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In vitro Development of Protocorms and Regeneration through Non-Symbiotic Seed Culture of a Medicinally Important Orchid of *Cymbidium aloifolium* (L.) Sw

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Key words: *Cymbidium aloifolium*, Medicinal value, Non-symbiotic seed, Orchid, Regeneration

Abstract

Different plant growth regulators were applied for *in vitro* development of protocorms, regeneration and mass multiplication using immature seeds of *Cymbidium aloifolium*. The germination ability and early development of protocorm-like bodies (PLBs) were evaluated on MS, ½MS, KC and VW media. In addition, different plant growth regulators, *viz*. BAP, Kn, NAA and IAA were used either individually or in combination with the medium. The maximum percentage of seed germination (97%) was recorded after initiation of cultures for 4-5 weeks. The highest number of protocorms was developed from germinated seeds after 5-7 weeks and seedlings were also developed after 15-16 weeks of culture initiation on MS basal medium without the phytohormones. The MS was found to be the most suitable medium for seed germination as well as seedling formation. Maximum shoot length (4.63 ± 0.56 cm) was recorded when MS medium was supplemented with BAP (1.0 mg/l) and NAA (0.5 mg/l). For root induction, ½MS medium containing NAA showed more superiority than other hormonal supplements. The highest number (5.62) of rooted plantlets and maximum root length (3.95 cm) were observed on ½MS medium containing 0.5 mg/l NAA. The well-rooted plantlets were acclimatized and successfully transferred to the pot containing soil. The protocol established under this study would be helpful for further research for the isolation of biochemical compounds for medicinal uses of this orchid and also for commercial cultivation purposes.

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Introduction

Cymbidium aloifolium (L.) Sw. is an epiphytic endangered orchid species in Bangladesh, having high medicinal and ornamental values. This orchid is also an endangered plant belonging to the family of Orchidaceae, which contains 800 genera distributed throughout the world (Chugh et al. 2009). The plant contains tannins, alkaloids, glucosides, β-sitosterol, γ-sitosterol, resins, fatty oils, coloring matters and long-chain aliphatic compounds. *Cymbidium aloifolium* is accounted for to have emetic and laxative properties (Das et al. 2008). The aerial roots of this orchid may be crushed with ginger and blended with water and this mixture used to cure chronic illness and paralysis. People are using their leaves and pseudobulbs for different medications. Paste of leaves and pseudobulbs is used on the fractured and dislocated bones and also used as a tonic (Pradhan et al. 2013). The leaf paste and extract are comprehensively used for anti-hemorrhagic properties for the treatment of boils and fevers by the local tribes (Huda 2000, Nongdam and Chongtham 2011). Small seeds of this orchid are used for curing wounds (Medhi and Chakrabarti 2009). The whole plant extract can also be used for the treatment of weakness of eyes, vertigo, burns, sores and also used as a tonic (Chowdhery 2001, Rasoanaivo et al. 2011, Stéphane et al. 2022, Balkrishna et al. 2022). Because of its elegant and nice flowers, this orchid is awfully demanded in the floriculture industry (Pradhan et al. 2013).

The agro-climatic circumstances in different parts of Bangladesh, especially Bandarban, Khagrachari, Rangamati, Chittagong, Sylhet and Sundarbans, are congenial to the vegetation of natural orchids. In Bangladesh, there are about 115 distinct orchid varieties accessible (Bhadra et al. 2002). But native orchids are facing extinction due to losses of habitat, deforestation and unsystematic collection by traders and hobbyists. Now orchid belongs to endangered extinct species due to habitat devastation and indiscriminate collection have also led to severe reduction of orchids in the Indian sub-continent, including Bangladesh (Bhattacharjee et al. 2014b, Bhattacharjee et al. 2015). Various biotech techniques have been widely used for improving orchids since the 1970s, such as *in vitro* micropropagation, genetic transformation and cryopreservation (Antony et al. 2014, Bhattacharjee and Islam 2015, Aung et al. 2022). Such techniques are key tools to fulfill the commercial requirement and preservation of rare, wild and important orchid species worldwide. Pierik (1987) noted that in nature, orchids generated more or less 1300 to 4 million seeds per capsule. But the natural germination rate of orchids is only 0.2-0.3 per cent (Singh 1992). Naturally, the seeds of orchids are very tiny in size due to a lack of endosperm. Therefore, during germination, they need external nutrient support for the proliferation of the embryo and to develop into a globular protocorm (Bindiya et al. 2013). The present investigation has been conducted to study the effects of different media and PGRs for *in vitro* germination of seeds, formation of protocorms and growth of seedlings and mass multiplication of *Cymbidium aloifolium.* The *in vitro* regeneration protocol has been standardized for the conservation of this plant as well as for biotechnological research in Bangladesh.

Materials and Methods

The immature capsules of *Cymbidium aloifolium* were collected from the natural habitat at Kaptai National Park, Kaptai, Rangamati, Bangladesh. These immature pods of *Cymbidium aloifolium* were used as a source of explants for the current study.

The capsules were washed with running tap water and detergent for 10 min to remove dust and microbes. They are surface sterilized by immersing them in 0.2% $HgCl_2$ solution for 5 min, 70% ethanol for 1 min, respectively. Finally, the capsules were washed with sterile distilled water 4-5 times and cut with a sterile surgical blade in aseptic conditions in the laminar air flow cabinet. The powdery seeds were taken out with the help of sterile forceps and were inoculated into the medium in culture vessels. The vessels were finally sealed with a lid and parafilm.

Four basal media *viz*. MS, ½MS, VW (Vacin and Went 1949) and KC (Knudson 1946) were used under this study to evaluate the seed germination status, protocorn-like bodies (PLBs) development, seedling growth and shoot elongation. Sucrose was used as the main carbohydrate source. 3% sucrose was used for MS and ½MS medium. For VW and KC medium, 2% sucrose was used. The p^H of all the media was adjusted to 5.4-5.8 before adding agar. 0.8% agar was used as a gelling agent and dissolved by boiling and about 50 ml of medium was dispensed into each culture vessel. The cultures were maintained in a growth room at 14/10 h continuous light and dark conditions illuminated with a fluorescent tube of 2000-3000 lux at 25 ± 2°C.

Four different basal media, *viz.* MS and ½MS, KC and VW were used for this present study. In every 30 days of culture initiation, different growth stages of PLBs were recorded. The percentages of seed germination were calculated by using the following formula:

$$\text{Germination (\%)} = \frac{\text{Number of seeds showing swelling of the embryo}}{\text{No. of total seeds}} \times 100$$

The seedlings continued to grow on the same medium very slowly after germination. For prompt elongation, the seedlings were evaluated on to MS and ½MS, KC and VW media with sixteen different treatments *viz.* M_0 = MS basal; M_1 = MS + BAP 1.0 mg/l + Kn 1.0 mg/l; M_2 = MS + BAP 1.0 mg/l + IAA 0.5 mg/l; M_3 = MS + BAP 1.0 mg/l + NAA 0.5 mg/l; HM_0 = ½MS; HM_1 = ½MS + BAP 1.0 mg/l + Kn 1.0 mg/l; HM_2 = ½MS + BAP 1.0 mg/l + IAA 0.5 mg/l; HM_3 = ½MS + BAP 1.0 mg/l + NAA 0.5 mg/l; V_0 = VW basal; V_1 = VW+ BAP 1.0 mg/l + Kn 1.0 mg/l; V_2 = VW + BAP 1.0 mg/l + IAA 0.5 mg/l; V_3 = VW + BAP 1.0 mg/l + NAA 0.5 mg/l; K_0 = KC basal; K_1 = KC + BAP 1.0 mg/l + Kn 1.0 mg/l; K_2 = KC + BAP 1.0 mg/l + IAA 0.5 mg/l; K_3 = KC + BAP 1.0 mg/l + NAA 0.5 mg/l.

The six-month old, elongated seedlings without roots were grown on full- and half-strength MS medium with three different levels (0.5, 1.0 and 1.5 mg/l) of auxins *viz*. IAA, IBA and/or NAA for the induction of a robust root system. The well-rooted plants were separated from the culture bottle and successfully hardened in the pot.

The experiments were designed according to a complete randomized design (CRD). Data on seed germination, PLBs formation, developmental stages of the seedling, shoot, and root growth were recorded. For all cases, ten (10) replicates were considered per treatment. Data were subjected to analysis of variance (ANOVA), and means were differentiated applying DMRT ($p \leq 0.05$) with one-way analysis of variance.

Results and Discussion

In this experiment, immature capsules were taken for *in vitro* seed germination and four kinds of culture media, *viz.* MS, ½MS, KC and VW were used without PGRs to evaluate their effect on the germination of seeds and protocorm development. The highest seed germination percentage was recorded in MS (97%), followed by ½MS (68%), KC (86%), and VW (51%) (Table 1). The germination was characterized by swelling and the emergence of the embryo from the testa, and within 4 to 5 weeks, spherules (an unequal shaped cell mass) were formed from amorphous embryos. After 1-2 weeks, these spherules turned green and formed round structures as protocorms (Fig. 1b). Protocorms became visible after 5 weeks of culture initiation and were shown at the vegetative apex stages (Fig. 1c-d). This was followed by the development of 1st leaf and root primordia when MS was taken at 10-11 weeks and 12-13 weeks, respectively, and the seedling also developed within 15-16 weeks (Table 2). Out of four media, MS showed the most effective on the leaf and root formation as well as seedling development (Table 2).

Table 1. Effects of various media on seed germination and PLBs development in *Cymbidium aloifolium.*

Media	Amount of seeds per culture vessel (mg/l)	Seed germination		PLBs development	
		Weeks	% of germination	Weeks	% of protocorms
MS	200	4 - 5	97.0^{d}	5 - 7	92.0^{d}
½MS	200	7 - 8	58.0^{b}	8 - 9	62.0^{b}
KC	200	5 - 6	86.0^{c}	6 - 7	83.0^{c}
VW	200	6 - 7	51.0^{a}	7 - 8	41.0^{a}

Different superscripts, small letters in a column, indicate significant differences at 5% level according to DMRT.

Table 2. Effects of different culture media on the duration of seedling growth in *Cymbidium aloifolium.*

Culture media	Time required (weeks) for differentiation		
	1st leaf primordia	1st root primordia	Seedling
MS	10 - 11	12 - 13	15 -16
½MS	13 - 14	15 - 16	18 -19
KC	11 - 12	13 - 14	17 - 18
VW	12 - 13	14- 15	17 - 18

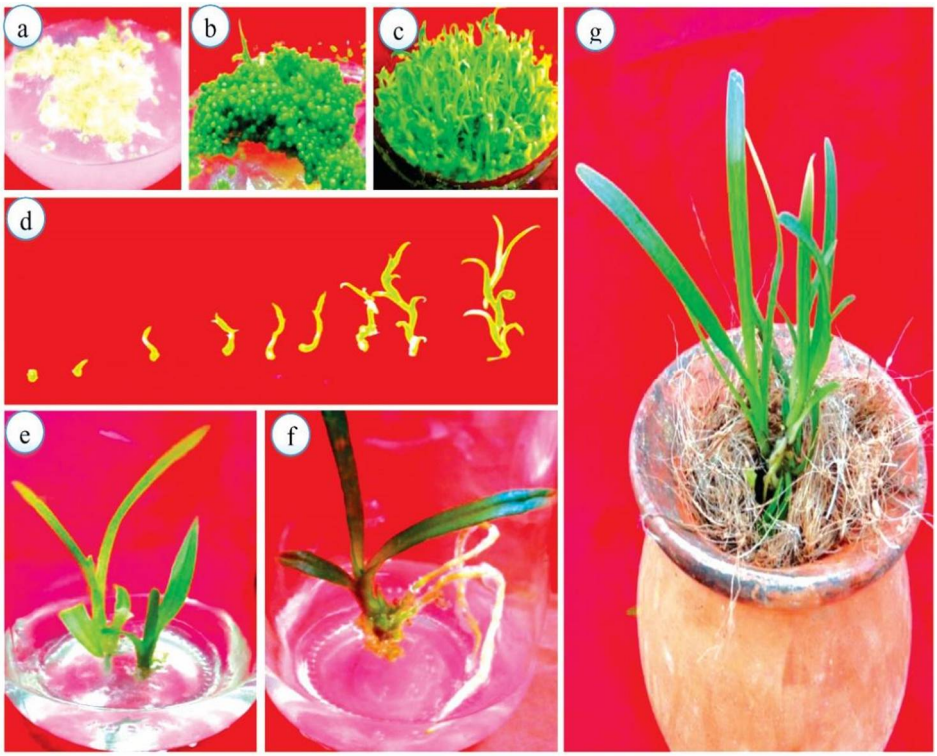

Fig. 1 (a-g). *In vitro* seed germination and seedling development of *C. aloifolium*: (a) inoculated seed for germination, (b) Protocorms formation, (c) Protocorms converted to PLBs and seedlings, (d) Different stages of seedling development from the PLBs, (e) Elongated plantlets, (f) Well rooted plant and (g) Hardened plant transfer to pot.

The seedlings continued to grow very slowly on the same medium after germination. Then the seedlings were transferred to four basal media with various combinations of PGRs for prompt elongation. The highest increased length of shoots (4.63 cm) was observed (Fig. 1e) on MS medium supplemented with 1.0 mg/l BAP and 0.5 mg/l NAA in comparison to all other combinations and concentrations of BAP, NAA, IAA and Kn within VW, KC and ½MS media. The shoot lengths are significantly varied among the four tested media (Fig. 2). Moreover, MS was the most suitable medium for seedling elongation.

To enhance the number of roots and their length, the plantlets were cultured in two strengths of MS media fortified with various concentration of auxins (Fig. 3). Half strength MS with agar (0.8% w/v) and sucrose (1.5% w/v) performed better than full strength MS basal medium both in terms of increased root length and greater number of roots (Fig. 1f). The highest number (5.62) of rooted plant was observed from 0.5 mg/l NAA in ½MS and the lowest (2.52) was noted in 1.5 mg/l NAA (Fig. 3). On the other hand, the maximum root length (3.95 cm) was found ½MS medium augmented with 0.5

mg/l NAA where in MS with 0.5 mg/l NAA was 3.53 cm and minimum (1.88 cm) was recorded MS basal (Fig. 3). As a result, NAA has been more efficient than IAA and IBA for root formation and elongation (Fig. 3). Therefore, significant differences were observed on the number of roots per plant among the combined effect of media and different levels of PGRs.

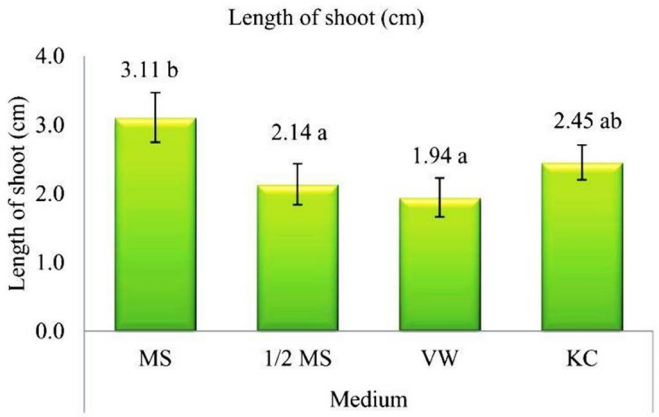

Fig. 2. Effects of various culture media on shoot elongation. Error bars indicate ± Standard Error (SE). Different small letters above the bar indicate significant differences at 5% level according to DMRT.

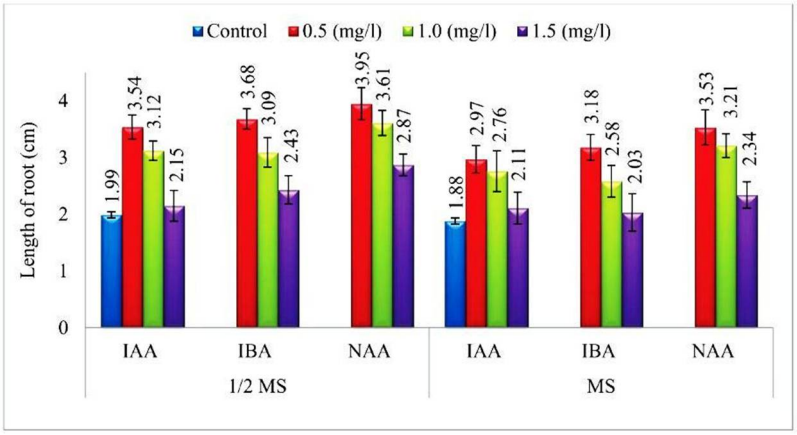

Fig. 3. Effect of two strengths of MS media with different concentrations of three auxins on root growth. Error bars indicate ± Standard Error (SE).

The different composts used for hardening of *in vitro* grown plantlets of *C. alloifolium* were found to be suitable for survivability and normal growth of the plants. However, the highest percentage survivability (76.0%) with maximum length (5.1 cm) of *C. alloifolium* hardened plants were obtained on substratum containing in Category-II: brick,

charcoal, decaying litter, coconut husk (1 : 1 : 1 : 1) with a layer of moss (Table 3 and Fig. 1g). The layer of moss on top proved to be favorable to higher retention of moisture content. Feeding the plantlets with dilute MS was found beneficial to the developing hardened plantlets of *C. alloifolium*. This protocol of protocorm regeneration, shoot formation, and *ex vitro* establishment of *C. alloifolium* can be successfully used for its mass multiplication and, therefore, conservation of this medicinally important endangered epiphytic orchid.

Table 3. Establishment of plants after 90 days in pots.

Category	Composition of substratum	No. of plants	Survival rate (%)	Plant height (cm)
I	a) Brick + charcoal (1 : 1) + moss (10.0 g/pot)	45	48.0 ± 82	2.7 ± 12
	b) Brick + charcoal + decaying litter (1 : 1 : 1) + moss (10.0 g/pot)	40	67.0 ± 85	4.1 ± 07
II	a) Brick + charcoal + decaying litter + saw dust (1 : 1 : 1 : 1) + moss (10.0 g/pot)	45	56.0 ± 78	3.0 ± 18
	b) Brick + charcoal + decaying litter + coconut husk (1 : 1 : 1 : 1) + moss (10.0 g/pot)	44	76.0 ± 80	5.1 ± 08
	c) Brick + charcoal + decaying litter + cow dung (1 : 1 : 1 : 1) + moss (10.0 g/pot)	40	61.0 ± 92	3.3 ± 13

The diameter of each pot was 8.0 cm.

In orchids, seed germination and the growth of the seedling are remarkably different from other flowering plants. Orchid seeds do not have endosperm and which limits their natural germination. Orchid seeds generally germinate symbiotically with certain species-specific mycorrhiza (sort of symbiotic fungus) that provides nourishment for the germinating undifferentiated orchid embryo (Rahman et al. 2009). Presently, the horticultural market depends on the wild orchid population, but the majorities are not properly propagated. For efficient germination and/or seedling growth in nature and *in vitro*, orchid seeds prefer and require external nutrients or growth substances (Teixeira da Silva 2015). Nutrient requirements are assumed to be genotype-specific in orchid seed germination (Arditti and Ernst 1984, Kauth et al. 2008). Consequently, it was not surprising that the various basal media used in the present study varied in their suitability for *in vitro* germination with their different compositions and concentrations of mineral salts, organic supplements, and vitamins. In this research, it was noted that seed germination was prompted by the MS medium. Nitrogen is an extremely essential plant growth component, and Stewart and Kane (2006) have reported that its source affects the germination of the various orchid species. The medium MS stimulated germination of seeds by containing ammonium and nitrogen (Popova et al. 2003). It was observed from

this investigation that MS is the most suitable medium for seed germination and PLB formation in *C. alloifolium.* Similar findings were also reported by Hossain (2013) in *Dendrobium aggregatum* and by Bhattacharjee and Islam (2014a and 2022) in *Vanda tessellata*. On conversely, Hossain (2008) reported that 75% of the *Eulophia ibaguense* seeds germinated on MS medium supplemented with 1 mg/l BAP, but with increasing concentrations of BAP, the seed germination percentage decreased. In case of *Cypripedium* sp. BAP enhanced seed germination (Zeng et al. 2014).

The present study showed that seedling elongation responded significantly higher in MS supplemented with 1.0 mg/l BAP and 0.5 mg/l NAA than in other media combinations. Similar results were observed in *Phalaenopsis cornorerris* (Bhattacharjee and Islam 2014b), *Cymbidium finlaysonianum* Lindl. (Islam SMS et al. 2015), *Vanilla planofolia* (Zuraida et al. 2013), *Alstroemeria* (Seyyedyousefi et al. 2013), Yoong et al. (2019), *Xanthosoma sagittifolium* (Bansal et al. 2023) and *Cymbidium* and *Cattleya* (Nagaraju and Mani 2005). Latha (1999) also reported that the addition of 1.0 mg/l BAP to the medium is effective in inducing the highest shoot elongation in *Habenaria crinifera*. On the other hand, the rapid seedling elongation was achieved by Bhattacharjee and Islam (2014b) in *Acampe prernorsa* and *Agrostophyllum khasianum* when 1.0 mg/l BAP and 0.1 mg/l picloram were supplemented in MS medium.

For the induction of a strong and stout root system, NAA was found to be the most superior auxin for promoting root formation and increase of root length, and it was also found that ½MS + 1.5% (w/v) sucrose was the best for root development. The positive impact of auxin in the event of *Dendrobium transparens was* also noted by Sunitibala and Kishor (2009). On the contrary, Bhadra et al. (2002) reported that ½MS + 1.5% (w/v) sucrose without auxin showed better performance in the case of root formation and increase of root length in *Dendrobium aphyllum*. However, in the compost of brick pieces, charcoal, decaying litter and coconut husk (1 : 1 : 1 : 1) with a layer of sphagnum moss, the highest percentage of survival of hardened plants was shown. Due to higher retention of moisture content, the layer of moss on top proved beneficial. As previously reported in *Dendrobium fimbriatum* var. *Oculatum* by Kumaria and Tandon (1994), feeding the plantlets with dilute MS nutrient salt solution was found to be favorable to the development of hardened plantlets of *Cymbidium devonianum*. This research obviously showed that the above-mentioned culture conditions were efficient for seed germination, protocorm growth, and large-scale seedling production from immature seeds of *Cymbidium alloifolium*, and this protocol will be beneficial to commercial growers for mass propagation and for the conservation of this orchid species in Bangladesh and elsewhere.

From this study, it may be concluded that MS was the best means of germinating the seed and forming the protocorm from immature seeds of *C. alloifolium* compared to the performance of other media used. Similarly, BAP (1.0 mg/l) and NAA (0.5 mg/l) supplemented MS medium were found to be the ideal medium for protocorm regeneration to plantlet development. This result suggests that the nutritional requirement in *in vitro* culture is species and medium-specific. On the other hand, NAA

was the most efficient phytohormone to stimulate the formation of the root and increase its length. Consequently, the results demonstrated that the above-mentioned culture condition was the most effective for mass multiplication of *C. alloifolium* and this protocol will be useful to commercial growers for mass multiplication. These techniques and protocols are helpful for the improvement of the orchids in Bangladesh for further research for isolation of biochemical compounds for medicine, plant genetic transformation and the conservation of these endangered orchids.

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