



Energy Efficient Micropropagation of *Dendrobium cruentum* Rchb. f.: Chemical Sterilization, Net House Culture, Sucrose Optimization, and Acclimatization Substrate Selection

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Abstract: *Dendrobium cruentum* Rchb. f., a threatened native orchid of southern Thailand, faces extinction from habitat loss and over-collection; however, conventional micropropagation protocols remain economically inaccessible for most conservation programs due to high energy costs. This study developed an energy-efficient micropropagation protocol by evaluating sterilization methods, culture environments, sucrose concentrations, and acclimatization substrates. Six sterilization treatments were assessed: conventional autoclaving (positive control), four concentrations of Haiter® bleach (NaOCl; 0.5, 1.0, 1.5, and 2.0 mL/L), and a non-sterilized negative control. Seedlings were cultured under controlled-room (25 ± 2°C, 16-h photoperiod) or net house (ambient temperature, natural light, 70% shade) conditions with sucrose at 0, 20, or 40 g/L on half-strength MS medium. Acclimatization performance was evaluated using sphagnum moss, chopped coconut husk, and charcoal substrates. Chemical sterilization with 2.0 mL/L Haiter® achieved an acceptably low contamination rate of 10.00% versus 6.67% for autoclaving, while eliminating autoclave capital (USD 2,000–10,000), maintenance (USD 200–500/year), and energy costs (2–3 kWh/cycle), reducing sterilization expenditure by 60–70%. Net house culture combined with 40 g/L sucrose produced superior plantlets with 2.63 shoots, 14.33 roots, 3.60 cm height, and 1.14 g fresh weight, representing numerically more shoots (21%, $p > 0.05$), significantly more roots (68%, $p \leq 0.01$), greater height (41%), and higher fresh weight (75.4%) than controlled-room culture, while substantially reducing operational electricity costs associated with climate-controlled culture rooms. Charcoal substrate achieved the highest acclimatization survival rate (85%). This integrated, low-cost protocol offers a practical, cost-effective tool for conservation and commercial production of this threatened native orchid.

Keywords: Micropropagation; chemical sterilization; net house culture; sucrose concentration; acclimatization

1. Introduction

Dendrobium cruentum Rchb. f. (the "bird-beak orchid"), belonging to section Formosae, is native to southern Thailand, where wild populations inhabit lowland and hill forests on the southern peninsula; its precise distribution across the Thai-Malay Peninsula remains incompletely documented. The species faces threats of extinction from deforestation, habitat loss, and unsustainable collection driven by high ornamental demand [1, 2].

Despite the CITES Appendix I listing [3], which prohibits the commercial trade in wild specimens, populations of *D. cruentum* continue to decline due to unsustainable collection and inadequate in situ assessment. Although an IUCN Red List assessment is lacking [4], this evidence underscores the urgent need for ex situ conservation interventions, including genetically verified micropropagation protocols [5].

The natural propagation of *D. cruentum* is severely limited by inherent biological constraints. Like all Orchidaceae, *D. cruentum* produces dust-like, endosperm-free seeds that require obligate mycorrhizal associations to supply nutrients for seedling development [6]. These reproductive limitations, compounded by ongoing habitat degradation and population fragmentation, make reliable in vitro propagation essential to prevent irreversible genetic erosion of remaining wild populations. Asymbiotic seed germination offers a well-established route for large-scale orchid propagation, bypassing mycorrhizal dependency while preserving genetic fidelity [7–10]. However, published protocols for *D. cruentum* are scarce, comprising only two reports: shoot induction on Vacin and Went (VW) medium [1] and asymbiotic germination on VW medium with organic additives [2]. The lack of optimized, resource-efficient protocols limits the conservation of this species through micropropagation.

Although commercial tissue culture facilities for *Dendrobium* and *Phalaenopsis* are established in Thailand, the high costs of conventional micropropagation — including growth media, sterilization equipment, and air-conditioned culture rooms [11, 12] — remain prohibitive for small-scale growers and conservation programs. Energy consumption is a major contributor to these costs, particularly through the energy-intensive autoclaving required for media sterilization and the continuous operation of air-conditioned culture rooms [13, 14]. Cost-reduction strategies have included reformulating the medium, substituting analytical-grade chemicals [15], using low-cost gelling agents [16], and adopting chemical sterilization methods instead of autoclaving [17–21]. However, the potential of ambient net house systems — using natural light and passive ventilation — as alternatives to controlled-room conditions has received little attention in orchid micropropagation. Ferreira et al. [22] demonstrated that *Cattleya warneri* cultured under variable greenhouse conditions produced greater biomass and incurred lower electricity costs than in constant-condition growth rooms. Similarly, Kodym and Zapata-Arias [23] found that *Musa acuminata* shoots grown under fluctuating natural light (up to $570 \mu\text{mol m}^{-2} \text{s}^{-1}$, 23–30°C) produced more shoots than those under constant artificial light ($65 \mu\text{mol m}^{-2} \text{s}^{-1}$) in controlled chambers. Nasiruddin et al. [24] reported 100% hardening survival in banana (*Musa* sp., cv. Robusta) shoots rooted under shadehouse conditions, with reduced electricity consumption. Although low-cost strategies have been explored for commercial *Dendrobium* species [25–28], no study has examined ambient net house conditions for threatened native species such as *D. cruentum*.

This study aimed to develop a comprehensive, cost-effective micropropagation protocol for *D. cruentum* by investigating three aspects: (1) medium sterilization techniques that minimize contamination and energy inputs; (2) effects of culture environment (controlled-room vs. net house) and sucrose concentration on plantlet growth, morphology, and biomass; and (3) the effect of acclimatization substrate type, in combination with pre-transfer sucrose concentration, on ex vitro survival rates; and (4) a comparative cost analysis of the operational cost implications of the optimized protocol relative to conventional micropropagation approaches. This research addresses the need for accessible, low-cost propagation tools to support both ex situ conservation of this CITES-listed orchid and sustainable production among resource-limited, small-scale growers in tropical regions.

2. Materials and Methods

2.1 Plant material and explant preparation

Plant material was obtained from six cultivated accessions of *Dendrobium cruentum* Rchb. f. maintained under nursery net-house conditions at the Biology Section, Faculty of Science and Technology, Surattthani Rajabhat University, Surat Thani Province, southern Thailand. All accessions were cultivated and maintained under standard nursery conditions for at least 2 years before experimentation; no wild specimens were collected for this study. Hand pollination was performed on each of the six maternal plants. Seed pod development was monitored from the date of pollination, and pods were harvested at 5 months post-pollination (approximately 150 days) for use in green pod culture. At this stage, capsules of *Dendrobium* species are characterized by a firm, fully closed green pericarp that has not yet undergone dehiscence, a stage

recognized as optimal for asymbiotic green pod culture in *Dendrobium* [29, 30]. Pods selected for use were uniformly green, firm, and undehiscent, with intact funicle attachment; pods showing discoloration, physical damage, pathogen infection, or size variation among maternal plants were excluded (Figure 1A). Before seed inoculation, seed viability was assessed using the 2,3,5-triphenyltetrazolium chloride (TTC) test following the modified protocol of Zainuddin et al. [31]; seeds exhibiting uniform red-to-deep-red embryo staining were included in the assessment. For explant preparation, pods were surface-sterilized by sequential washing with 10% Teepol® detergent, tap water, and distilled water, followed by immersion in 70% ethanol (3–5 min) and 95% ethanol with surface flaming (2–3×) to achieve surface sterility. All subsequent steps were performed in a laminar-flow cabinet (horizontal airflow) under aseptic conditions. To standardize seed inoculation density across treatments, seeds from each capsule were suspended in 5 mL of sterile distilled water after the capsule was split longitudinally. A 0.1 mL aliquot of the resulting seed suspension was pipetted onto the surface of solidified medium in each culture vessel, ensuring uniform seed distribution across all replicates.

2.2 Culture medium sterilization treatments

This experiment investigated the effects of six culture medium preparation treatments on contamination rates during asymbiotic seed germination of *D. cruentum*. Treatments differed only in the method used to sterilize or supplement the culture medium, while the seed source, inoculation method, and all other experimental conditions remained identical across treatments. The six treatments were as follows: (A) culture medium sterilized by conventional autoclaving at 121°C for 20 minutes (positive control); (B) culture medium with no sterilization applied (negative control); and (C–F) non-autoclaved culture medium supplemented with Haiter® commercial bleach (NaOCl, 6% w/w active ingredient; Lion Corporation, Japan) at four concentrations of 0.5, 1.0, 1.5, and 2.0 mL/L, equivalent to approximately 0.003%, 0.006%, 0.009%, and 0.012% active chlorine (w/v), respectively. For Treatments C–F, half-strength Murashige and Skoog (1/2 MS) salts [32], sucrose (30 g/L), and agar (8 g/L) were dissolved in distilled water. The pH was adjusted to 5.7 ± 0.1 with 0.1 N NaOH or 0.1 N HCl before Haiter® addition, and the agar was fully dissolved by heating to near boiling in a microwave oven. The medium was then cooled to approximately 50–55°C, and Haiter® was added at the designated concentration, then gently mixed to ensure uniform distribution. The final pH was verified to be within 5.5–6.0 after the addition of Haiter®. The supplemented medium was dispensed immediately into sterile culture vessels and allowed to solidify at room temperature. For Treatment A, the medium of identical composition was autoclaved at 121°C for 20 minutes in the conventional manner. All media were prepared fresh on the day of seed inoculation. Seeds for inoculation were obtained from the pre-sterilized pods described in Section 2.1. All seed handling and inoculation procedures were performed entirely within a laminar-flow cabinet (horizontal airflow). Under aseptic conditions, the pod was re-flamed with 95% ethanol, split longitudinally, and approximately 0.1 g of seeds was evenly distributed onto the medium surface using a sterile inoculation loop. The identical inoculation procedure was used for all six treatments. Culture vessels were sealed with Parafilm and incubated under standard controlled-room conditions ($25 \pm 2^\circ\text{C}$, 16-h photoperiod, $40 \mu\text{mol m}^{-2} \text{s}^{-1}$). Contamination rates were recorded after four weeks of culture. Following this experiment, seeds from aseptic cultures were allowed to germinate and develop into protocorms (Figure 1B).

2.3 Culture conditions and sucrose concentrations

All subsequent culture media were autoclaved at 121°C for 20 min. Stage 3 protocorms (bearing rhizoids and early leaf primordia, ~1.0–1.5 mm diameter) were allowed to develop into seedlings (~1.0 cm height) before transfer. One seedling per vessel was cultured on 1/2 MS medium supplemented with sucrose at 0, 20, or 40 g/L (20 seedlings per treatment per replication) under two contrasting environments. Seedlings were then transferred to 1/2 MS medium supplemented with three sucrose concentrations (0, 20, and 40 g/L), with 20 seedlings per treatment combination per replication, and cultured under the same two contrasting environments as described below. All culture vessels contained 25 mL of medium and were sealed with non-vented polypropylene caps. *Controlled-room conditions*: Temperature was maintained at $25 \pm 2^\circ\text{C}$ using an air-conditioning system. A 16-hour photoperiod was provided by cool-white fluorescent lamps at $40 \mu\text{mol m}^{-2} \text{s}^{-1}$. Relative humidity was maintained at 60–70% with forced air circulation. Electricity consumption (kWh) was continuously monitored using a digital energy meter. *Net house conditions*: Culture vessels were placed in

an ambient-temperature net house covered with a 70% shade cloth. Air temperature fluctuated between 24 and 33°C over the daily cycle, and natural daylight transmitted through the shade cloth provided a midday PPFD of approximately 300–500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at the vessel level. The natural photoperiod during the experimental period ranged from approximately 12 to 13 h of light per day, consistent with the study site's tropical latitude (approximately 9°N). Relative humidity ranged from 50% to 85% in response to natural diurnal variation, and ventilation was entirely passive. No electricity was consumed for climate control under these conditions. Plantlets were cultured for two months under both conditions. At harvest, the following parameters were recorded for each plantlet: number of new shoots, number of leaves, number of roots, plantlet height (cm), leaf width (cm), leaf length (cm), root length (cm), and fresh weight (g). Electricity consumption data from the controlled-room system were used to calculate the per-cycle difference in operational cost between the two culture environments.

2.4 Ex vitro acclimatization

Plantlets from the 20 and 40 g/L sucrose treatments were selected for acclimatization; those from sucrose-free medium (0 g/L) were excluded due to insufficient shoot and root development (Table 3). Only plantlets meeting the following minimum morphological criteria were selected for transplanting: at least two fully expanded leaves, at least three visible roots, and a height of at least 1.5 cm. Three substrate types were evaluated: Sphagnum moss, chopped coconut husk, and charcoal. Before transplanting, residual agar was gently removed from the roots under running distilled water. Plantlets were individually transplanted into plastic pots (7 cm diameter) containing the respective substrates and maintained in a shade net nursery covered with 70% shade cloth at an ambient temperature of 25–32°C and a relative humidity of 60–80%. Plantlets were irrigated twice weekly with distilled water (pH 6.5–7.0, EC < 0.1 mS/cm) and fertilized once every two weeks with a diluted balanced liquid fertilizer (20-20-20 NPK, 1 g/L). Survival rates were assessed after 2 months of acclimatization, with survival defined as the retention of at least 1 turgid, green leaf and at least 1 intact root in contact with the substrate.

2.5 Experimental design and statistical analysis

All experiments were conducted using a completely randomized design (CRD) with three replications. Sterilization experiments comprised six treatments, three replications, and 10 vessels per treatment (30 vessels in total). Culture condition experiments comprised six treatment combinations (2 environments $\times$ 3 sucrose levels) $\times$ 20 explants/replication (60 explants/combination). Acclimatization studies comprised six treatment combinations (3 substrates $\times$ 2 sucrose levels) $\times$ 20 plantlets/replication (60 plantlets/combination). Acclimatization studies used 20 plantlets per substrate-sucrose combination per replication, with three substrate types (Sphagnum moss, chopped coconut husk, and charcoal) evaluated at two sucrose concentrations (20 and 40 g/L), yielding six treatment combinations and 60 plantlets per combination across all replications. Data were analyzed using one-way ANOVA for sterilization treatments. Culture condition and acclimatization experiments were analyzed using two-way ANOVA; fixed factors were culture environment $\times$ sucrose concentration, and substrate type $\times$ sucrose concentration, respectively. Mean separation was performed using Duncan's Multiple Range Test (DMRT) at $p \leq 0.05$ or $p \leq 0.01$, as indicated in the respective tables. All statistical analyses were performed using SPSS Statistics version 26.0 (IBM Corp., Armonk, NY, USA). All data are presented as means $\pm$ standard error (SE).

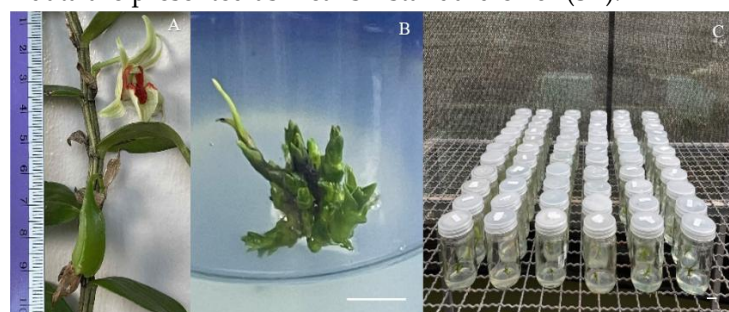


Figure 1. The 5-month-old, hand-pollinated seed pod of *D. cruentum* Rchb. f. (A) and protocorm development (B), and the plantlet cultured on 1/2 MS medium under net house conditions (C) (bar = 1 cm).

3. Results and Discussion

3.1 Effect of sterilization technique on contamination rate

The sterilization method significantly affected the contamination rate of *D. cruentum* seed cultures ($p \leq 0.01$; Table 1). Conventional autoclaving (Treatment A) resulted in the lowest contamination rate, at $6.67 \pm 0.46\%$, which was significantly lower than those of all chemical sterilization treatments. Among chemical methods, Haiter® at 2.0 mL/L (Treatment F) yielded the lowest contamination ($10.00 \pm 0.59\%$), with rates increasing progressively at lower concentrations (26.70%, 53.30%, and 56.70% at 1.5, 1.0, and 0.5 mL/L, respectively). The control treatment without Haiter® (Treatment B) showed complete contamination ($100.00 \pm 0.00\%$), underscoring the need for proper sterilization procedures. Although autoclaving remained the most effective sterilization method, chemical sterilization with 2.0 mL/L Haiter® achieved acceptable contamination rates while eliminating the energy consumption associated with autoclaving, estimated at 2–3 kWh per cycle. This finding aligns with previous reports demonstrating the effectiveness of sodium hypochlorite for plant tissue culture sterilization [17–21], although optimal concentrations vary by species. The 10% contamination rate, while higher than that of autoclaving, represents a reasonable trade-off given substantial energy savings and the elimination of autoclave-related operational costs for resource-limited facilities. A comparative cost analysis further substantiates this trade-off (Table 2). NaOCl-based sterilization has demonstrated economic viability across multiple species, including *Eucalyptus benthamii*, *Gerbera hybrida*, and *Anthurium* sp. [17, 33, 34], achieving contamination rates comparable to autoclaving while reducing infrastructure requirements, including a potential reduction in dependence on laminar flow cabinets via the CSUP technique [Peiris et al., 2012]. In the context of small-scale orchid conservation programs in Thailand, eliminating the need for autoclaves could reduce sterilization-related costs by an estimated 60–70%, making this approach particularly transformative for community-based production.

The safety of chlorine residues at the effective concentration used in this study (2.0 mL/L Haiter®) warrants discussion, as NaOCl is a potent oxidizing agent that can cause phytotoxic injury at elevated concentrations. NaOCl is a potent oxidant; its active species, hypochlorous acid (HOCl), can cause protein denaturation and membrane disruption at high concentrations, leading to phytotoxic effects, including disruption of meristematic cell division and chromosomal anomalies, as shown in dose-dependent bioassays [[35–37]]. Phytotoxic effects are strongly concentration-dependent, and the critical threshold beyond which NaOCl impairs tissue viability varies by species, explant type, exposure duration, and temperature [35, 38]. In the present study, the 2.0 mL/L Haiter® treatment did not produce visible phytotoxic symptoms in *D. cruentum* seeds or developing protocorms. Successful germination and normal plantlet development in non-contaminated cultures confirmed that residual active chlorine concentrations after rinsing were below phytotoxic thresholds. This finding aligns with a growing body of literature showing that NaOCl at carefully optimized, low concentrations does not cause significant phytotoxic injury to plant tissues. Supporting evidence from diverse species confirms that low NaOCl concentrations are biologically safe: Bui et al. [39] reported high survival with minimal tissue damage in *Litsea cubeba*; Rodrigues et al. [40] demonstrated superior shoot production in two orchid species at $1,200 \text{ mg L}^{-1}$; and [41] achieved 94.53% germination in rice at 4%, with phytotoxicity appearing only above this threshold. Park et al. [42] further reported that 1% NaOCl improved germination in the endangered orchid *Cypripedium guttatum*, supporting the use of dilute NaOCl as a germination-compatible pre-treatment. Collectively, these findings confirm that 2.0 mL/L Haiter® is a biologically safe and effective sterilization alternative for *D. cruentum*, provided rinsing steps are maintained to limit residual chlorine carry-over.

Table 1. Effects of sterilization technique on the contamination rate of *D. cruentum* seeds.

	Sterilize technique	Contamination rate (%) $\pm$ SE ¹
A	Autoclave	6.67 $\pm$ 0.46 ^e
B	0.0 mL/L Haiter®	100.00 $\pm$ 0.00 ^a
C	0.5 mL/L Haiter®	56.70 $\pm$ 0.94 ^b
D	1.0 mL/L Haiter®	53.30 $\pm$ 0.95 ^b
E	1.5 mL/L Haiter®	26.70 $\pm$ 0.87 ^c
F	2.0 mL/L Haiter®	10.00 $\pm$ 0.59 ^d

¹ Data are presented as means $\pm$ SE. Different letters in a column indicate statistically significant differences ($p \leq 0.01$) by DMRT.

Table 2. Comparative cost analysis of autoclave sterilization versus chemical sterilization with Haiter® (NaOCl) for micropropagation medium sterilization.

Cost Parameter	Autoclave Sterilization	Chemical Sterilization (2.0 mL/L Haiter®)
Equipment purchase	USD 2,000–10,000+ (laboratory autoclave)	Negligible (no specialized equipment)
Equipment maintenance	Regular servicing required; USD 200–500/year	None
Energy per cycle	2–3 kWh/cycle ¹ (121°C, 20 min)	0 kWh
Electricity cost per cycle (at ~USD 0.10/kWh, Thai rate)	~USD 0.20–0.30/cycle ²	USD 0.00
Monthly energy cost (20 cycles/month)	~USD 4–6/month	USD 0.00
Annual energy cost	~USD 48–72/year	USD 0.00
Reagent cost per cycle	Distilled water only (~USD 0.01)	Haiter® ~1 mL/500 mL medium; ~USD 0.01–0.02/cycle
Contamination rate	6.67%	10.00%
Laminar flow cabinet required	Yes	Can be reduced (see CSUP technique)
Skill requirement	Trained operator	Minimal training
Overall relative cost	High (baseline)	~60–70% lower

¹ Autoclave capital and energy data from O'Callaghan et al. (2023) [ref. no.].

² Thai commercial electricity tariff (USD 0.10–0.14 per kWh) based on the Provincial Electricity Authority of Thailand (PEA) rate schedule (2023).

3.2 Effects of culture condition and sucrose concentration on shoot and root development

3.2.1 Shoot multiplication

Both sucrose concentration and culture environment significantly influenced shoot production ($p \leq 0.01$), and their interaction was also significant ($p \leq 0.05$), indicating that the promotive effect of sucrose on shoot number was modulated by culture environment. Root formation was similarly influenced by sucrose concentration, culture environment, and their interaction ($p \leq 0.01$ each), confirming that sucrose effects on root development were disproportionately enhanced under net house conditions (Table 3; Figures 2 and 3). In sucrose-free medium (0 g/L), no new shoots formed regardless of culture condition, with both controlled-room and net house treatments yielding 0.00 ± 0.00 shoots per plantlet. The addition of sucrose promoted shoot proliferation, with 20 g/L yielding 1.65 ± 0.18 shoots in controlled-room and 1.95 ± 0.20 shoots in net house conditions. The highest number of new shoots (2.63 ± 0.15) was obtained with 40 g/L sucrose under net house culture, which was significantly superior to all other treatments except the controlled-room culture at the same sucrose concentration (2.15 ± 0.18 shoots).

3.2.2 Root formation

Root formation responded markedly to both sucrose concentration and culture condition. In the absence of sucrose, root development was minimal, with only 1.27 and 1.87 roots per plantlet under controlled-room and net house conditions, respectively. Increasing sucrose to 20 g/L substantially enhanced root formation (4.50 and 4.87 roots for the controlled-room and net house, respectively). The most pronounced root proliferation occurred at 40 g/L sucrose in net house culture, yielding 14.33 roots per plantlet — significantly higher than all other treatments and nearly twice that of controlled-room culture at the same sucrose level (8.50 roots). Collectively, these results demonstrate that sucrose concentration was the dominant factor for both shoot and root development. Culture environment and its interaction with sucrose were significant determinants of root organogenesis. The mechanistic basis of this interaction is discussed in the following sections. Net house culture at 40 g/L sucrose produced more shoots (2.63 vs. 2.15; 22% increase, $p \leq 0.05$) and significantly more roots (14.33 vs. 8.50; 69% increase, $p \leq 0.01$) than controlled-room culture at the same sucrose concentration. This superior performance reflects the combined benefits of enhanced light quality and intensity, improved CO₂ availability, and diurnal temperature fluctuations characteristic of the ambient outdoor environment [43–45]. The robust root systems produced under net house conditions are particularly advantageous for subsequent *ex vitro* acclimatization, as well-developed root architecture is a key determinant of post-transfer survival in orchid micropropagation. Eliminating air-conditioned culture rooms delivers concurrent economic and agronomic benefits by removing the electricity costs associated with climate control in controlled-room culture.

The superior shoot and root responses observed at 40 g/L sucrose under net house conditions can be explained mechanistically by a synergistic interaction between exogenous carbon supply and the enhanced photosynthetic environment provided by the net house. Under controlled-room conditions (40 $\mu\text{mol m}^{-2} \text{s}^{-1}$, limited CO₂), plantlets operate predominantly heterotrophically, relying on exogenous sucrose as the primary carbon source. In contrast, the net house environment (300–500 $\mu\text{mol m}^{-2} \text{s}^{-1}$, ~400–450 $\mu\text{mol mol}^{-1}$ CO₂, passive ventilation) promotes concurrent CO₂ fixation and sucrose absorption, thereby establishing photomixotrophic growth with total carbon input exceeding that of either source alone [46, 47]. Root development benefited particularly from photomixotrophic conditions: sucrose acts as a primary substrate and signal for root sink organs via SUT/SWEET transporters [48, 49], while photosynthetically fixed carbon provided additional carbohydrate for root proliferation. Together, these mechanisms explain the 69% increase in root number (14.33 vs. 8.50) at 40 g/L sucrose in the net house. Consistent with this, Gago et al. [46] demonstrated up to 2-fold more shoots when sucrose supplementation was combined with elevated CO₂ and high light in bioreactor-cultured *Prunus domestica*.

Consistent with this interpretation, Gago et al. [46] demonstrated in bioreactor-cultured plum (*Prunus domestica*) that combining sucrose supplementation with elevated CO₂ and high light intensity produced up to 2-fold more shoots and 1.7-fold greater shoot length than sucrose under low light and ambient CO₂, confirming that carbon source and environment interact multiplicatively rather than additively in photomixotrophic systems. The superior performance of net house culture reflects three key environmental advantages over sealed controlled rooms: (1) broad-spectrum natural light (300–500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ through 70% shade cloth) saturates orchid photosynthesis, exceeding the capacity of conventional fluorescent lamps (40 $\mu\text{mol m}^{-2} \text{s}^{-1}$) [50, 51]; (2) continuous passive ventilation maintains ambient CO₂ (~400–450 $\mu\text{mol mol}^{-1}$), preventing the depletion that limits photosynthesis in sealed vessels — elevated CO₂ has been shown to increase Fv/Fm by 14% and enhance Rubisco activity in *Phalaenopsis* [52]; and (3) diurnal temperature fluctuation (24–33°C) promotes stomatal cycling and carbohydrate metabolism pathways suppressed under constant temperature [45]. Having established the environmental basis for net house superiority, it is important to recognize that sucrose itself plays multiple distinct physiological roles that are independent of, yet synergistic with, the photomixotrophic framework.

Beyond its role in photomixotrophic carbon supply, sucrose exerts four distinct physiological functions: (1) carbon and energy supply — hydrolysis to hexoses fuels glycolysis, ATP synthesis, and meristematic activity [53, 54]; (2) root organogenesis — sucrose loading and unloading via SUTs/SWEETs activates root meristem cell cycle progression [48]; (3) osmotic regulation — sucrose lowers the medium's

osmotic potential, driving turgor-driven cell expansion and fresh weight accumulation [55]; and (4) sugar signalling — glucose and fructose interact with HXK1, TOR, and T6P pathways to regulate auxin biosynthesis and developmental transitions [56]. Collectively, sucrose acts as a multidimensional regulator of plantlet development, not merely a carbon source. These sugar signals modulate the expression of genes governing auxin biosynthesis and transport — which are essential for both shoot meristem activation and root organogenesis — explaining why sucrose concentration critically determines both the number and developmental quality of shoots and roots at this stage. Collectively, these four functions mean that sucrose concentration in the culture medium is not simply a variable carbon source but a multidimensional regulator of plantlet development, and optimizing sucrose supply is therefore a prerequisite for achieving maximum organogenic responses regardless of culture environment.

These results align with reports indicating that net houses reduce energy consumption by 0.18–0.26 kWh m⁻² day⁻¹ [57, 58] and that air conditioning is the second-largest cost in commercial orchid tissue culture. Chen [13] confirmed that net house culture provides both economic and agronomic benefits for *D. cruentum*. The dramatic quantitative differences in root formation are clearly illustrated in Figure 3, which shows representative root development across all treatment combinations after two months of culture. Plantlets cultured without sucrose under both controlled-room (Figure 3A) and net house (Figure 3D) conditions produced only sparse, thread-like roots with minimal branching, consistent with their low root counts (1.27 and 1.87 roots per plantlet, respectively; Table 3). At 20 g/L sucrose, root systems were more developed in both environments (Figure 3B and E), with visibly longer and more numerous roots, though net house plantlets (Figure 3E) appeared to produce slightly more branched root structures compared to controlled-room counterparts (Figure 3B). The most striking visual contrast was observed between the controlled-room treatment at 40 g/L sucrose (Figure 3C) and the net house treatment at the same concentration (Figure 3F): net house plantlets in Figure 3F displayed markedly denser root systems characterized by a substantially higher number of individual roots (14.35 vs. 8.55; Table 3), visibly thicker root axes reflecting greater radial growth consistent with the 1.30 cm root length recorded in Table 3, and a notably pale-white to greenish coloration. The green tinge observed in roots of net house plantlets under high sucrose (Figure 3F) is a well-documented characteristic of orchid aerial roots exposed to natural light, attributable to the differentiation of chloroplast-containing cells in the root velamen and cortex, which represents a photosynthetically functional adaptation of epiphytic orchid roots to ambient illumination [59, 60]. Additionally, root branching density appeared visually greater in the Figure 3F treatment, suggesting that the photomixotrophic carbon surplus available under net house conditions actively promoted lateral root initiation — a process that is positively regulated by local sucrose accumulation in root tissues [49]. These morphological characteristics collectively indicate that the net house and 40 g/L sucrose combination produces root systems with superior architectural complexity, which directly underlie the substantially higher acclimatization survival rates observed in plantlets from this treatment. The significant interaction term confirms that the effect of sucrose concentration on plantlet development was not independent of the culture environment, but was substantially modulated by whether plantlets were grown under controlled-room or net house conditions — consistent with the photomixotrophic framework described above and with reports of multiplicative sucrose × environment interactions in other micropropagation systems [61, 62].

3.3 Effects of culture condition and sucrose concentration on plantlet morphology

Culture conditions and sucrose concentration significantly influenced plantlet height, leaf dimensions, and root length ($p \leq 0.01$) (Table 4). Plantlet height ranged from 1.50 ± 0.50 cm to 3.60 ± 0.20 cm across treatments. The shortest plantlets were observed in controlled-room culture without sucrose (1.50 ± 0.50 cm). Net house culture consistently produced taller plantlets than controlled-room culture at all sucrose concentrations. The tallest plantlets (3.60 ± 0.20 cm) were obtained with 40 g/L sucrose under net house conditions, followed by 20 g/L sucrose in the net house (3.30 ± 0.10 cm). Both were significantly taller than the controlled-room treatments. An exception to the general pattern of net house superiority was observed in leaf length, where controlled-room plantlets at 20 g/L sucrose produced the longest leaves of any treatment (2.20 ± 0.10 cm; Table 4), exceeding even the net house + 40 g/L sucrose combination (1.52 ± 0.07 cm). This apparent paradox can be explained by the dual physiological role of sucrose at elevated concentrations: while higher

sucrose levels promote lateral cell expansion, fresh weight accumulation, and root organogenesis through enhanced carbon supply and osmotic water uptake, the elevated osmotic potential of the 40 g/L sucrose medium may simultaneously restrict turgor-driven longitudinal cell elongation in leaf mesophyll and epidermis, thereby limiting leaf length despite promoting leaf width and biomass accumulation [63, 64]. This concentration-dependent trade-off between leaf elongation and other growth parameters has been documented in other in vitro systems, where sucrose concentrations above the physiological optimum generate osmotic conditions that suppress shoot elongation while maintaining or enhancing lateral growth [65]. The net house environment at 40 g/L sucrose therefore optimizes most plantlet quality parameters — including height, root number, root length, leaf width, and fresh weight. By contrast, leaf length was maximized under moderate sucrose (20 g/L) combined with net house conditions (1.89 ± 0.04 cm), suggesting that an intermediate carbohydrate concentration best supports longitudinal leaf cell elongation in this environment. Figure 2 visually confirms these quantitative differences. Net house plantlets at 40 g/L sucrose (Figure 2D–F) displayed darker green leaves, consistent with enhanced chlorophyll accumulation under broad-spectrum natural light [66, 67], and visibly broader leaves and thicker stems, reflecting greater cell expansion and photomorphogenic development (leaf width data in Table 4). Sucrose-free plantlets (Figure 2A, D) were noticeably etiolated, consistent with their minimal biomass and the absence of shoot formation (Tables 3, 5). Future studies should verify chlorophyll differences spectrophotometrically. In contrast, plantlets maintained without sucrose under both culture conditions (Figure 2A and D, 0 g/L) were noticeably etiolated, with pale yellow-green, narrow leaves and weak stems, visually consistent with their severely limited biomass accumulation (0.50 and 0.72 g, respectively; Table 5) and the complete absence of new shoot formation (Table 3).

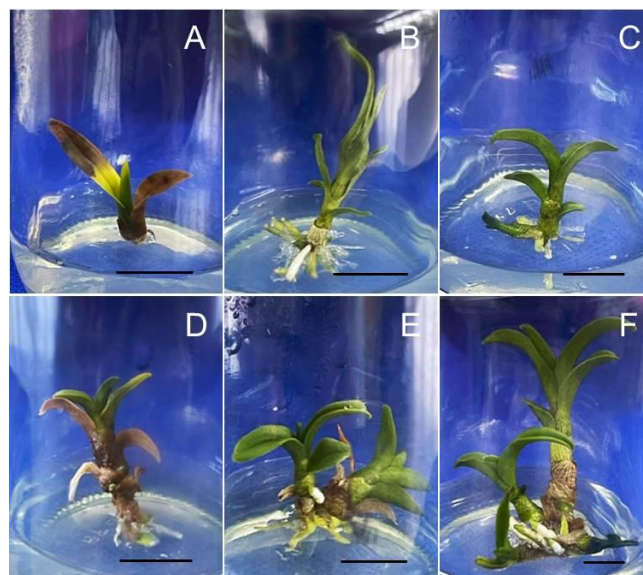


Figure 2. Shoot explants of *D. cruentum* were cultured on 1/2MS agar medium supplemented with 0, 20, and 40 g/L of sucrose in both controlled room (A–C) and net house (D–F) environments for a duration of two months. (bar = 1 cm).

Table 3. Number of shoots, leaves, and roots of *D. cruentum* after 2 months of culture.

Sucrose concentration (g/L)	Number of new shoots		Number of leaves		Number of roots	
	Culture environment (CE)	Average (SC)	Culture environment (CE)	Average (SC)	Culture environment (CE)	Average (SC)
	CR	NH	NH	CR	NH	CR
0	0.00 ± 0.00 ^c	0.00C	4.67 ± 0.57	4.33 ± 0.57	1.27 ± 0.31 ^d	1.87 ± 0.23 ^d
20	1.65 ± 0.15 ^b	1.80B	5.50 ± 1.32	4.83 ± 1.04	4.50 ± 0.5 ^c	4.87 ± 0.99 ^c
40	2.15 ± 0.18 ^b	2.39A	4.50 ± 0.5	4.17 ± 0.29	8.50 ± 0.86 ^b	14.33 ± 0.58 ^a
Average (CE)	1.27B	1.52A	4.89	4.44	4.78B	7.02A
SC	**		Ns		**	
CE	**		Ns		**	
SCxCE	*		Ns		**	
C.V. (%)	9.04		18.45		8.24	

¹ Data are presented as means ± SD. Different letters in a column and row indicate statistically significant differences ($p \leq 0.01$) by DMRT.

Table 4. Height, size of leaf, and root of *D. cruentum* plantlets cultured under controlled room and net house after culture for 2 months.

Sucrose Con. (g/L)	Plantlet height (cm)		Leaf width (cm)		Leaf length (cm)		Root length (cm)	
	Culture environment (CE)	Average (SC)	Culture environment (CE)	Average (SC)	Culture environment (CE)	Average (SC)	Culture environment (CE)	Average (SC)
	CR	NH	NH	CR	NH	CR	NH	CR
0	1.50 ± 0.50 ^c	2.70 ± 0.10 ^b	0.30 ± 0.01 ^c	0.32 ± 0.01 ^c	1.07 ± 0.05 ^e	1.33 ± 0.05 ^d	0.30 ± 0.02 ^d	0.50 ± 0.05 ^c
20	2.36 ± 0.05 ^b	3.30 ± 0.10 ^a	0.41 ± 0.01 ^b	0.51 ± 0.01 ^a	2.20 ± 0.10 ^a	1.89 ± 0.04 ^b	0.59 ± 0.01 ^c	0.90 ± 0.05 ^b
40	2.57 ± 0.05 ^b	3.60 ± 0.20 ^a	0.42 ± 0.02 ^b	0.49 ± 0.02 ^a	1.57 ± 0.05 ^c	1.52 ± 0.07 ^c	0.93 ± 0.01 ^b	1.30 ± 0.1 ^a
Average (CE)	2.14B	3.20A	0.37B	0.44A	1.61	1.58	4.78B	7.02A
SC	**		**		**		**	
CE	**		**		ns		**	
SCxCE	*		**		**		**	
C.V. (%)	8.63		3.91		4.45		7.15	

¹ Data are presented as means ± SE. Different letters in a column and row indicate statistically significant differences ($p \leq 0.01$) by DMRT, ns = not significantly different

Taken together, these morphological data confirm that net house culture combined with 40 g/L sucrose produced the highest-quality plantlets across most measured parameters, except leaf length, as discussed above. These advantages can be attributed to diurnal temperature fluctuations, a broader light spectrum, and enhanced CO₂ availability inherent to the net house environment [58]. Differential responses to sucrose concentration across culture environments further demonstrate that carbohydrate availability and the photosynthetic environment interact to modulate distinct developmental programs, consistent with the photomixotrophic framework described in Section 3.2 [[46, 62]. These morphological patterns reflect the photomixotrophic advantage of net house culture [22, 68]. The visual phenotypes in Figures 2 and 3 align directly with the quantitative data in Tables 3–5: dark green, broad-leafed plantlets with dense root systems in the net house + 40 g/L treatment show the highest fresh weight (1.14 g), root count (14.33), and plantlet height (3.60 cm), whereas etiolated, sucrose-free plantlets show minimal growth across all parameters. This alignment between visual morphology and quantitative metrics validates the experimental observations and confirms that the net house + 40 g/L sucrose combination produces the highest-quality plantlets across most measurable parameters, except for leaf length, as noted above.

3.4 Effects of culture condition and sucrose concentration on fresh weight accumulation

Fresh weight of plantlets was significantly affected by the interaction between culture conditions and sucrose concentration ($p \leq 0.01$) (Table 5). The lowest fresh weight (0.50 ± 0.02 g) was recorded in controlled-room culture without sucrose. Net house culture without sucrose produced plantlets with a moderately higher fresh weight (0.72 ± 0.02 g), presumably reflecting the partial contribution of photosynthetically fixed carbon under ambient light conditions — a finding consistent with the photomixotrophic advantage of the net house environment described in Section 3.2. Adding 20 g/L sucrose increased fresh weight to 0.63 ± 0.03 g in controlled-room and 0.78 ± 0.04 g in net house conditions. The highest fresh weight accumulation (1.14 ± 0.08 g) was achieved with 40 g/L sucrose under net house culture, which was significantly superior to all other treatments, representing a 2.3-fold increase compared to controlled-room culture without sucrose and a 1.75-fold increase compared to controlled-room culture at the same sucrose concentration. As noted in Section 3.2, sucrose also contributes to fresh weight via osmotic regulation: lowering the medium water potential drives turgor-driven cell expansion [55, 63]. The 0.42 g difference in fresh weight between sucrose-free and 40 g/L net house treatments, under otherwise identical conditions, is consistent with this osmotic mechanism. The substantial fresh weight accumulation observed in net house culture, a 75.4% increase over controlled-room conditions at 40 g/L sucrose, underscores the synergistic effect of optimal carbohydrate supply and favorable environmental conditions on overall plantlet vigor and biomass production. Sucrose concentration critically influenced plantlet development: the 40 g/L treatment, twice the conventionally applied 20 g/L concentration, supported markedly enhanced biomass accumulation under photomixotrophic net house conditions [46, 47, 61, 62]. This progressive improvement confirms heterotrophic dependence at this developmental stage, as growth was severely limited in the absence of sucrose. The progressive increase in fresh weight with sucrose concentration ($0 \rightarrow 20 \rightarrow 40$ g/L) reflects the obligate dependence on exogenous carbon in conventional in vitro systems, where low light and restricted CO₂ suppress photoautotrophy [62]. Utami et al. [69] similarly reported optimal growth in *D. lineale* at 20 g/L sucrose, with complete removal preventing normal development. The 75.4% increase in fresh weight at 40 g/L sucrose under net house conditions (1.14 vs. 0.65 g) reflects the net house environment's capacity to enhance carbon utilization through photomixotrophic metabolism, an advantage absent in sealed controlled-room vessels.

Table 5. Fresh weight of *D. cruentum* plantlets cultured under controlled room and net house after culture for 2 months.

Sucrose concentration (SC) (g/L)	Fresh weight (g)		Average (SC)
	Culture environment (CE)		
	CR	NH	
0	0.50 ± 0.02 ^e	0.72 ± 0.02 ^c	0.61C
20	0.63 ± 0.01 ^d	0.78 ± 0.02 ^b	0.70B
40	0.65 ± 0.01 ^d	1.14 ± 0.04 ^a	0.89A
Average (CE)	0.59B	0.88A	
SC	**		
CE	**		
SCxCE	**		
C.V. (%)	3.47		

¹ Data are presented as means ± SD. Different letters in a column and row indicate statistically significant differences ($p \leq 0.01$) by DMRT

3.5 Effect of substrate type and sucrose concentration on ex vitro survival

Two-way ANOVA confirmed that substrate type exerted a significant main effect on acclimatization survival rate ($p \leq 0.01$) (Table 6), whereas the sucrose concentration of the preceding in vitro culture (20 vs. 40 g/L) did not produce a statistically significant main effect ($p > 0.05$). Plantlets previously cultivated on media supplemented with 20 or 40 g/L sucrose showed comparable responses across all three substrates. Sphagnum moss failed to support adequate plantlet survival, yielding only $15.00 \pm 5.00\%$ and $20.00 \pm 5.00\%$ for plantlets from 20 and 40 g/L sucrose media, respectively. Chopped coconut husk resulted in moderate survival rates of $70.00 \pm 8.66\%$ and $75.00 \pm 5.00\%$ for plantlets cultured on media containing 20 and 40 g/L sucrose, respectively. Charcoal substrate yielded the highest survival rates: $80.00 \pm 5.00\%$ for plantlets grown in 20 g/L sucrose medium and $85.00 \pm 5.00\%$ for those grown in 40 g/L sucrose medium. Survival on charcoal was significantly higher than on coconut husk and Sphagnum moss. The superior performance of charcoal as an acclimatization substrate (80–85% survival) reflects the specialized requirements of epiphytic orchids [26]. This finding aligns with previous work by Samala et al. [70], who reported an 80% survival rate using a foam substrate under greenhouse conditions, confirming that well-aerated, moisture-permeable substrates with adequate drainage are critical determinants of post-transfer survival in this species. Charcoal's excellent drainage and aeration, adsorption of phytotoxic metabolites, near-neutral pH, and structural stability make it well-suited to the epiphytic growth requirements of *D. cruentum*, achieving 80–85% survival, comparable to that of a foam substrate [2]. Poor survival on Sphagnum moss (15–20%) likely reflects its excessive moisture retention, its compact structure when wet, and its inherently acidic pH (3.5–4.5), which are incompatible with the near-neutral substrate conditions of *Dendrobium*'s natural epiphytic habitat [71]. The moderate survival rates achieved on coconut husk substrate (70% and 75% for the 20 and 40 g/L treatments, respectively) confirm its utility as a practical alternative substrate, although its susceptibility to gradual decomposition may necessitate more frequent repotting than with charcoal. The marginal improvement in plantlet survival from the 40 g/L sucrose medium (85% vs. 80%) suggests that elevated internal carbohydrate reserves confer a metabolic advantage during the critical transition from heterotrophic in vitro conditions to autotrophic *ex vitro* nutrition, consistent with established relationships between pre-acclimatization carbohydrate status and post-transfer survival in orchid micropropagation. Although somaclonal variation is generally low in seed-derived cultures, genetic fidelity under the non-standard conditions employed here (net house, 40 g/L sucrose) has not been verified. This limitation, along with the recommended marker approaches (RAPD, ISSR, SCoT), is discussed in Section 3.6.

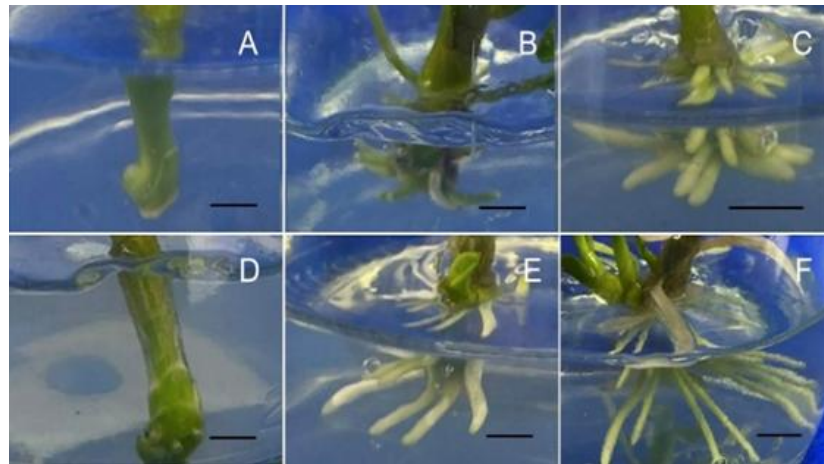


Figure 3. After being cultured on 1/2MS agar medium supplemented with 0, 20, and 40 g/L sucrose, rooting of *D. cruentum* Rchb.f. was observed in both controlled room (A-C) and net house (D-F) conditions for a period of two months. (bar = 1 cm).

Table 6. Survival rate of transplanted plantlets after 2 months.

Substrates (ST)	Survival rate		Average (ST)
	Sucrose concentration (SC) (g/L)		
	20	40	
Sphagnum Moss	15.00 ± 5.00 ^c	20.00 ± 5.00 ^c	17.5C
Chopped coconut husk	70.00 ± 8.66 ^b	75.00 ± 5.00 ^{ab}	72.5B
Charcoal	80.00 ± 5.00 ^{ab}	85.00 ± 5.00 ^a	82.5A
Average (SC)	55.00	60.00	
ST	**		
SC	ns		
ST xSC	*		
C.V. (%)	10.16		

¹ Data are presented as means ± SD. Different letters in a column and row indicate statistically significant differences ($p \leq 0.01$) by DMRT, ns = not significantly different

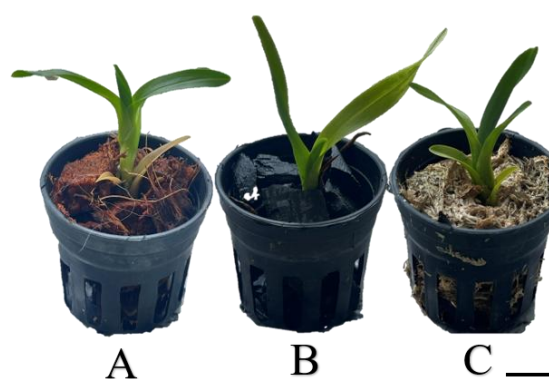


Figure 4. *D. cruentum* plantlets, previously cultured in bottles with 1/2MS agar medium supplemented with 40 g/L of sucrose in a net house, were acclimatized by transferring them to pots filled with three different substrates (bar = 1 cm), A, Chopped coconut husk; B, charcoal; C, sphagnum moss.

3.6 Limitations and future directions.

Two important limitations of the present study merit acknowledgment. First, the genetic fidelity of plantlets produced under net house conditions and at the elevated sucrose concentration of 40 g/L has not

been verified in this study. However, somaclonal variation is generally considered low in seed-derived cultures; the non-standard culture conditions employed here warrant verification through molecular marker analyses (e.g., RAPD, ISSR, SCoT, or SSR) in future studies [5, 25]. Second, the net house performance documented in this study reflects the tropical climate of southern Thailand, where ambient temperatures, light intensity, and CO₂ availability are favorable year-round; the applicability of this protocol in temperate or seasonally variable climates may require modification, particularly with respect to temperature management and supplemental lighting during cooler months.

4. Conclusions

This study developed a comprehensive, cost-effective micropropagation protocol for the CITES Appendix I-listed orchid *D. cruentum*, addressing both ex situ conservation and commercial production needs. Chemical sterilization with 2.0 mL/L Haiter® controlled contamination to 10% eliminated the need for autoclaving, reducing sterilization-related costs by an estimated 60–70% (per-cycle cost: USD 0.01–0.02 vs. USD 0.20–0.30 for autoclaving). Net house culture with 40 g/L sucrose produced superior plantlets compared with controlled-room culture after two months, yielding 2.63 new shoots, 14.33 roots, 3.60 cm height, and 1.14 g fresh weight per plantlet — representing improvements of 22%, 69%, 41%, and 75.4%, respectively. Charcoal substrate supported the highest acclimatization survival (85%), confirming its suitability for this species' epiphytic root characteristics. The integrated protocol reduces overall operational costs by 40–70% across sterilization and climate control components, making micropropagation economically accessible to small-scale growers and community-based conservation programs in tropical regions. Genetic fidelity verification and validation under non-tropical climates remain priorities for future research. This approach offers a transferable model for other threatened tropical orchids where ambient net house conditions can substitute for energy-intensive infrastructure.

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Conflicts of Interest: The authors declare no conflict of interest.

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