a	18.00±27.41 ^b

Note: Means of 10 replicates and mean values followed by the same letter (s) within each column are not significantly different at $p < 0.05$, according to Duncan's Multiple Range Test. mT: Meta-Topoline; BAP: 6-Benzylaminopurine.

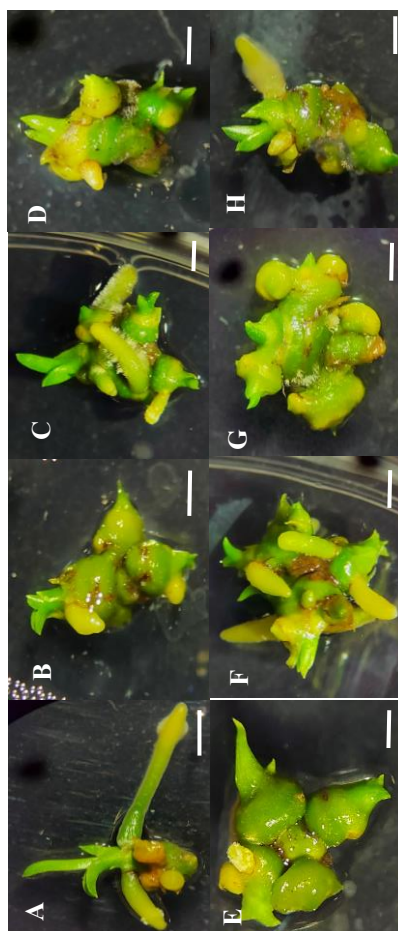


Fig. 3. *C. chloranthum* protocorm development and proliferation at 90 DAC in MS medium supplement with: (A) MS control, (B) 1.0 mg/L mT, (C) 0.5 mg/L mT, (D) 1.0 mg/L BAP, (E) 0.5 mg/L BAP, (F) 0.5 mg/L mT + 0.5 mg/L BAP, (G) 10% CW (v/v), and (H) 5% CW (v/v). Bar= 2 mm

Table 3. Effects of PGRs on seedling and plantlet development in treatment using MS basal medium under 12h photoperiod at 25±2°C after 90 DAC.

Treatment	Number of Leaf (±SD)	Leaf Length (cm ±SD)	Number of Root (±SD)	Root Length (cm ±SD)	Survival (%±SD)	Seedling Proliferation (%±SD)	Number of New PLBs (±SD)
Control	2.97±0.53 ^a	0.79±0.29 ^a	3.83±1.07 ^b	2.78±1.18 ^a	100.00±0.00 ^a	86.67±27.59 ^a	2.27±0.96 ^a
Seedling 0.5 mg/L mT	2.40±0.87 ^a	0.45±0.23 ^b	3.37±1.84 ^b	1.08±0.56 ^b	90.00±31.62 ^a	90.00±31.62 ^a	2.87±1.31 ^a
0.5 mg/L mT + 0.5 mg/L BAP	2.50±0.91 ^a	0.46±0.18 ^b	5.80±3.12 ^a	1.01±0.52 ^b	83.33±36.00 ^a	90.00±31.62 ^a	3.50±2.85 ^a
Control	2.70±0.29 ^a	0.57±0.30 ^a	3.63±1.33 ^b	1.38±0.76 ^a	100.00±0.00	100.00±0.00 ^a	2.77±1.21 ^a
Plantlet 0.5 mg/L mT	2.27±0.31 ^b	0.27±0.07 ^b	2.90±0.79 ^b	0.75±0.22 ^b	100.00±0.00	96.67±10.54 ^a	3.63±1.39 ^a
0.5 mg/L mT + 0.5 mg/L BAP	2.40±0.44 ^{ab}	0.29±0.06 ^b	5.83±1.98 ^a	0.93±0.20 ^b	100.00±0.00	100.00±0.00 ^a	3.83±1.17 ^a

Note: Means of 10 replicates and mean values followed by the same letter (s) within each column are not significantly different at $p < 0.05$, according to Duncan's Multiple Range Test.
 mT: Meta-Topoline; BAP: 6-Benzylaminopurine.

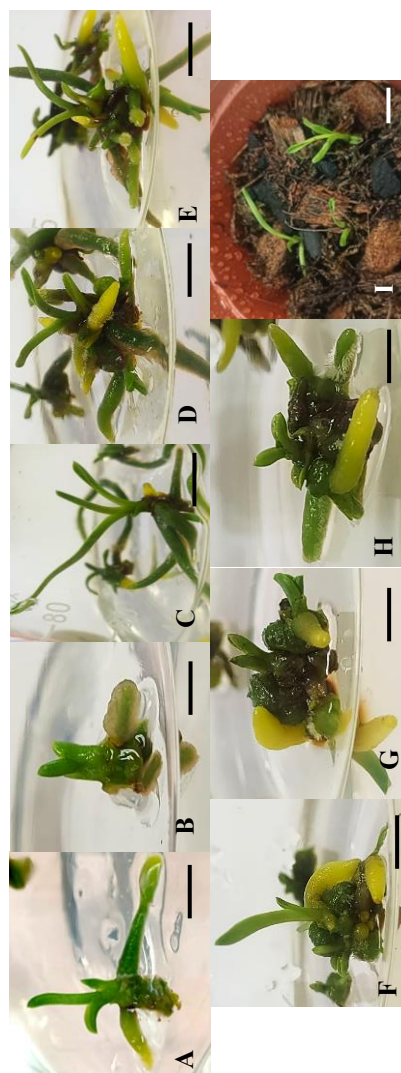


Fig. 4. Further development of *C. chloranthum* seedling and plantlet. (A) Stage-6 seedling used as explant; (B) Stage-6 plantlet regenerated from PLBs used as explant; (C) Seedlings at 90 DAC in MS control; (D) MS + 0.5 mg/L mT; (E) MS + 0.5 mg/L mT + 0.5 mg/L BAP; (F) Plantlet at 90 DAC in MS control; (G) MS + 0.5 mg/L mT; (H) MS + 0.5 mg/L mT + 0.5 mg/L BAP; (I) Acclimatized seedlings. Bar (A – C): 4 mm; (D – E): 5 mm; (F – H): 3 mm; (I): 6mm.

3.4 Acclimatization

After 180 days of culture, seedlings of *C. chloranthum* were randomly selected and transplanted into potted media containing mixtures of charcoal, coconut husk chip and sphagnum moss (1:1:1) (Fig. 4I). After four weeks in the acclimatization phase, the seedlings obtained survival rates of 66.67%. Charcoal was added to the potting mixtures because it provides a highly porous structure that may enhance porosity, and water-holding capacity, thus promoting the proliferation of beneficial microorganisms [20]. The coconut husk chips functionally retain water and provide aeration for root respiration, and finally, sphagnum moss has a low pH and absorbs water and mineral nutrients for seedlings [28]. The fully acclimatized *C. chloranthum* seedlings were reintroduced into their natural habitat in Tenom Orchid Centre, Lagud Seberang.

4 Conclusion

The ornamental potential of *C. chloranthum* remains underexplored due to its rare and declining status. This study has successfully established an efficient in vitro regeneration protocol for *C. chloranthum* protocorm via PLBs induction. Two regeneration pathways of *C. chloranthum* were observed: seedlings regeneration on medium without PGR; and plantlet regeneration from PLBs on media with PGRs. The combination of 0.5 mg/L BAP + 0.5 mg/L mT was most effective in promoting the highest number of secondary protocorms. These findings are significant, offering promising potential for the mass propagation of *C. chloranthum* for both horticultural purposes and essential conservation efforts.

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