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RESEARCH ARTICLE

In vitro Regeneration and *Ex vitro* Acclimatization of *Aerides crispa* Lindl., A Commercially Important orchid

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Abstract

Aerides crispa Lindl., an epiphytic orchid native to the Western Ghats of India, holds significant ornamental and medicinal values. Unfortunately, several orchid species are recognized as threatened due to habitation loss and lack of inefficient natural propagation. This study aimed to develop a reliable and reproducible *in vitro* regeneration protocol for *A. crispa* through asymbiotic seed germination and tissue culture techniques. Green, un-dehisced seedpods harvested 60-63 days after pollination were surface sterilized and inoculated on Murashige and Skoog medium. The highest seed germination (91.67%) occurred on hormone-free MS medium, indicating that exogenous plant growth regulators are not essential for germination. However, 1.0 µM/L BAP significantly enhanced protocorm-like body (PLB) formation (93.33%) and shoot development (90%). Optimal microshoot multiplication (15.52 ± 0.17 shoots/explant) was achieved with 2.0 µM/L BAP + 1.0 µM/L IBA, while maximum shoot elongation occurred at 1.0 µM/L BAP. Rooting was most effective with 4.0 µM/L IBA, producing 93.33% rooting and an average of 4.21 roots/shoot. Among acclimatization substrates, the mixture of garden soil, cocopeat, and charcoal (1:1:1) supported 100% *in vivo* survival. This study provides a suitable plant propagation approach that might be useful for the conservation of *A. crispa*, thereby contributing to *ex situ* conservation of the threatened orchid species and their further commercial cultivation.

Keywords: *Aerides crispa*, asymbiotic seed germination, *in vitro* propagation, PLBs, shoot multiplication, rooting, acclimatization.

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Introduction

The Orchidaceae family is one of the largest and most diverse families of angiosperms, comprising over 25,000 species and more than 100,000 hybrids worldwide (Arditti and Ernst 1993). Orchids are renowned for their ecological complexity, economic significance, and ornamental value. Among these, *Aerides crispa* Lindl., a monopodial epiphytic orchid endemic to the Western Ghats and surrounding regions of India, has garnered attention for its attractive, fragrant flowers and medicinal applications in traditional systems of medicine (De and Pathak 2015). However, *A. crispa*, like many orchid species, faces significant conservation challenges due to overexploitation, habitat destruction, and inherently inefficient natural propagation mechanisms. Orchid seeds are minute and lack endosperm, necessitating a symbiotic relationship with mycorrhizal fungi for successful germination in nature (Rasmussen 1995). Moreover, vegetative propagation is slow, producing limited numbers of plantlets and offering little scope for meeting commercial demands or conservation goals (Hossain et al. 2010). To address these constraints, *in vitro* propagation has emerged as a promising tool for the mass multiplication and conservation of rare, threatened, and commercially important orchid species (Baskaran and Jayabalan 2005). Tissue culture techniques enable the production of large numbers of uniform, disease-free plantlets under controlled conditions, independent of seasonal limitations and environmental factors (Martin and Madassery 2006,

Deb and Pongener 2011). Such biotechnological approaches are not only valuable for conserving genetic diversity but also provide a sustainable means of supporting floriculture and pharmaceutical industries.

Previous studies on orchid micropropagation have demonstrated the effectiveness of asymbiotic seed germination and shoot tip culture for several genera, including *Dendrobium*, *Phalaenopsis*, and *Vanda* (Roy and Banerjee 2003, Kaur et al. 2010, Rahman et al. 2023). However, there is limited literature available on optimized tissue culture protocols specific to *A. crispa*, necessitating further investigation. The present study aims to develop an efficient and reproducible *in vitro* regeneration protocol for *Aerides crispa* using tissue culture techniques. By optimizing culture media and regeneration conditions, this work seeks to contribute to the conservation and commercial utilization of this ecologically and economically significant orchid species.

Materials and Methods

Sixty to sixty-three days after pollination, the green, undehisced seedpods or capsules were harvested from the mother plant's native habitat in Bangladesh's Satkhira area (Fig. 1A). Approximately six to ten seedpods were removed from the plants and washed under running water. After that, those were put in a conical flask with distilled water in it. Savlon (0.3% v/v chlorhexidine gluconate and 3% w/v cetrimide, marketed in Bangladesh by ACI) was used to surface sterilize the explants. To ensure that the sterilizing agents were entirely removed, the components were thoroughly rinsed four or five times with distilled water before being moved to a 500 ml sterilized conical flask under a laminar air-flow cabinet.

A laminar air-flow cabinet was used to sterilize the surface. For six to twelve minutes, the explants were gently agitated while immersed in a 0.1% $HgCl_2$ solution in sterile conical flasks. Sterilized distilled water was then used to rinse the samples three or four times to eliminate $HgCl_2$. Following this procedure, the seedpods were put in a sterile Petri dish. The seed coat was cut lengthwise with a scalpel for inoculation after the tips of the seedpod were removed using a pair of tiny forceps. Because orchid seeds are tiny and powdery, they are spread out on a petri dish with Murashige and Skoog (1962) medium to germinate (Fig. 1B-D).

Protocorm developmental phases were documented weekly following a two-week inoculation period. Protocorms were aseptically removed from culture containers and placed in other culture vessels with the same germination medium. The protocorm regenerated young plantlets with one or two leaves and one or two weak roots. The plantlets were sub-cultured again at various intervals of varying lengths of time. The number of plantlets per vessel was decreased before each subculture. To create secondary protocorms, *in vitro* grown protocorms were utilized as explants. PGRs were added to agar-solidified MS medium in varying quantities, either singly or in combination, to evaluate their impact on secondary protocorm growth and development. Various cytokinin and auxin combinations and concentrations were employed as PGRs in the study. The control group was MS medium without PGRs. The ability of a single primary protocorm to produce several protocorms and their capacity to produce plantlets each week after culture initiation served as the basis for the data collection.

In order to establish roots, recently formed adventitious shoots were placed in the rooting medium. To evaluate the effects of three plant growth regulators on root development, we utilized NAA, IBA, and NAA at 2.0, 4.0, and 6.0 µM/L in MS medium. The plantlets that were successfully rooted *in vitro* were able to harden in the various substrates.

Three different kinds of acclimatization substrates were used in this study: i) coco peat, ii) garden soil, cocopeat, and charcoal (1:1:1), and iii) garden soil, sand, and compost (2:1:1). For eight weeks, the plantlet was primarily grown *in vivo*, and the *in vivo* conditions were assessed. The average number of leaves, average leaf length (cm), and plant survival percentage were among the metrics evaluated.

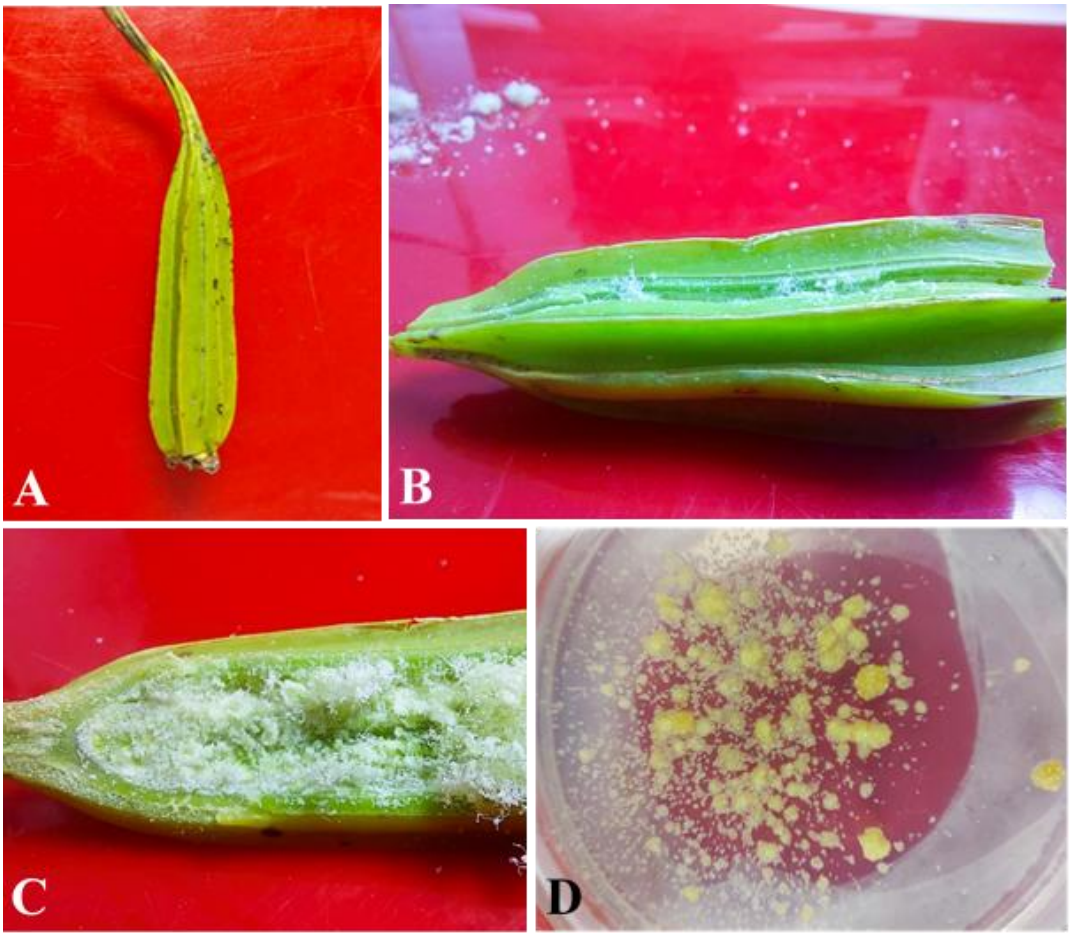

Fig. 1(A-D): Photographs showing the morphology and culture of seedpod of *Aerides crispa.* A: Sterilized seed pod, B-C: Dissected seedpod with powdery seeds, D: Cultured seeds on hormone-free MS medium.

All media were prepared using 3% (w/v) sucrose and 0.8% (w/v) agar. The pH of the medium was adjusted to 5.7 ± 1.0 before autoclaving it at 121°C for 20 minutes at 1.2 kg/cm^2 pressure. A cool-white fluorescence tube lamp with a light intensity of 50 µM/Lol·m^{-2}·s^{-1} was used to illuminate the cultures throughout a 16-hour photoperiod at 25 ± 1°C. After the culture was incubated for 4-8 weeks, all experimental results were documented. In all investigations, 10 to 20 explants were used and each experiment was conducted three times. The mean and standard deviation were calculated for every numerical data set. The mean data for each treatment was compared using the Duncan's Multiple Range Test (DMRT) with a p-value of 0.05%.

Results and Discussion

Surface sterilization of seed pods

The effectiveness of surface sterilization in *Aerides crispa* seed pods was evaluated using varying treatment durations of 0.1% mercuric chloride ($HgCl_2$). The aim was to identify a treatment regime that minimized contamination while preserving explant viability and responsiveness *in vitro.* The data clearly indicated a treatment-dependent trend in contamination rates and explant survival (Fig. 2).

Short exposure times of 5 and 7 minutes were insufficient, resulting in 100% and 80% contamination, respectively, and no significant explant response. As the treatment duration increased to 8 and 9 minutes, contamination rates decreased to 65% and 45%, with corresponding increases in explant responsiveness to 35% and 55%, respectively. The optimal outcome was recorded at a 10-minute exposure, which resulted in only 10% contamination and 90% explant responsiveness. However, an extended treatment of 12 minutes, although completely eliminating contamination, led to total tissue necrosis, likely due to the toxic effects of prolonged $HgCl_2$ exposure.

These results suggest that 10 minutes of sterilization with 0.1% $HgCl_2$ is optimal for *A. crispa* seed pods, effectively balancing microbial control with tissue viability. This is consistent with other studies in orchid micropropagation, where similar concentrations and exposure durations have been found effective for sterilizing explants while minimizing phytotoxicity (Arditti and Ernst 1993, Martin 2003).

Mercuric chloride remains a widely used sterilizing agent due to its strong antimicrobial properties, especially for hard-to-sterilize orchid tissues. However, its narrow safety margin necessitates precise optimization, as even slight overexposure can be lethal to delicate tissues. The current findings underscore the importance of empirically determining species-specific protocols to ensure the success of subsequent *in vitro* culture processes.

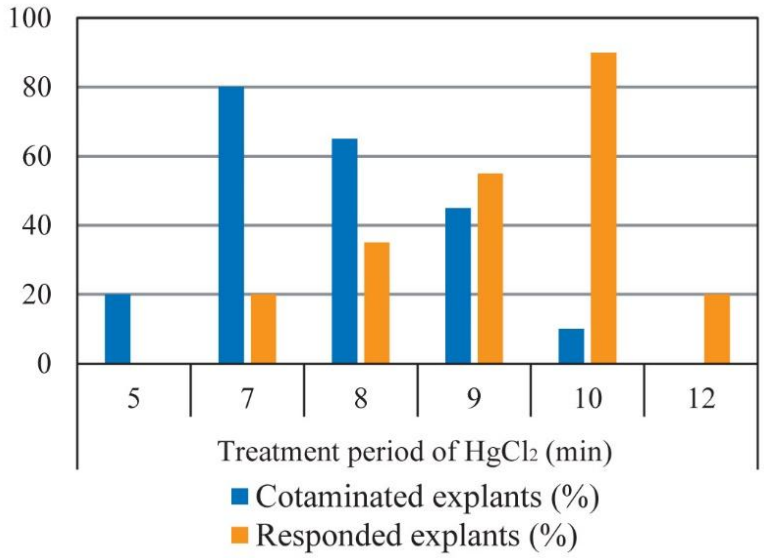

Fig. 2: Figure shows the effect of different treatment durations with mercuric chloride ($HgCl_2$) on explant sterility and response.

In vitro seed germination under different PGRs

The *in vitro* seed germination response of *A. crispa* varied markedly with different concentrations and combinations of plant growth regulators (Table 1). The highest germination percentage (91.67%) was observed in the control (hormone-free MS medium), indicating that exogenous application of plant growth regulators (PGRs) may not be essential for seed germination in this species and could even have inhibitory effects (Fig. 3A).

Among the single PGR treatments, BAP (6-Benzylaminopurine) at 1.0 µM/L resulted in a relatively high germination rate (83.33%) with slight callusing. However, increasing the BAP concentration 4.0 µM/L drastically reduced germination to 41.67% and promoted intensive callus formation (+++). This inverse relationship suggests a possible threshold beyond which cytokinin activity inhibits embryogenic development, possibly due to the redirection of cellular activity toward undifferentiated callus growth rather than organized seedling formation. Similar inhibitory effects of high cytokinin concentrations on seed germination have been reported in other orchid species (Pathak et al. 2023, Sharma and Singh 2022).

Table 1: Effects of different PGRs in MS medium on *in vitro* seed germination of *Aerides crispa*. Seeds were cultured on 10-12 petri dishes for each treatment and data ($\bar{X}$+SE) were recorded every 4 weeks after culture initiation.

PGRs	Conc. of PGRs (µM/L)	Germination (%)	Intensity of callus growth*
HO	0.0	91.67	-
BAP	1.0	83.33	+
	2.0	75.00	++
	4.0	41.67	+++
BAP + IBA	1.0 + 5.0	72.73	+++
	2.0 + 1.0	66.67	++++
	4.0 + 2.0	38.46	++++
BAP + NAA	1.0 + 5.0	54.55	+++
	2.0 + 1.0	40.00	++++
	4.0 + 2.0	27.27	++++

*Intensity of callusing (-) = No callusing; (+) = Slight callusing; (++) = Considerable callusing; (+++) = Intensive callusing; (++++) = Massive callusing.

In combination treatments, BAP with IBA showed progressively declining germination with increasing concentration: from 72.73% (1.0 + 5.0 µM/L) to 38.46% (4.0 + 2.0 µM/L). A similar trend was observed for BAP + NAA combinations, where germination decreased from 54.55% (1.0 + 5.0 µM/L) to 27.27% (4.0 + 2.0 µM/L), accompanied by increasingly massive callus formation (++++) at higher concentrations (Table 2). These results suggest a synergistic but counterproductive interaction between cytokinins and auxins at elevated levels, leading to excessive callogenesis that competes with or inhibits organogenic pathways essential for germination (Rajeswari et al. 2020).

The negative correlation between callus intensity and germination percentage across all PGR treatments highlights a critical balance required between morphogenesis and callogenesis for successful *in vitro* propagation. While a certain degree of callus formation may be beneficial for subsequent organogenesis, excessive callusing appears detrimental at the initial germination stage. This observation aligns with recent findings on *Dendrobium* and *Vanda* species where optimal seedling regeneration was achieved at low to moderate PGR levels (Hoque et al. 2021, Lee et al. 2024).

Our study revealed that the control medium without PGRs was the most effective for germination of *A. crispa*, indicating that endogenous hormonal levels are likely sufficient for triggering germination *in vitro*. The application of PGRs, particularly at higher concentrations, induced callogenesis at the expense of germination, underscoring the need for precise hormonal regulation in micropropagation protocols for this species.

Formation of PLBs and shoots from *in vitro* germinated seeds

The current investigation assessed the effect of various PGRs, including individual and combined treatments of BAP, 2,4-D (2,4-Dichlorophenoxyacetic acid), NAA (α-Naphthaleneacetic acid), and IBA (Indole-3-butyric acid), on protocorm-like bodies (PLBs), shoot formation, number of shoots, callus growth, and shoot initiation time from *in vitro* germinated seeds of *A. crispa*. The highest PLB formation (93.33%) was observed with both hormone-free (control) and 1.0 µM/L BAP treatments (Fig. 3B). However, significant enhancement in shoot formation (90.00%) was recorded only in 1.0 µM/L BAP, suggesting its superior efficacy in shoot regeneration compared to other treatments (Fig. 3C; Table 2). This agrees with earlier findings by Baskaran and Jayabalan (2005), who noted BAP as a potent cytokinin for shoot proliferation in orchids.

With increasing BAP concentrations, both PLB and shoot formation rates decreased progressively. For example, at 4.0 µM/L BAP, PLB and shoot formation were reduced to 80.00% and 45.55%, respectively. This decline may be attributed to cytokinin-induced stress or hormonal imbalance at higher concentrations, as reported in *Dendrobium nobile* by Roy et al. (2023). Among auxins, 2,4-D at 2.0 µM/L showed moderate PLB formation (80%) but minimal shoot regeneration (10%), suggesting its limited role in direct organogenesis and a preference for

callogenesis, consistent with the observations in *Phalaenopsis* spp. (Zeng et al. 2022). The maximum number of shoots per explant (12.40 ± 0.26) was recorded in 1.0 µM/L BAP, followed by 1.0 + 0.5 µM/L BAP + NAA (9.55 ± 0.34), indicating the synergistic effect of low auxin and cytokinin concentrations in promoting shoot multiplication. This result corroborates with George et al. (2008), who emphasized optimal cytokinin-to-auxin ratios for enhanced shoot proliferation.

Table 2: Effects of different concentration of PGR on PLBs and shoots formation from *in vitro* germinated seeds of *Aerides crispa*. There were 10-15 explants for each treatment and data ($\bar{X}$+SE) were recorded after each 4 weeks of culture initiation.

PGRs	Conc. of PGRs (µM/L)	PLBs formation (%)	Shoot formation (%)	No. of Shoots/culture	Callus growth (fresh Wt. g)	Shoot initiation (days)
HO	-	60.00	93.33	15.21 ± 0.26^{a}	1.51 ± 0.12^{d}	7-8
BAP	1.0	93.33	90.00	12.40 ± 0.26^{b}	2.23 ± 0.17^{bc}	9-10
	2.0	90.91	75.00	8.33 ± 0.26^{d}	2.56 ± 0.11^{bc}	10-11
	4.0	80.00	45.55	6.74 ± 0.30^{e}	2.87 ± 0.21^{ab}	11-12
2, 4-D	1.0	64.29	15.38	2.52 ± 0.23^{g}	1.92 ± 0.15^{cd}	10-12
	2.0	80.00	10.00	1.51 ± 0.30^{h}	2.02 ± 0.20^{cd}	12-14
	4.0	54.55	6.67	1.10 ± 0.23^{h}	3.10 ± 0.20^{ab}	14-15
BAP+ NAA	1.0+0.5	66.67	60.00	9.55 ± 0.34^{c}	2.20 ± 0.15^{bc}	10-12
	2.0+1.0	70.00	53.85	7.22 ± 0.34^{e}	2.67 ± 0.18^{abc}	12-13
	4.0+2.0	50.00	36.36	5.33 ± 0.34^{f}	3.12 ± 0.20^{a}	13-14
BAP + IBA	1.0+0.5	71.43	63.64	4.60 ± 0.30^{f}	2.42 ± 0.23^{bc}	9-11
	2.0+1.0	75.00	57.14	3.10 ± 0.34^{g}	2.89 ± 0.16^{ab}	11-12
	4.0+2.0	60.00	38.46	2.50 ± 0.34^{g}	3.30 ± 0.34^{a}	12-14

The values are presented as Mean ± SE, indicating that values sharing the same letter are not significantly different according to Dunkan's multiple range test at the 0.05 probability level.

The callus growth (measured as fresh weight) varied across treatments, with the highest value (3.30 ± 0.34 g) recorded in the 4.0+2.0 µM/L BAP + IBA combination. In general, higher auxin concentrations, particularly when combined with cytokinins, favored increased callus formation, which aligns with the findings of Cruz et al. (2023) in orchid tissue culture systems. Shoot initiation occurred earliest (7-8 days) in the hormone-free medium, indicating the intrinsic regenerative potential of *A. crispa* seeds. However, while BAP-treated explants-initiated shoots slightly later (9-10 days), they developed significantly more shoots. Treatments involving 2,4-D showed delayed initiation (up to 15 days), further supporting its less direct role in shoot development.

Combinations of BAP with auxins (NAA and IBA) showed moderate performance in both shoot number and PLB induction. Among them, 1.0 + 0.5 µM/L BAP+NAA produced better outcomes than higher concentrations, highlighting the importance of hormone balance. Similar trends have been noted in other orchids like *Vanda coerulea* and *Oncidium* hybrids (Pathak et al. 2020).

This study demonstrates that 1.0 µM/L BAP alone is optimal for efficient shoot regeneration and PLB formation in *Aerides crispa*. While auxin combinations enhanced callogenesis, they were less effective in shoot multiplication. These findings provide valuable insights for refining micropropagation protocols of this ornamental orchid species and can aid in its conservation and commercial production.

Micro-shoot induction from protocorm like bodies (PLBs)

The study investigated the effects of varying concentrations and combinations of cytokinins (BAP) and auxins (NAA, IBA) on the shoot multiplication and elongation from protocorm-like bodies (PLBs). Substantial variation in both shoot number and length was observed across treatments (Table 3), highlighting the influence of

plant growth regulators (PGRs) on micropropagation efficiency. The combination of 2.0 µM/L BAP with 1.0 µM/L IBA was consistently the most effective treatment across all time points, producing 7.47 ± 0.32 shoots at 4 weeks, 11.12 ± 0.15 shoots at 8 weeks, and 15.52 ± 0.17 shoots at 12 weeks (Fig. 3D). These values were significantly higher than all other treatments, indicating a strong synergistic effect between BAP and IBA on shoot induction. This supports previous findings by Lee et al. (2023) and Kumar et al. (2022), who reported enhanced shoot proliferation in orchids using similar hormonal combinations.

The second-best performing treatment was 2.0 µM/L BAP + 1.0 µM/L NAA, which yielded 5.67 ± 0.44, 8.36 ± 0.21, and 11.52 ± 0.25 shoots at 4, 8, and 12 weeks, respectively. Although NAA also contributed to increased shoot number, its effect was generally less pronounced compared to IBA. This aligns with Rahman et al. (2023), who suggested that IBA is more effective in stimulating shoot initiation due to its slower metabolism and sustained activity in tissue cultures. Interestingly, BAP alone at 2.0 µM/L produced a moderate response (7.32 ± 0.24 shoots at 12 weeks), whereas a higher concentration (4.0 µM/L) resulted in significantly lower shoot numbers (5.20 ± 0.16), indicating potential cytokinin toxicity or feedback inhibition at supra-optimal levels, a phenomenon well-documented by George et al. (2023).

In terms of shoot elongation, the longest average shoot length (5.61 ± 0.11 cm) was observed in the 1.0 µM/L BAP treatment after 12 weeks, although this treatment only produced 6.25 ± 0.17 shoots, showing a potential trade-off between shoot number and elongation. Treatments such as BAP 2.0 µM/L and BAP + IBA 1.0 + 0.5 µM/L also resulted in relatively long shoots (4.35 ± 0.30 cm and 4.34 ± 0.24 cm, respectively), suggesting that moderate cytokinin levels support elongation when not counteracted by excessive callus formation or shoot clustering.

Table 3: Effects of different concentrations of cytokinin and auxin on shoot multiplication developed from PLBs. There were 10 explants for each treatment and data ($\bar{X}$+SE) were recorded after every 4 w of culture.

PGRs	Conc. of PGRs (µM/L)	Avg. No. of shoot (4 w)	Avg. No. of shoot (8 w)	Avg. No. of shoot (12 w)	Avg. shoot length after 12 w (cm)
BAP	1.0	2.98 ± 0.26^{ef}	4.75 ± 0.23^{d}	6.25 ± 0.17^{ef}	5.61 ± 0.11^{a}
	2.0	3.78 ± 0.30^{de}	5.98 ± 0.42^{cd}	7.32 ± 0.24^{de}	4.35 ± 0.30^{b}
	4.0	2.14 ± 0.14^{f}	3.66 ± 0.34^{e}	5.20 ± 0.16^{f}	3.11 ± 0.23^{cd}
BAP + NAA	1.0 + 0.5	3.56 ± 0.23d^{e}	5.89 ± 0.11^{cd}	8.21 ± 0.33^{cd}	3.87 ± 0.21^{bc}
	2.0 + 1.0	5.67 ± 0.44^{b}	8.36 ± 0.21^{b}	11.52 ± 0.25^{b}	3.12 ± 0.15^{cd}
	4.0 + 2.0	2.97 ± 0.13e^{f}	4.65 ± 0.32^{de}	6.32 ± 0.19^{ef}	2.00 ± 0.19^{e}
BAP + IBA	1.0 + 0.5	5.12 ± 0.15b^{c}	7.56 ± 0.14^{bc}	10.24 ± 0.25^{bc}	4.34 ± 0.24^{b}
	2.0 + 1.0	7.47 ± 0.32^{a}	11.12 ± 0.15^{a}	15.52 ± 0.17^{a}	4.01 ± 0.13^{bc}
	4.0 + 2.0	4.56 ± 0.23^{cd}	6.32 ± 0.19^{cd}	8.61 ± 0.24^{cd}	2.14 ± 0.17^{de}

The values are presented as Mean ± SE, indicating that values sharing the same letter are not significantly different according to Dunkan's multiple range test at the 0.05 probability level.

Conversely, treatments with high auxin concentrations (e.g., BAP + IBA 4.0 + 2.0 µM/L) resulted in shorter shoots (2.14 ± 0.17 cm), which may be attributed to increased callogenesis or suppressed elongation, as previously observed in orchid cultures by Nayak et al. (2022).

These outcomes are consistent with the hormonal balance model proposed by George et al. (2023), which emphasizes that a low-to-moderate cytokinin-to-auxin ratio favors organogenesis, while a high auxin level skews the response towards callus formation or root induction.

The superior performance of the 2.0 µM/L BAP + 1.0 µM/L IBA treatment highlights its potential as a standardized protocol for efficient micropropagation of this orchid species. The data underscore the necessity of fine-tuning PGR concentrations to match the physiological needs of the explant, reinforcing insights from Chen et al. (2024), who emphasized species-specific and stage-specific responses to PGRs in orchid tissue culture.

Root induction of *in vitro* grown micro-shoots

Root induction from *in vitro* grown micro-shoots is a crucial phase in micropropagation, significantly influenced by the type and concentration of auxins in the culture medium. The present study evaluated the effect of three major auxins-NAA, IBA, and IAA (indole-3-acetic acid) on adventitious root formation using MS medium. The data revealed distinct responses in root formation percentages, number of roots per shoot, root length, and days to root emergence across different treatments (Table 4).

IBA at 4.0 µM/L showed the highest root formation (93.33%) (Fig. 3E), followed by IAA at 6.0 µM/L (85.33%) and NAA at 4.0 µM/L (85.71%). The control (hormone-free) medium yielded 80.0% root formation, suggesting endogenous auxin activity was sufficient to support moderate root induction. The lowest response was noted in IAA at 2.0 µM/L (53.85%). Significant variation was observed among treatments. IBA at 4.0 µM/L was the most effective, producing the highest average number of roots per shoot (4.21 ± 0.42), followed by NAA at 4.0 µM/L (3.11 ± 0.29). These results align with findings by Gopi et al. (2023), who reported enhanced rooting potential with IBA due to its higher stability and effective transport in plant tissues compared to other auxins.

Conversely, IAA at 6.0 µM/L resulted in a significantly lower root number (1.04 ± 0.23), suggesting suboptimal rooting efficiency possibly due to IAA's rapid degradation in culture conditions, as discussed by Sharma et al. (2022). Root length differed significantly among treatments. The control treatment exhibited the longest roots (4.82 ± 0.07 cm), closely followed by IBA at 2.0 µM/L (4.30 ± 0.32 cm). This suggests that while IBA can enhance root proliferation, higher concentrations may compromise elongation, possibly due to hormonal imbalance or early lignification. Similar trends were reported in Phalaenopsis hybrids by Kim and Lee (2021).

Treatments with higher concentrations of NAA and IAA produced shorter roots, with the lowest root length recorded in IAA at 6.0 µM/L (1.87 ± 0.17 cm). These findings correspond with Ahmed et al. (2021), who reported inhibitory effects of high auxin concentrations on root elongation due to increased ethylene synthesis. The earliest root emergence (15-20 days) was recorded in NAA (4.0 µM/L) and IBA (4.0 µM/L), reflecting the synergistic effect of optimal auxin concentration on root induction kinetics. In contrast, the longest duration (30-35 days) was required for root emergence in IAA at 2.0 µM/L, reinforcing the notion of its limited effectiveness in this system.

Table 4: Effects of different concentrations of auxins on adventitious roots formation from *in vitro* grown microshoots in MS medium. Each treatment consisted of 10-15 micro-shoots and data ($\bar{X}$+SE) were recorded after 8 weeks of culture.

PGRs	Conc. of PGRs (µM/L)	Root formation (%)	Avg. No. of roots/shoot	Avg. length of roots/shoot (cm)	Days to immergence of roots
HO	0.0	80.00	1.80 ± 0.20^{de}	4.82 ± 0.07^{a}	25-30
NAA	2.0	64.29	1.89 ± 0.37^{de}	3.12 ± 0.42^{c}	20-25
	4.0	85.71	3.11 ± 0.29^{b}	2.45 ± 0.29^{d}	15-20
	6.0	71.43	1.45 ± 0.30^{ef}	1.98 ± 0.09^{de}	18-25
IBA	2.0	76.92	2.60 ± 0.12^{bc}	4.30 ± 0.32^{ab}	20-25
	4.0	93.33	4.21 ± 0.42^{a}	3.87 ± 0.19^{b}	15-20
	6.0	85.71	2.30 ± 0.14^{cd}	2.55 ± 0.42^{d}	20-25
IAA	2.0	53.85	1.23 ± 0.23^{fg}	2.10 ± 0.26^{de}	30-35
	4.0	66.67	2.12 ± 0.15^{d}	2.89 ± 0.35^{cd}	25-30
	6.0	85.33	1.04 ± 0.23^{g}	1.87 ± 0.17^{e}	25-30

The values are presented as Mean ± SE, indicating that values sharing the same letter are not significantly different according to Dunkan's multiple range test at the 0.05 probability level.

IBA at 4.0 µM/L emerged as the most effective treatment overall, yielding the highest root formation percentage and number of roots per shoot, coupled with moderately long roots and early emergence. These results substantiate IBA's superiority in routing protocols, particularly for woody and ornamental plant species, a finding

consistent with studies on other orchids and ornamental taxa (Bhattacharyya and Kumaria 2020). In contrast, although IAA at 6.0 µM/L showed relatively high rooting percentage, it was less effective in producing a desirable root number and length. NAA also proved effective at 4.0 µM/L but was comparatively inferior to IBA in overall rooting quality.

This study underscores the critical role of auxin type and concentration in optimizing *in vitro* rooting. IBA at 4.0 µM/L is recommended for efficient root induction in the studied plant, balancing both quality and quantity of roots. Future studies may explore synergistic combinations with cytokinins or alternative rooting substrates to further enhance acclimatization success.

Acclimatization of *in vitro* regenerated plantlets

Acclimatization represents a critical phase in micropropagation, determining the survival and subsequent growth performance of *in vitro* regenerated plantlets. The success of this process largely depends on the selection of appropriate substrates that support root aeration, moisture retention, and nutrient availability (Mitra et al. 2022). In this study, three different substrates were evaluated for their effectiveness in acclimatizing *Aerides crispa* plantlets over 10 weeks.

Among the three tested substrates, the combination of garden soil + cocopeat + charcoal (1:1:1) demonstrated the highest survival rate, achieving 100% survival under *ex vitro* conditions and 90% under *in vivo* conditions (Fig. 3F). Cocopeat alone also supported relatively high survival (90% *ex vitro*, 80% *in vivo*). The lowest survival was observed in the garden soil + sand + compost mixture (80% *ex vitro*, 70% *in vivo*) (Table 5).

Table 5: Effects of different substrates on the acclimatization of *Aerides crispa.* Each treatment consisted of 10 plantlets and data ($\bar{X}$+SE) were recorded after 10 weeks of transplantation.

Substrates	Survival rate (%)		Avg. No. of leaf		Avg. length of leaf (cm)
	Ex vitro	*In vivo*	*Ex vitro*	*In vivo*	*In vivo*
Coco peat	90	80	5.20 ± 0.29a	6.21 ± 0.38a	4.87 ± 0.30b
Garden soil + cocopeat + charcoal (1:1:1)	100	90	5.50 ± 0.42a	7.20 ± 0.35a	5.56 ± 0.34a
Garden soil + sand + compost (2:1:1)	80	70	3.70 ± 0.26b	5.00 ± 0.42b	3.51 ± 0.28c

The values are presented as Mean ± SE, indicating that values sharing the same letter are not significantly different according to Dunkan's multiple range test at the 0.05 probability level.

These findings align with those of Lakshanthi and Seran (2019), who reported superior survival of orchid plantlets in substrates containing cocopeat and charcoal due to their high porosity, water retention, and microbial suppression capabilities. Charcoal, in particular, improves substrate aeration and adsorbs phenolic compounds released during root development, which may otherwise be toxic to developing plantlets (Luan et al. 2021).

The results also indicate that substrates containing cocopeat and charcoal supported higher leaf production. This is consistent with observations by Singh and Kalita (2022), who reported that cocopeat provides a favorable microenvironment for leaf emergence due to its high moisture-holding capacity and good drainage. Plantlets transferred to the garden soil + cocopeat + charcoal substrate produced significantly longer leaves compared to those in other treatments. Leaf length is a reliable indicator of photosynthetic capacity and overall plant vigor. This result supports findings by Rahman et al. (2023), who showed that substrates with better aeration and nutrient availability enhance vegetative growth during orchid acclimatization.

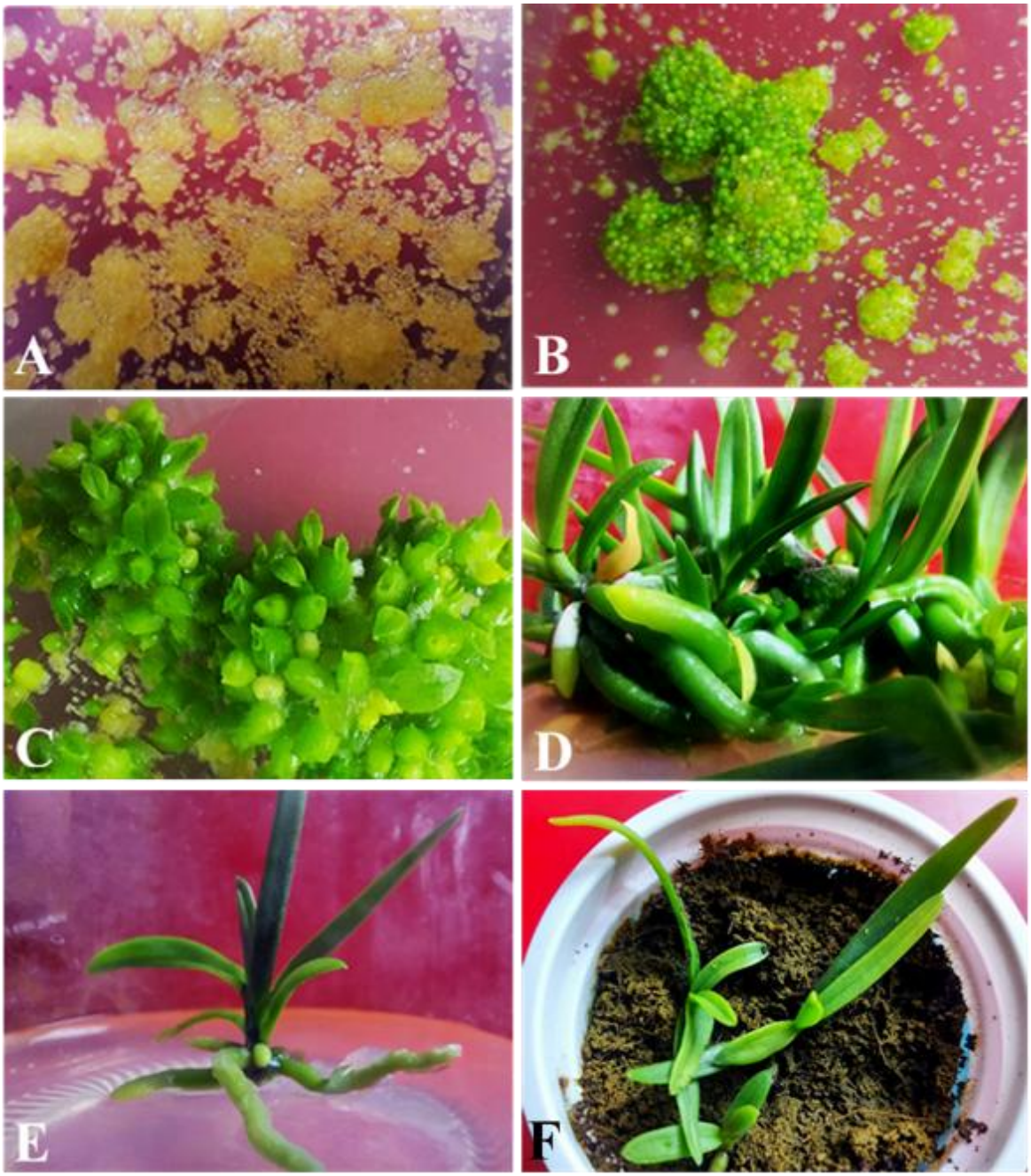

Fig. 3 (A-F): Photographs showing seed germination, regeneration and acclimatization of *Aerides crispa*. A: Cultured seeds germinated on hormone-free MS medium, B: PLBs formed on 1.0 µM BAP containing MS medium, C: Microshoots initiated from PLBs on MS medium containing 1.0 µM BAP, D: Shoot's multiplication and elongation of the same medium, E: Elongated shoot formed roots on 4.0 µM IBA, F: Rooted shoots acclimatized on garden soil + cocopeat + charcoal (1:1:1).

The superior performance of the garden soil + cocopeat + charcoal (1:1:1) substrate across all growth and survival parameters suggests that this combination offers optimal physical and biochemical conditions for post-transplant establishment of *Aerides crispa*. The presence of charcoal likely contributed to root health by buffering pH and reducing microbial load, while cocopeat provided moisture retention and aeration. These factors are essential for orchids, which are naturally adapted to epiphytic environments (Bhattacharjee et al. 2020).

On the contrary, the garden soil + sand + compost mixture showed consistently inferior results, possibly due to reduced porosity and water retention capacity, which may have led to higher transplant shock and limited root development. This study confirms that substrate composition plays a crucial role in the successful

acclimatization of *Aerides crispa*. The mixture of garden soil, cocopeat, and charcoal significantly enhances survival, leaf production, and leaf elongation, making it an ideal choice for large-scale orchid acclimatization. The findings provide valuable insight into substrate optimization for ex situ conservation and commercial orchid propagation.

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