

***In vitro* Mass Propagation of *Cymbidium aloifolium* (L.) Sw.**

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Abstract

Cymbidium aloifolium (L.) Sw. is an epiphytic orchid distributed locally in Bangladesh. Natural populations of this orchid are under threat due to its high commercial value in the floriculture industry by over exploitation. Mass propagation provides an alternative means of satisfying the demand. In this study, half strength of MS supplemented with BAP or different combinations with NAA was evaluated for seed germination and early protocorm development of *C. aloifolium* (L.) Sw. The maximum percentage of seed germination (98) was obtained on modified MS (half strength of micro and macro nutrients and full strength of vitamin) supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA after three weeks of culture. Maximum number of shoots (9.6 shoots per single shoot) were developed from shoot tip explants on half strength of MS supplemented with 1.0 mg/l BAP and 0.5 mg/l NAA. The most effective auxin source promoting root production (3.2 roots per shoot) was 1.0 mg/l IBA in combination with 0.5 mg/l BAP. The seedlings were successfully acclimatized (survival percentage 99) in a nethouse using a rooting medium of crushed sterile brick, cocopit and charcoal (1 : 1 : 1 v/v).

Introduction

Orchidaceae plays an important role in the evolution of monocots and is one of the most popular families of flowering plants, as it is evident from its extensive distribution and numerous species (25000 - 30000) (Chowdhery 2001). Increased popularity of cut flower in Bangladesh has huge prospects in floriculture trade. Among them, *Cymbidium* comprises more than 70 natural species and 100s of manmade hybrids. This rich variety has contributed significantly to the development of the international trade in orchid cut flowers (Vij et al. 2004).

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Cymbidium aloifolium (L.) Sw. is an economically important epiphytic orchid of Bangladesh (Hossain et al. 2013). Its attractive long-lasting beautiful flowers explain its high commercial value. *C. aloifolium* (L.) Sw. is now on the verge of extinction from its natural habitat due to deforestation, over exploitation for trade and absence of pollinators due to indiscriminate use of pesticides or other modifications of the biome (Chugh et al. 2009). Another reason for its decline is the slow germination rate in nature as they require specific mycorrhizal fungi for successful seed germination (Mohanraj et al. 2009). Seed germination and propagation of this orchid in nature is very slow due to ecological constraints. The *Cymbidiums* are conventionally propagated through separation of pseudobulbs. Unfortunately, conventional propagation is slow and difficult and proliferation rate is very low. An alternative source of these plants is clearly needed and mass propagation/*ex situ* conservation offers a solution (Pant and Swar 2011).

Micropropagation of plants through tissue culture has become a significant and informative technique to reproduce and make the availability of crops, orchids and ornamental plants (Kannan 2009). For mass propagation, regeneration from tissue-cultured explants is superior to seed culture due to year round availability of plant materials and an exponential propagation rate (Pant and Swar 2011). In the recent years tissue culture technique has been extensively exploited for the large-scale propagation as well as *ex situ* conservation of *Cymbidiums* (Chang and Chang 2000, Jamir et al. 2002, Pant and Pradhan 2010). The plants produced through micropropagation provide much export potential as they are shipped internationally with limited quarantine restrictions and it has the prospective for developing new cultivars of *Cymbidium* species (Hyndman 1987).

Although *C. aloifolium* faces rapid destruction due to exploitation and also urbanization and habitat loss, there is no report on *in vitro* mass propagation of *C. aloifolium* from Bangladesh. Present authors have addressed this shortcoming by developing an efficient protocol for rapid propagation of *C. aloifolium* starting with *in vitro* asymbiotic seed germination, leading to protocorm induction followed by plantlet development and successful *ex vitro* acclimation. The present study constitutes a simple, efficient and reproducible protocol suitable for mass propagation of *Cymbidium aloifolium*.

Materials and Methods

Young capsules of *Cymbidium aloifolium* L. Sw. (Fig. 1A) were collected from the plants grown in orchid house of Department of Botany, University of Dhaka and used for *in vitro* seed germination and subsequent plant development.

For *in vitro* seed germination and protocorm culture, the harvested immature green capsule was rinsed with detergent in running water for 10 minutes to remove the adhering soil particles. The capsule was surface sterilized with 70% ethanol for 1 min and then with 0.1% (w/v) mercuric chloride solution for 5 min. The capsule was rinsed with

sterile distilled water and allowed to air dry. Green capsule was dissected longitudinally with sterile surgical blade. The immature seeds scooped out from the sterilized capsule were germinated on the solidified half strength of MS (Half the strength of micro and macro-salts and full strength of vitamin) supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA.

The medium was solidified with agar at a concentration of 0.8% (w/v). The pH of the medium was adjusted to 5.8 and the cultures were maintained at $25 \pm 2^\circ\text{C}$ under a 16/8 hrs light/dark cycle photoperiod provided by cool white fluorescent lamps. Ten per cent (v/v) coconut milk was used as additive. Protocorms developed from germinated seeds were used as explant for further multiplication.

For the clonal mass propagation, protocorms were cultured in the culture tubes containing half strength of MS supplemented with different concentrations of BAP and NAA (Table 1) either alone or in combinations. Multiple shoot formation and root initiation was examined at the given cultured condition. Shoots were subcultured on to fresh media once every eight weeks. The young plantlets were maintained *in vitro* in the culture condition as mentioned above. As additive 0.2 g/l charcoal and 15% (v/v) coconut milk were added to half strength of MS along with 10 gm of sucrose. After 130 days of culture, mean number of shoots and mean shoot lengths were recorded.

The multiple shoots obtained from the above experiment were cultured on half strength of MS supplemented with different concentrations of BAP and IBA, 0.2 g/l charcoal, 15% (v/v) coconut milk and 10 g of sucrose (Table 2). Gelrite was used as solidifying agent. The cultures were incubated for 2 months under the conditions described for the previous experiment. The micropropagated plantlets were acclimatized using cocopit for 2 - 3 weeks and finally transplanted to the small earthen pots containing coal and small pieces of brick. Rooted plantlets were able to grow into normal plantlets in *ex vitro* condition after a short period of acclimatization (Fig. 1G).

Results and Discussion

In vitro germination and mass propagation of *Cymbidium aloifolium* (L.) Sw. was carried out on half strength MS supplemented with various concentrations of BAP and NAA. The regeneration competence of the protocorm explants seems to be markedly influenced by different growth regulators in nutrient media (Paudel and Pant 2012, Vajrabhaya 1978). MS with or without growth regulators was found to be effective for the germination of immature seeds (Pant and Swar 2011). In the present study, immature seeds developed into green coloured oval protocorms after 3 weeks of culture on half strength of MS supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA. *In vitro* grown protocorms obtained from immature seeds of *C. aloifolium* was used as explants for further multiplication in this experiment. Addition of growth regulators resulted in vigorous growth of the protocorms.

Different concentration and combinations of NAA and BAP were found to be effective for the growth and multiplication of shoots from the shoot tip explants (Fig. 1C). Effect of different concentrations of NAA and BAP on shoot tip explants has shown in Table 1. Highest number of multiple shoots was 9.6 per shoot cultured was observed for the combination of 1.0 mg/l BAP and 0.5 mg/l NAA. On the other hand lowest number of multiple of shoots found on 2.0 mg/l BAP and 0.5 mg/l NAA. Increase of BAP concentration showed inhibitory effect, which is in agreement with the result of Pant and Swar (2011) who worked with *Cymbidium iridioides*. The longest shoot length 3.52 cm (Table 1) was observed on protocorms when cultured with 0.5 mg/l of NAA and this result was analogous to the report found on *Esmeralda clarkei* (Paudel and Pant 2012). On the other hand shortest shoot length was observed on half strength of MS supplemented with 0.5 mg/l BAP. Root induction was observed on all the combinations of BAP and NAA in cultured protocorms except half strength of MS supplemented with 0.5 mg/l BAP.

Table 1. Effect of different concentrations of BAP and NAA in *in vitro* plant regeneration from protocorm explants of *Cymbidium aloifolium* (L.) Sw.

Medium	Hormonal supplements (mg/l)		Mean no. of shoots (\pm SE)	Mean length of shoots (cm) (\pm SE)	Mean no. of roots (\pm SE)	Mean length of roots(cm) (\pm SE)
	BAP	NAA				
Half strength of MS	0.5	-	5.5 \pm 0.89	2.06 \pm 0.15	-	-
	0.5	0.5	7.4 \pm 0.54	3.16 \pm 0.12	2.6 \pm 0.90	1.6 \pm 0.65
	1.0	0.5	9.6 \pm 0.90	3.44 \pm 0.32	5.2 \pm 0.83	3.8 \pm 0.57
	1.5	0.5	7.0 \pm 0.71	3.06 \pm 0.05	3.4 \pm 0.90	3.0 \pm 0.61
	2.0	0.5	4.7 \pm 0.71	3.52 \pm 0.03	3.2 \pm 1.48	3.6 \pm 0.41

Half strength of MS supplemented with different concentrations of BAP and IBA were tested for the *in vitro* rooting of shoots (Fig. 1F). The well-developed un-rooted shoot was removed from the culture tubes and transferred to the rooting media supplemented with various concentrations of BAP and IBA (Table 2). All media responded positively for root formation of well-developed roots (86.6%). Half strength of MS supplemented with 0.5 mg/l BAP and 1.0 mg/l IBA showed highest root formation (3.2) and mean length of roots was also highest (3.6 cm) in the same combination. According to Pant and Swar (2011) IBA (1.0 mg/l) was more effective for rooting which supports the results of the present experiment. Bannerjee et al. (1999) found that 2.0 mg/l NAA was most appropriate in inducing 3 - 4 roots in 2 months in *Cymbidium*s. However, in the present experiment it showed least number of roots in increasing concentrations of NAA and IBA.

Table 2. Effect of different concentrations of BAP and IBA in induction of root on shoot in *Cymbidium aloifolium* (L.) Sw.

No. of roots per flask	Percentage of root formation (%)	Average percentage of roots	Hormonal supplements (mg/l)		Mean no. of roots (\pm SE)	Mean length of Roots (cm) (\pm SE)
			BAP	IBA		
17	85		0.5	0.5	1.6 \pm 0.9	1.6 \pm 0.41
16	80	86.6%	0.5	1.0	3.2 \pm 1.3	3.6 \pm 0.55
19	95		0.5	2.0	2.8 \pm 1.09	2.52 \pm 0.42

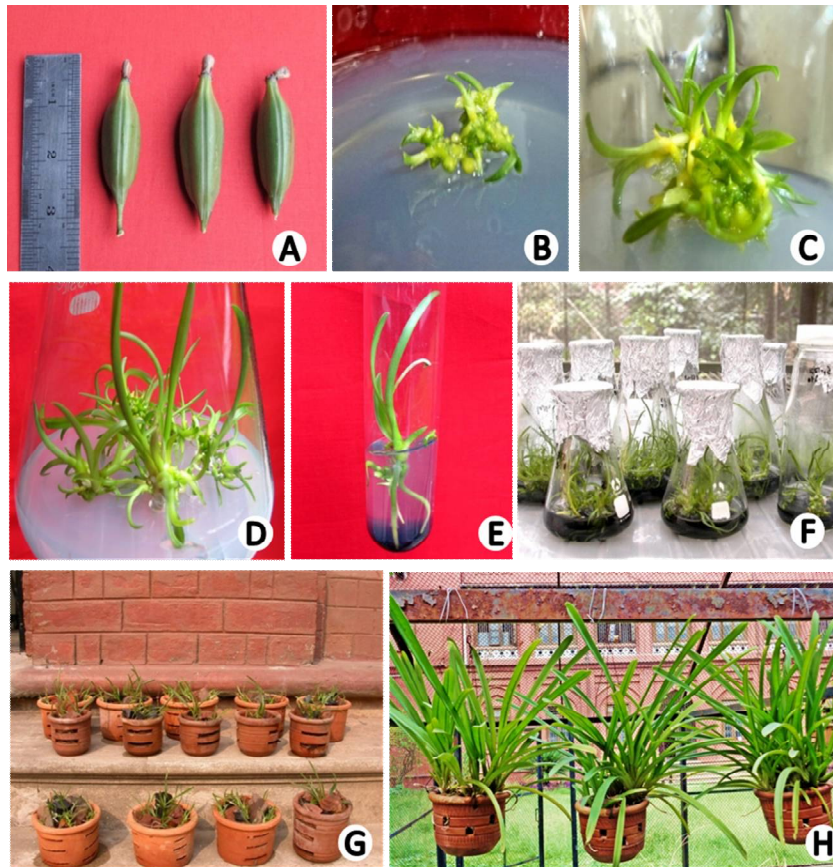


Fig. 1. Different stages of micropropagation of *Cymbidium aloifolium* (L.) Sw. A. Young capsules of *Cymbidium aloifolium*. B. *In vitro* plant regeneration from protocorm culture. C. Development of plantlets from protocorms. D. Multiple shoots developed from shoot tip explants on MS with BAP (1 mg/l) + NAA (0.5 mg/l). E. Fully developed plants with leaves and roots. F. Well-rooted plants in nethouse for hardening. G. Acclimatized plants of *Cymbidium aloifolium* in earthen pots. H. Regenerated plants of *Cymbidium aloifolium* growing in nethouse.

In vitro initiation and development of protocorm largely depends on the activity of growth regulators on seed germination medium. Moreover, 15% coconut milk was used as additive in this experiment. Islam et al. (2014) reported plantlet development from the protocorm of *Vanda roxburghii* using MS supplemented with 15% coconut water. According to Utami et al. (2017), in *Dendrobium lasianthera* 15% coconut water showed improved shoot development, with well-developed roots and leaves compared to the other treatments. On the other hand, 0.2 g/l charcoal was supplemented in the medium because root development was the best when activated charcoal was added into the medium. Similar findings were also reported by Vij et al. (1994).

Cymbidium aloifolium is a commercially important endangered epiphytic orchid. Since this plant is not commonly available an alternative source of plants and flowers is needed to satisfy commercial demands. This protocol for *in vitro* mass propagation was based on the germination of seeds from fruit capsule and propagating them through protocorm production. Micropropagation using protocorm can be applied for the commercial production of this species and in this way this species may be conserved in nature more effectively.

The results of this study allow the establishment of a protocol for *in vitro* mass propagation of *Cymbidium aloifolium* (L.) Sw. Half the strength of MS supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA may be recommended for the effective *in vitro* germination and protocorm development. The seedlings exhibited vigorous growth and root development on half strength of MS with the addition of 0.5 mg/l BAP and 1.0 mg/l IBA. This protocol is an efficient means for the large-scale propagation of *Cymbidium aloifolium* (L.) Sw., which may be applicable for other *Cymbidium* species.

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