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In vitro Propagation and Forest Reestablishment of *Cymbidium finlaysonianum* Lindl., an Endangered Medicinal Orchid

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Abstract

An *in vitro* plant regeneration protocol was successfully established for *Cymbidium finlaysonianum* Lindl., an epiphytic endangered medicinal orchid, by culturing seeds from 4-month-old green self-pollinated fruits. Tetrazolium (TZ) viability test indicated a mean embryo viability of 97.80 %. Three asymbiotic orchid seed germination media, namely Knudson C medium (KC), New Dogashima medium (ND), and Vacin and Went medium (VW), were studied to select a suitable medium for seed germination. The highest seed germination percentage was 83.8 % on VW medium. Protocorm-like bodies (PLBs) of *C. finlaysonianum* Lindl. induced from protocorm segments cultured in VW liquid medium supplemented with 8.84 µM 6-benzylaminopurine (BAP) gave the highest % PLB formation and number of PLBs per explant, at 85.7 and 5.2, respectively. VW medium supplemented with 0.2 % (w/v) activated charcoal (AC) gave the highest number of roots per plantlet and root length, at 6.4 roots and 5.2 cm, respectively. The highest percentage of plantlet survival recorded at 24 months after reestablishment in the forest was 71.6 %.

Keywords: *In vitro* propagation, *Cymbidium finlaysonianum*, acclimatization, forest reestablishment, boat orchid

Introduction

Orchid species are facing a serious threat, and an uncertain future, because of unscrupulous collection for commercial usage [1]. This vulnerability could also result from their highly specialized life cycles, such as their long juvenile period, and the fact that seed germination in nature is dependent on association with mycorrhizal fungi [2]. *In vitro* propagation techniques have been widely used for the conservation of threatened orchid species [3-5]. Although this approach has been used for a long time, there is limited scientific research on the *in vitro* germination of orchid seeds, especially when considering the large number of species in the family. Moreover, most of the published studies have focused on those partial to terrestrial habitats and/or temperate orchid species.

Cymbidium finlaysonianum Lindl. is well distributed in Vietnam, Cambodia, Thailand, Sumatra, Java, Borneo, the Philippines, and Malaysia [6]. *Cymbidium*, or ''boat orchid'', is a popular orchid grown commercially worldwide [7]. Today, orchids, such as *Cymbidium*, *Dendrobium*, *Oncidium*, and

Phalaenopsis, are marketed globally, and the orchid industry contributes substantially to the economy of many South East Asian countries, such as Thailand, with exports of orchid cut-flowers and plants worth over one billion US dollars each year.

In *Cymbidium*, plantlets were regenerated in *in vitro* using green capsules [8,9], protocorm-like bodies (PLBs) [10], thin cell layers of PLBs [11], and through somatic embryogenesis [12]. *Cymbidium* (Orchidaceae) is an economically important orchid which develops *in vitro* and is now being well-studied [13-15]. Protocorm-like bodies (PLBs) are the best clonal propagules, as they are somatic embryos [16] that can be multiplied through the induction of new PLBs. Thus, the goal of the present study was to establish an effective propagation system by asymbiotic germination, PLB formation and proliferation, plantlet regeneration, greenhouse acclimatization, and reestablishment in forest. Such a protocol would allow for large-scale propagation to meet commercial needs and to directly conserve this threatened orchid species by reducing wild collection.

Materials and methods

Seed collection and viability estimation

Four-month-old green capsules from self-pollination were collected from 5-year-old plants of *C. finlaysonianum* Lindl. in Songkhla, Thailand (**Figure 1**). Seed viability (**Figures 2A** and **2B**) was evaluated by using the Tetrazolium test [17]. This method uses a solution of 2, 3, 5-triphenyl tetrazolium chloride (TTC), in which viable embryos are stained. Seeds were immersed in a 1 % (w/v) TTC solution and stored in the dark for 24 h at 30 °C. Samples of 800 seeds were analyzed under an optical microscope. Percentage of viable seeds was calculated by dividing the number of viable embryos by the total number of analyzed embryos.

Plant collection, capsule preparation and sterilization

Each capsule of *C. finlaysonianum* Lindl. was cleaned by washing with running tap water for a few minutes, subsequently soaked in 95 % ethanol, and flamed. Seeds from the surface sterilized capsules were taken out by longitudinally splitting the capsule with a sharp sterilized surgical blade (**Figure 2A**).

Effects of basal media on germination *in vitro*

Three asymbiotic orchid seed germination media, namely Knudson C medium (KC) [18], New Dogashima medium (ND) [19], and Vacin and Went medium (VW) [20], were tested to select a suitable medium for seed germination. The germination percentage of seeds at 60 days was calculated by the number of germinated seeds or protocorms divided by the number of seeds cultured and multiplied by 100. The seed derived protocorms were used in the next experiments related to PLB induction and proliferation. All experiments were performed in 3 independent replicates, with 10 culture Petri dishes per replication.

The culture medium was solidified with 0.75 % agar (commercial grade). The pH of the media was adjusted to 5.7 with 1 N KOH or 1 N HCl prior to autoclaving for 15 min at 121 °C. Whole seeds were placed on the media and maintained at 25 ± 2 °C under a 16 h photoperiod with light supplied by cool-white fluorescent lamps at an intensity of 10 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD). The percentages of seed germination were compared statistically after 60 days of culture.

Effects of BAP and coconut water on formation of PLBs from protocorms *in vitro*

Protocorms derived from seeds germinated for 90 days were transferred to VW liquid medium for proliferation of PLBs. The basal medium used in this study was VW liquid medium supplemented with different concentrations of the cytokinins, 6-benzylaminopurine (BAP; 0, 2.21, 3.80, 4.42, 8.84, and 13.26 μM) and coconut water (CW; 0 and 15 % (v/v). BAP were used alone or in combination with CW and added to culture medium prior to autoclaving. The pH of VW liquid medium was adjusted to 5.0 with 1 N KOH or 1 N HCl prior to autoclaving for 15 min at 121 °C. The cultures were incubated at 25 ± 2 °C under a 16 h photoperiod with light supplied by cool-white fluorescent lamps at an intensity of 10 μmol

m^{-2} s^{-1} photosynthetic photon flux density (PPFD) in culture room conditions. The percentage of PLB formation and the mean number of PLBs per explant were compared statistically after 60 days of culture.

Effects of concentrations of activated charcoal on root development

PLBs derived shoots were transferred to VW medium without any plant growth regulators but supplemented with activated charcoal (AC) at different concentrations (0, 0.1, 0.2 % w/v). The AC was added prior to autoclaving. All culture media were supplemented with 15 % (v/v) coconut water. The culture media were solidified with 0.75 % (w/v) agar (commercial grade). The pH of the media was adjusted to 5.0 with 1 N KOH or 1 N HCl prior to autoclaving for 15 min at 121 °C. All explants were placed on the surfaces of VW medium and maintained at 25 ± 2 °C under a 16 h photoperiod with light supplied by cool-white fluorescent lamps at an intensity of 10 µmol m^{-2} s^{-1} PPFD. The numbers of roots per plant and root length per plant were compared statistically after 60 days of culture.

Transplantation of plantlets/greenhouse acclimatization and forest reestablishment and reintroduction

After 2 months of culture*, in vitro* rooted plantlets were taken out from culture bottles and rinsed thoroughly with tap water to remove residual nutrients and agar from the plantlets. The plantlets were then transplanted to pots containing sterilized coconut husks. All plantlets were grown in the greenhouse with 70 - 80 % relative humidity and about 12 h photoperiod, 300 - 400 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) (shaded sunlight), and 33 ± 1 to 30 ± 1 °C day/night temperature. The young plants were sprayed with water twice a day for 3 months. The hardened plants were reintroduced into the Rusamilae forest, Pattani province, for *in situ* conservation. The percentage of survival rate was scored at 24 months after reestablishment in the forest.

Experimental design and statistical analysis

All the experiments were conducted using a completely randomized design (CRD), with 5 replicates per treatment, and the experiments were repeated 3 times. The results are expressed as mean ± SE of 3 experiments. The data were analyzed by ANOVA using SPSS version 17.5, and the mean values were separated using Duncan's multiple range test (DMRT) at a 5 % probability level.

Results and discussion

Seed collection and viability estimation

Seed viability of *C. finlaysonianum* Lindl. was evaluated by Tetrazolium (TZ) viability test. TZ viability test (**Figure 2B**) indicated a mean embryo viability of 97.80 %.

Viability tests, such as the triphenyl tetrazolium chloride (TTC) reduction assay [21] and the fluorescein diacetate (FDA) staining technique [22], have been largely explored for seeds, protocorms, and PLBs. The TTC assay, qualitative for large tissues and organs, is often used for orchid seeds and embryos, because TTC reduction assay and regrowth observations used in the assessment of seedlings growth or plantlets survival are correlated [23-26]. Dehydrogenases, through respiration in mitochondria, reduce colorless TTC to red triphenylformazan or reduced TTC [27].

Figure 1 (A) The flower of *C. finlaysonianum* Lindl. in a natural habitat in Songkhla, Thailand, and (B) 4-month-old green pods from self-pollination of *C. finlaysonianum* Lindl. (Scale bar, A = 2 cm and B = 10 cm).

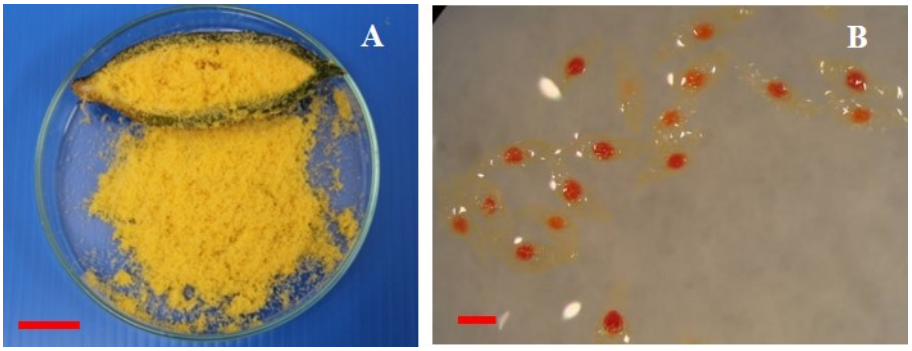

Figure 2 (A) Seeds of *C. finlaysonianum* Lindl., and (B) viable seeds of *C. finlaysonianum* Lindl. evaluated by TTC staining; red stained embryos (Scale bar, A = 1 cm and B = 50 µm).

Effects of basal media on germination *in vitro*

An *in vitro* plant regeneration protocol was successfully established for *C. finlaysonianum* Lindl. by culturing seeds from green pods. Seeds germinated on all the media tested (**Table 1**). The percentage of germinated seeds varied in different media. After 60 days of culture, the highest seed germination (83.8 %), a significant increase, was observed on VW medium, compared to KC and ND media (**Table 1**). The embryos enlarged and occupied the entire seed coat after 45 days of culture (**Figure 3**).

The growth index of protocorms could be evidenced only on VW medium (**Figure 4**). Germination as evidenced by enlargement of the embryos was first observed on VW medium. At the beginning of germination, the undifferentiated embryos swelled by imbibing water at the fourth week (**Figure 3**). The embryos repeated cell division and emerged, rupturing the testa, at the fifth week.

The embryos swelled and developed into globular protocorms, with rhizoid growth at the basal part that turned into green protocorms, at stage 3, within 8 weeks after sowing (**Figure 4A**). The upper part of the protocorms formed the shoot apexes, with leaf primordia that continued to enlarge. Young leaves developed at week 10 - 12 (**Figure 4B**). A pair of leaves emerged from the surface of the protocorms at week 12 - 13 (**Figure 4C**). Roots were seen after 13 weeks, which ultimately developed into seedlings (**Table 2**).

Seed germination and protocorm development was significantly higher on VW medium (83.8 %), followed by ND medium (69.7 %) and KC medium (48.4 %), after 60 days of culture (**Table 1**). However, protocorm formation occurred on ND and KC medium and remained green for 2 to 3 months but failed to develop further into seedlings. Protocorms germinated on ND and KC medium became yellowish and eventually turned brown after 3 months from sowing. These results suggest that ND and KC medium did not promote differentiation of germinated orchid seeds. Therefore, VW medium was the most appropriate basal medium for seed germination and subsequent protocorm development of *C. finlaysonianum* Lindl. among all media tested.

Of the 3 media tested in this study, VW medium showed significantly higher (83.8 %) percentage of seed germination after 60 days of culture initiation. Besides VW medium, the other 2 media also supported moderate germination (69.7 % in ND and 38.4 % in KC); however, germination was delayed, and the germinated seeds failed to differentiate into either PLBs or plantlets.

Table 1 Effects of different culture media on seed germination of *Cymbidium finlaysonianum* Lindl. after culture for 60 days.

Media	Germination (%)
KC	38.4[c]
ND	69.7[b]
VW	83.8[a]

Similar letters within the same columns mean no significant difference at $p \leq 0.05$ by DMRT.

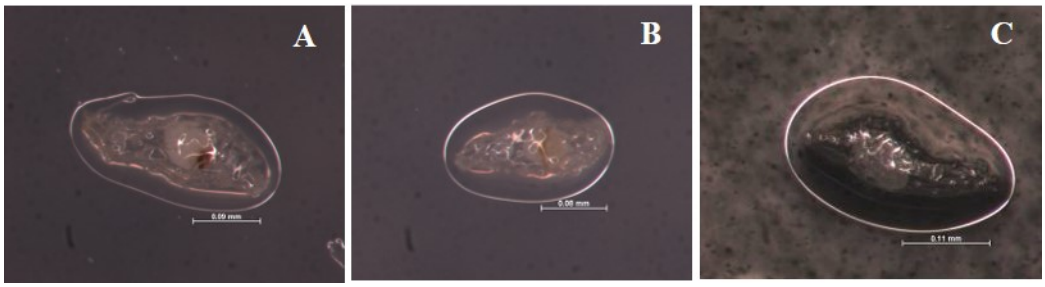

Figure 3 Germination of *C. finlaysonianum* Lindl. seeds after 30 days of culture: (A) on KC medium, (B) on ND medium, and (C) on VW medium.

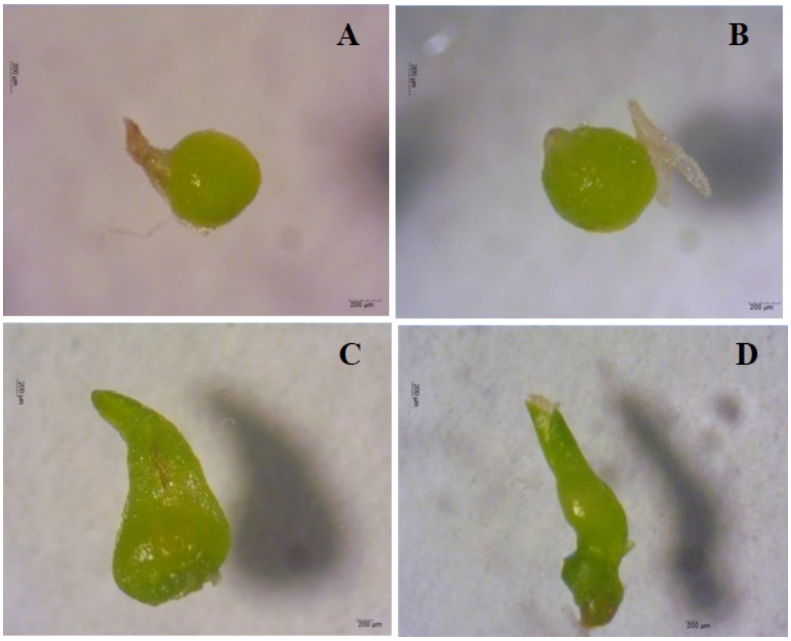

Figure 4 Asymbiotic seed germination and development of protocorms at different stages of growth index: (A) Globular embryo, rhizoids present, (B) protocorm at Growth index 3 (GI3); 8 weeks showing development into globular protocorm, with rhizoid growth at the basal part, which turned into a green protocorm at stage 3, (C) protocorm at GI4 with one leaf; 10 - 12 weeks, and (D) protocorm at GI5 with 2 leaves; 12 - 13 weeks (Scale bar = 200 μm).

Table 2 Seed germination and protocorm developmental categories in *C. finlaysonianum* Lindl. (modified from Suzuki *et al*. [28]).

Stage	Description
0	Non - germinated seed, seed with intact seed coat
1	Swollen embryo, seed coat ruptured by the enlargement of embryo (=germination)
2	Globular embryo, rhizoids present
3	Globular protocorm, with rhizoid growth at the basal part, which turned into a green protocorm, appearance of protomeristem
4	Protocorm bearing one leaf
5	Protocorm bearing 2 or more leaves
6	Emergence of root (seedling stage)

In vitro seed germination has been suggested as a suitable propagation method for the conservation of orchids [29]. According to Hartman [30], specific endogenous growth promoting and inhibiting compounds are involved directly in control of seed development, dormancy, and germination. To initiate seed germination, 3 conditions must be fulfilled, which are seed viability, appropriate environmental conditions, and the overcoming of primary dormancy. In the present study, successful seed germination and protocorm formation was influenced to a great extent by the quality of basal medium.

The nutrient regime for orchid culture is species specific, and no single culture medium is universally applicable for all orchid species. For example, MS medium for *Coelogyne suaveolens* [31]

and *Cymbidium aloifolium* [9], P723 medium for *Eulophia alta* [32], New Dogashima medium for *Calanthe tricarinata* [5], and Lindemann orchid medium (LO) for *Cymbidium bicolor* [33] were reportedly most suitable over other nutrient media.

Asymbiotic seed germination of orchids is greatly influenced by factors like seed age, nutrient media composition, and addition of PGRs [31]. *Cymbidium finlaysonianum* is an epiphytic orchid, which usually germinates within about 4 weeks. In contrast, terrestrial orchids are recalcitrant to germinate, and may have a complicated dormancy pattern, making them one of the most difficult groups of plants to grow from seeds [34].

Both germination rates and protocorm development are affected by asymbiotic culture media. Mineral salts in media vary not only in their concentrations, but also in their available forms. The germination and protocorm development of several orchid species are greatly influenced by different culture media [3,4,35]. For *Cyrtopodium punctatum*, Dutra *et al*. [3] observed that the VW medium provided the highest germination percentage (26.1 %), compared to the half-strength MS and KC media (12.1 and 10.0 %, respectively). The highest germination rate was obtained in the Lindemann orchid (LO) medium for *Cymbidium bicolor* Lindl. [33]. These results showed that the composition of the culture medium is important for the successful germination of orchid seeds, and that the results vary significantly from one species to another. Therefore, it is necessary to determine the most suitable nutritional composition for each species to attain optimal multiplication rates. This will facilitate the subsequent production of a larger number of plants to contribute to the conservation of threatened and endangered species.

A varied response in terms of seed germination percentage has been observed in different media used. Species-specific media for the germination of seeds have been reported in many orchids [36,37].

Out of these 3 media, VW medium showed a significantly higher percentage of seed germination than the other 2 counterparts (**Table 1**). This reaffirmed the fact that the selection of optimal nutrient medium is most crucial for asymbiotic seed germination in orchids, presumably because of the differences in the balance and supply of organic and inorganic nutrients [34,38].

The optimal conditions for orchid seed germination and seedling growth are specific to each genus, and sometimes for each species [39]. *In vitro* mineral nutrition studies on embryos have become particularly important for understanding the ontogenetic and biochemical processes involved in seedling development [40]. According to Stewart [41], orchids that responded to *in vitro* propagation can be divided into 2 broad groups based on their basic nutritional needs. The first group comprises orchids whose seeds germinate easily in a culture medium with a simple nutrient composition; for example, Knudson [18], Vacin and Went [20] media. The second group consists of species that require a more elaborate culture medium, richer in both macro- and micro-nutrients, such as Murashige and Skoog [42] medium.

Effects of BAP and coconut water on formation of PLBs from protocorms *in vitro*

Protocorms of *C. finlaysonianum* Lindl., germinated on VW medium, were sowed as explants (**Figure 5A**). Protocorms were cultured in VW liquid medium supplemented with varying levels of BAP and CW for the induction of PLBs. The combination of BAP and CW was found to be effective for PLB induction. After 60 days of culture, there was no PLB formation in all protocorm explants cultured in medium without BAP and CW (**Table 3**). When BAP was used alone at different concentrations (0, 2.21, 3.80, 4.42, 8.84, and 13.26 μM), PLBs could be induced from protocorm segments cultured in medium supplemented with 8.84 μM BAP, with the highest number of 5.2 PLBs per explant, and gave a significant difference in PLB formation at 85.7 %. (**Table 3** and **Figure 5B**). PLBs were directly induced from the seed derived protocorms. This culture medium was found to be the best for the induction of PLBs. Inclusion of 2.21 μM BAP alone in the medium was less successful for PLB formation, with an average of 2.2 PLBs per explant. The number of PLBs increased with increasing concentration of BAP, up to an optimal level of 8.84 μM in medium without CW, and the number of PLBs decreased at BAP concentrations higher than 8.84 μM. These findings demonstrate that BAP (8.84 μM), when applied singly, was efficient for PLB formation. In the addition of 15 % (v/v) CW in the culture media, the number of PLBs increased with increasing concentration of BAP up to an optimal level of 13.26 μM,

which gave the highest number of PLBs per explant at 5.0 (**Table 3**). The present study is the first report to show that PLBs can be induced from protocorm segments in VW liquid medium, and when supplemented with 8.84 µM BAP and without CW it is significantly different, compared to other treatments, with the highest number of 5.2 PLBs per explant.

Table 3 Effects of different concentrations of BAP and coconut water on PLB formation from seed-derived protocorms of *C. finlaysonianum* Lindl. after culture for 60 days.

VW media		PLB formation	No. of PLBs
BAP (µM)	CW (%)	(%)	(mean± SE)
0	0	0^{f}	0 ± 0^{d}
0	15	46.0^{c}	4.2 ± 0.4^{ab}
2.21	0	75.0^{b}	2.2 ± 0.2^{c}
2.21	15	19.7^{de}	3.0 ± 0.4^{bc}
3.80	0	80.0^{ab}	4.0 ± 0.5^{ab}
3.80	15	26.7^{d}	3.8 ± 0.4^{ab}
4.42	0	80.0^{ab}	4.8 ± 0.4^{a}
4.42	15	40.0^{cd}	4.0 ± 0.6^{ab}
8.84	0	85.7^{a}	5.2 ± 0.7^{a}
8.84	15	56.0^{bc}	4.4 ± 0.4^{ab}
13.26	0	79.0^{ab}	2.2 ± 0.4^{c}
13.26	15	50.0^{c}	5.0 ± 0.6^{a}

Similar letters within the same columns mean no significant difference at $p \leq 0.05$ by DMRT.

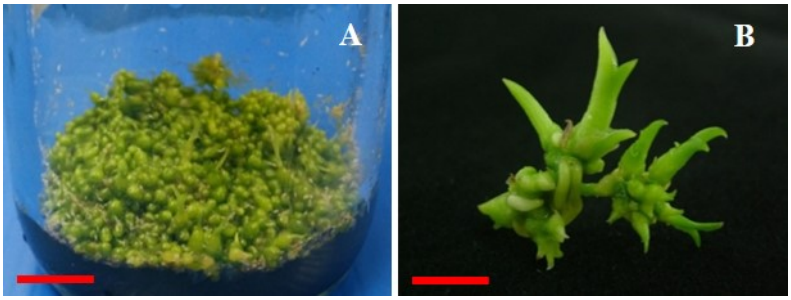

Figure 5 (A) Asymbiotic germination of seeds from capsules after 90 days of culture on VW medium, and (B) protocorms derived PLBs of *C. finlaysonianum* Lindl. after 60 days of culture in VW liquid medium supplemented with 8.84 µM BAP (Scale bar = 1 cm).

Between the BAP and CW tested, BAP was effective in PLB induction from protocorm segments of *C. finlaysonianum* Lindl. The types and concentrations of PGRs play an important role in the *in vitro* propagation of many orchid species [36]. BAP was also found to be suitable for protocorms and shoot multiplication in *Geoderum purpureum* [43], *Dendrobium tranparens* [44], *Eulophia nuda* [45], and *Vanda coerulea* Griff ex.Lindl. (Blue Vanda) [46], whereas CW was beneficial in *Cymbidium* Sleeping Nymph [47] and *Phalaenopsis cornu-cervi* [48].

Protocorms, which are but the intermediate structure between the embryos and the plants, were found to be excellent explants for regenerating PLBs, i.e., organs that resemble protocorms, which also lead to plantlet formation like protocorms do [49]. Apart from that, the cells of protocorms are highly

meristematic in nature, and thus can be used to enhance the proliferation and simultaneous production of orchid plantlets [50]. Because of this attribute, protocorms obtained from different plant parts are being frequently used by many workers as explants for the mass propagation of many rare orchid species, such as foliar explants of shoot-tips of *Cymbidium* hybrid [49], shoot-tips and leaves of *V. coerulea* [51], seeds of *Zygopetalum intermedium* [52], *Malaxis khasiana* [53], *Cymbidium giganteum* [8], etc. Above all, the main advantages of direct PLB formation without an intervening callus phase is that it saves time in regenerating uniform plantlets and it is economically viable. This opens another efficient way for mass propagation.

The formation of PLBs can be classified into 2 types. The first is the direct formation of PLBs from protocorms, shoot tips, root tips, and stem segments through direct embryogenesis [54,55]. The second is the formation of PLBs through callus [56-58]. In this present study, PLBs of *C. finlaysonianum* Lindl. developed directly from the protocorms without callus formation.

Effects of concentrations of activated charcoal on root development

For plantlets developed from PLBs, the clusters of PLBs derived from VW liquid medium supplemented with 8.84 μM BAP were separated and then cultured on VW medium without any plant growth regulators. Activated charcoal (AC) was evidenced to be crucial for the rooting process, and rooting efficiency was significantly improved with increasing concentrations of AC from 0 to 0.2 % (w/v). VW medium supplemented with 0.2 % (w/v) AC proved to be the best medium composition for rooting, which resulted into root induction from *C. finlaysonianum* Lindl. PLBs developed into plantlets when they were subcultured onto VW medium supplemented with 0.2 % (w/v) AC. All media induced rooting, including media without AC; however, in the absence of AC, fewer roots were induced, and those induced were much shorter, compared to those induced using AC treatments. The optimal rooting was observed on the medium containing 0.2 % (w/v) AC. The regenerated shoots developed an average of 6.4 roots per shoot, and an average root length of 5.2 cm, after culture for 60 days (**Table 4**).

Table 4 Effects of concentrations of activated charcoal (AC) on root development from PLBs of *C. finlaysonianum* Lindl. after culture for 60 days.

Activated charcoal (AC) (%)	Rooting (%)	No. of roots/explants	Root length (cm) (Mean ± SE)
0	100.0[a]	1.5[c]	1.4 ± 0.4[c]
0.1	100.0[a]	3.2[b]	3.3 ± 0.2[b]
0.2	100.0[a]	6.4[a]	5.2 ± 0.1[a]

Similar letters within the same columns mean no significant difference at $p \leq 0.05$ by DMRT.

This was contrary to the findings of the study carried out by Roy *et al*. [46], where the rooting response in *Vanda coerulea* Griff ex. Lindl. (Blue Vanda) was suppressed with an increase in AC. Similar findings were found in *Vanda coerulea* Griff ex. Lindl [51] and *Phalaenopsis* [59,60].

In plant tissue culture, AC was widely used to stimulate rooting of micropropagated shoots since it can adsorb both inhibitory substances and cytokinins in the medium. Moreover, it is suggested that AC favors the establishment of a balance of endogenous auxins and cytokinins that facilitate root formation by decreasing decomposition of endogenous IAA under light condition [61]. The beneficial effects of AC could be due to positive stimulation of many development processes [62] and its ability to absorb phenolics, which can injure living tissues.

Good growth and development of hybrid *Cymbidium* plantlets *in vitro* were obtained when culture media were supplemented with 0.1 % (w/v) AC [63]. Similar observations were also reported with *Vanda coerulea* Griff ex. Lindl [51] and *Phalaenopsis* [59]. AC increased the number and the length of roots. AC has beneficial and harmful effects in culture medium, depending upon the medium, explants, and

plant growth regulators used. The beneficial effects of AC on tissue responses *in vitro* could be attributed to providing a dark environment by darkening the medium, the adsorption of harmful substances produced by either the media or explant [64], the adsorption of plant growth regulators and other organic compounds [65], or releasing substances naturally present in or adsorbed by AC [66].

Transplantation of plantlets / greenhouse acclimatization and field establishment and reintroduction

Complete plantlets showed a 90 % survival rate during their gradual acclimatization to greenhouse conditions after the transfer of rooted plantlets to sterilized coconut husks and irrigation with water twice a day (**Figure 6A**). Plantlets without well-developed roots were unable to survive when transferred to the greenhouse. The hardened plants did not show detectable variation in morphological or growth characteristics, as compared to the field-grown parent plants. There have been no studies on the reintroduction of *C. finlaysonianum* Lindl. back into forest for *in situ* conservation. The highest percentage of plantlet survival recorded at 24 months after reestablishment in the Rusamilae forest, Pattani province, was 75.8 % (**Figure 6B**).

Figure 6 Plantlets derived from PLBs of *C. finlaysonianum* Lindl. after being cultured on VW medium supplemented with 0.2 % (w/v) AC for 8 weeks: (A) acclimatized plantlets of *C. finlaysonianum* Lindl., with well-expanded leaves in pots containing sterilized coconut husks after 3 months of transfer, and (B) establishment of *in vitro* plantlets in the wild for 24 months after reestablishment in the Rusamilae forest, Pattani province (Scale bar, A = 2 cm and B = 3 cm).

Conclusions

In conclusion, an efficient method for the *in vitro* germination of seeds and for the proliferation of a large number of plantlets from protocorms for *Cymbidium finlaysonianum* Lindl. has been described. The present results may hold the key to the mass multiplication and conservation of this highly important medicinal orchid, which is under severe threat from over-exploitation. TZ viability test indicated a mean embryo viability of 97.80 %. The highest seed germination percentage was 83.8 % on VW medium. Protocorm segments of *Cymbidium finlaysonianum* Lindl. cultured in VW liquid medium supplemented with 8.84 µM BAP showed the highest PLB formation at 85.7 %, with the highest number of 5.2 PLB per explant. VW medium supplemented with 0.2 % (w/v) AC gave the highest number of roots per plantlet and root length, at 6.4 roots and 5.2 cm, respectively, and the highest percentage of plantlet survival recorded at 24 months after reestablishment in the forest was 71.6 %. This is the first report that could be useful for mass-scale propagation, as well as *ex situ* conservation of this commercially important and threatened orchid species.

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