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Research Article

Cost effective protocol for *in vitro* mass propagation of *Cymbidium aloifolium* (L.) Sw.– a medicinally important orchid

In the present study, four nutrient media *viz*., Murashige and Skoog (MS), Phytamax (PM), Mitra *et al.* (M) and Knudson 'C' (KC), were evaluated for seed germination and early protocorm development in *Cymbidium aloifolium* (L.) Sw., a medicinally important orchid of Bangladesh. Besides, effect of peptone, activated charcoal, plant growth regulators *viz*., 6-benzylaminopurine (1.0–2.0 mg/L) and 2,4-dichlorophenoxy acetic acid (1.0–2.0 mg/L), and light and dark conditions were also studied. Varied response in terms of per cent seed germination was observed in different media. Mitra *et al.* medium supplemented with 2.0 g/L activated charcoal (AC) showed 100% seed germination and effective for induction of significantly large size protocorms (1.64 mm in dia.). Protocorms turned necrotic under dark condition. Within two weeks of culture spherules emerged out due to cracking of the seed coat. The spherules developed into protocorms with a leaf primordium at apical portion after 3-4 weeks and gradually produced complete seedlings. A strong and stout root system was induced in *in vitro* seedlings upon transfer to half strength PM and M media fortified with 0.5 mg/L indole-3-acetic acid (IAA). Well-rooted seedlings were transferred to a green house with 95% survival. Based upon cost analysis it was suggested that Mitra *et al.* medium supplemented with AC (2.0 g/L) was more effective and economic for high frequency germination of seeds and seedlings development. IAA (0.5 mg/L) in Mitra *et al.* medium was suitable for development of stout root system which helped in high survival of seedlings under greenhouse condition.

Keywords: Seed germination / Embryo morphogenesis / Protocorm

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1 Introduction

Cymbidium aloifolium (L.) Sw. an orchid, is pronounced as a high value medicinal herb in the indigenous system of medicine. The plant is said to be emetic and purgative. It contains two substituted bibenzyls, a dihydrophenanthrene and a phenanthraquinone (Cymbinodin-A) which are biologically active phytochemicals [1]. Leaves, roots and even whole plant are used for treatment of different ailments. Dried root powder with equal volume of dried ginger and half volume of black pepper are mixed thoroughly. Half spoon of this mixed powder with a cup of cow milk is given twice a day for two months to cure paralysis [2]. Tribal people of Chittagong hill tracts use leaf extract for treating boils and fever. Pasted aerial roots are used for joining fractured bones. Whole plant is used as tonic and in treating ear-ache, chronic illness, weakness of eyes, vertigo, burns and sores [3]. Besides its medicinal importance it has a demand in cut flower trade for its exquisite and perpetual flowers [4].

Unfortunately it is disappearing at an alarming rate due to continuous destruction of natural habitats, over exploitation for medicinal purposes, unauthorized trade and ruthless collection by orchid lovers. Multipurpose utility of this orchid undoubtedly led to an increased emphasis on mass propagation and conservation in nature. Major obstacles for mass propagation of economically important orchids for commercial purposes as well as conservation are: (a) non availability of efficient and reliable protocols for seed germination, (b) a clear understanding of early seedling growth and development, (c) high mortality of seedlings during transplantation, and (d) obligate mycorrhizal association for natural seed

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germination. Ever since the development of a method for asymbiotic germination of orchid seeds by Knudson [5], the techniques have been routinely used for large scale propagation of a number of orchid species and their hybrids [6–11] but a very few studies critically investigated on the peculiarities of seed germination and protocorm development. Furthermore, modification of traditional tissue culture medium by adding specific plant growth regulators (PGRs), activated charcoal (AC), peptone (P) and changing culture conditions are reported to enhance germination percentage and subsequent development of protocorms in many orchids [12–17]. Therefore, the present studies were undertaken with a view to i) study the effect of PGRs (BAP and 2,4-D), AC, P, light and dark conditions on seed germination, ii) develop an efficient and cost effective protocol for *in vitro* germination of seeds, and iii) establish the *in vitro* raised seedling in outside environment. A special attention was also paid to investigate the mode of morphogenesis of embryo during formation of protocorm and seedling development in *Cymbidium aloifolium*. The present investigation has the potential of providing a source for mass supply of planting material according to pharmaceutical and ornamental demands.

2 Materials and methods

2.1 Initiation of aseptic cultures

Six to seven months old green capsules of *C. aloifolium* were collected from the naturally grown populations from the hilly forest of Cox-S-Bazar district (200 m above mean sea level) of Bangladesh. These were washed under running tap water and 20% Teepol, a commercial detergent (Qualigens Fine Chemicals, Mumbai, India) for 5–7 min. The capsules were then surface sterilized by 0.04% (w/v) solution of both bavistin (BASF, India) and streptomycin sulfate (Sigma-Aldrich, Germany) for 20 min and washed three times with sterile distilled water (SDW). Subsequently these were treated with 0.1% (w/v) $HgCl_2$ solution with 1–2 drops of teepol as wetting agent for 10 minutes with occasional agitation and washed thoroughly with SDW. Finally the capsules were dipped in 70% ethanol for 30 seconds followed by flaming for 2–3 sec. The sterilized capsules were split longitudinally with a sterile surgical blade and the dust-like seeds were inoculated on the surface of agar (0.8%, w/v) gelled nutrient media.

2.2 Culture medium and incubation conditions for seed germination

Four different media *viz.* Knudson C (KC) [18], Murashige and Skoog (MS) [19], Mitra *et al.* (M) [20] and Phytamax (PM, Sigma Chemical Co. USA) (Table 1) supplemented with BAP (1.0–2.0 mg/L) and 2,4-D (1.0–2.0 mg/L), either individually or in combinations and two additives such as peptone (2.0 g/L) (HiMedia, Mumbai, India) and activated charcoal (2.0 g/L) (particle size 80–100 mesh; Qualigens Fine Chemicals, Mumbai, India) were used for seed germination. The pH of the medium was adjusted at 5.8 prior to gelling with 0.8% (w/v) agar (HiMedia, Mumbai, India). 100 ml of the medium was dispensed in 250 ml Erlenmeyer flasks (Borosil, India) and autoclaved at 121°C for 20 minutes at 0.103 MPa (1.05 kg/cm^2). Culture vessels with inoculated seeds were maintained in a culture room where a cycle of 14/10 h light-dark at 60 µmol $m^{-2}s^{-1}$ provided by cool white fluorescent lamps (Philips Truelight 36w/86 6500° K B7, Philips India), and 60% RH at 25±2°C. In one set of experiment, culture flasks were wrapped with aluminum foil for maintaining continuous dark conditions and kept in the same culture room.

2.3 Per cent germination and seedling development

After two weeks of inoculation, some of the seeds were taken out and dispersed in one drop of water on a glass slide and observed under light microscope. Per cent germination was calculated employing following formula:

$$\%\text{germination} = \frac{\text{No. of seeds showing swelling of the embryo} \times 100}{\text{Total No. of seeds}}$$

Once the spherules were formed, observations were recorded at one week interval to trace different stages of protocorm development. These were observed using stereozoom microscope (Nikon SMZ1500, Japan). After protocorm development these were subcultured at 4-5 week interval.

2.4 Statistical analysis

The experiments were designed following Complete Randomize Design (CRD). Three replicates were taken per treatment for seed germination and protocorm size whereas for rooting 10 replicates were taken. The effects of different media on germination of seeds, protocrm size, and induction of roots in the *in vitro* experiments were tested applying Duncan's multiple range test ($P = 0.05$) in one way ANOVA. The statistical analyses were performed using the programme package Statistica ver. 7 (Statsoft, Tulsa, USA). The experiments were repeated thrice.

2.5 Histological studies

Protocorms were fixed in formalin : acetic acid : 50% ethanol (FAA,1:1:18; v/v/v) for one week and preserved in 70% ethanol until used. These were dehydrated in a tertiary butyl alcohol series and embedded in paraffin wax. Longitudinal sections (12 µm thick) were cut with the help of a microtome (Shandon Finsse ME, Thermo Electron Corp., UK), stained with safranin-fast green combination and mounted in DPX (mixture of distyrene, a plastilizer and xylene) mountant [21]. The sections were observed under light microscope (Labophot, Nikon Corp., Japan) and photographs were captured using digital camera (Nikon DXM1200, Nikon Corp., Japan).

Table 1. Compositions and cost* (per litre) of different medium components.

Constituents	MS	Cost	PM	Cost	M	Cost	KC	Cost
Macronutrients (mg/L)								
KNO_3	1900	0.627	950	0.3145	180	0.0594	–	–
NH_4NO_3	1650	0.396	825	0.198	–	–	–	–
NH_4SO_4			–		100	0.017	500	0.085
$NaH_2PO_4 \cdot 2H_2O$			–		150	0.556	–	–
KH_2PO_4	170	0.0816	85	0.0408	–	–	250	0.12
$MgSO_4 \cdot 7H_2O$	370	0.10846	90.35	0.0262	250	0.0725	250	0.725
$CaCe_2 \cdot 2H_2O$	440	0.3423	166	0.129	–	–	–	–
$Ca(NO_3)_2 \cdot 4H_2O$			–	–	200	0.0556	1000	0.278
Iron source								
Na_2-EDTA	37.3	0.106708	37.3	0.106708	22.3	0.06379	37.3	0.106708
$FeSO_4 \cdot 7H_2O$	27.8	0.01404	27.8	0.01404	16.7	0.0084	27.8	0.01404
Micronutrients (mg/L)								
KI	0.83	0.0044	0.415	0.0022	0.03	0.00016	–	–
$MnCl_2 \cdot 4H_2O$			–	–	0.4	0.000148	–	–
$MnSO_4 \cdot 4H_2O$	22.3	0.0146	8.45	0.0055			–	–
$ZnSO_4$	–	–	–	–	0.05	0.00040	–	–
$ZnSO_4 \cdot 7H_2O$	8.6	0.0036	5.30	0.0029		0.00002	0.35	0.001463
H_3BO_3	6.2	0.00041	3.1	0.0002	0.6	0.00040	0.6	0.0039
$CuSO_4 \cdot 5H_2O$	0.025	0.000021	0.0125	0.00001	0.05	0.000041	0.04	0.00032
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.0026	0.125	0.00131	0.05	0.00522		
MoO_3							0.02	0.00021
$CoCl_2 \cdot 6H_2O$	0.025	0.00021	0.0125	0.00011				
$CoNO_3 \cdot 6H_2O$	–	–	–	–	0.05	0.00012		
Vitamins and organics (mg/L)								
Glycine	2	0.054	–	–	–	–	–	–
Thiamine-HCl	0.50	0.00108	1.00	0.0107	0.3	0.00032	–	–
Pyridoxine-HCl	0.50	0.0188	0.50	0.0188	0.3	0.0113	–	–
Nicotinic acid/Niacin	0.50	0.0013	0.50	0.0013	1.25	0.00032	–	–
Riboflavin	–	–	–	–	0.05	0.00078	–	–
D-Biotin	–	–	–	–	0.05	0.00354	–	–
Folic acid	–	–	–	–	0.3	0.0057	–	–
Inositol	100	0.796	–		–		–	–
Sucrose	30 000	16.80	20 000	11.00	20 000	11.00	20 000	11.00
Agar	8000	26.1400	8000	26.1400	8000	26.1400	8000	26.1400
Total cost (Basal medium)		**45.5131**		**38.0122**		**38.0011**		**38.4746**
Peptone (P)	2000	37.4400	2000	37.4400	2000	37.4400	2000	37.4400
Activated charcoal (AC)	2000	0.8600	2000	0.8600	2000	0.8600	2000	0.8600
Cost (Basal medium +P)		**82.9531**		**75.4522**		**75.4411**		**75.9146**
Cost (Basal medium +AC)		**46.3731**		**38.8722**		**38.8611**		**39.3346**

* According to HiMedia Price List 2008–09, HiMedia Laboratories Pvt. Limited, Mumbai, India, cost presented in Indian Rupees (Rs).

2.6 Rooting and transfer of seedlings to greenhouse

Half and full strength PM and M basal medium fortified with 0.5–1.0 mg/L IAA were used for induction of stout root system. The seedlings (3–4 cm in size) with 2–3 leaves were individually grown in test tube (1.5 × 15 cm, Borosil, India) containing 50 ml medium for 30 days. Well-rooted seedlings were taken out from culture tubes, washed the roots thoroughly under running tap water to remove agar and then transferred to plastic pots (10 × 10 cm, Axiva, India) in greenhouse (temperature range 25–30°C and RH 60–70%). The potting mixture used for transplanting of seedlings was prepared with sterilized small brick pieces, charcoal pieces, vermiculite and peat moss at 1:1:0.5:0.5.

3 Results and discussion

3.1 Influence of basal media and plant growth regulators on seed germination

The seed germination percentage varied on different basal media *viz*., KC, MS, M and PM. The comparative effect of media compositions on seed germination have been shown in Table 2. It was 85% in PM basal medium followed by 80% in M, 65% in MS and 50% in KC. There are many reports of species-specific media for germination of orchid seeds [22]. All the presently employed media differ from one another in their chemical compositions (Table 1). MS is highly enriched with macro and micro elements, PM contained approximately half of MS, M contained low amount of both macro and micro elements but enriched with different vitamins than MS or PM, whereas KC contained comparatively low amount of both macro and micro nutrients and lacked vitamins. Maximum per cent germination of seeds in M medium could be attributed to the fact that this medium is enriched with different vitamins. Addition of various vitamins into the medium was reported to be promotive for seed germination and seedling growth of *Cymbidium elegans* and *Coelogyne punctulata* [23]. Mariat [24] reported that vitamin-B favoured germination and differentiation in *Cattleya* seedlings; thiamine, nicotinic acid and biotin are most effective in *Cattleya* hybrids. Pyridoxine was essential for chlorophyll synthesis and combination with nicotinic acid and biotin favoured better germination of *Orchis laxiflora* seeds [25].

Both PM and M media supplemented with 1.0 mg/L BAP showed 100% seed germination but growth of protocorms was better in M medium (Figs.1A, B). Fascinatingly, a few protocorms became brown and finally died in subsequent subcultures in BAP supplemented media. BAP is known to enhance germination frequency in *Cypripedium* spp. [7], *Eulophia dabia* and *Pachystoma senile* and stimulated protocorm

Table 2. Comparative effect of culture media on asymbiotic germination of seeds of *Cymbidium aloifolium*.

Medium	PGRs (mg/L)		Additives (2g/L)[a)]	Spherule formation (Time wks)	Protocorm formation (Time wks)	Seed germination (%) (mean ± SEm)[b)]
	BAP	2,4-D	AC / P			
MS	–	–	–	9–10	12–13	65.32 ± 5.41^{fgh}
	–	1.0	–	9–10	12–13	49.76 ± 2.42^{hi}
	1.0	–	–	7–8	9–10	76.14 ± 4.85^{bcdefg}
	2.0	2.0	–	7–8	9–10	50.00 ± 0.00^{hi}
	–	–	P	7–8	8–9	79.26 ± 0.74^{bcdefg}
	–	–	AC	7–8	8–9	76.14 ± 4.85^{efg}
PM	–	–	–	6–7	9–10	85.83 ± 3.00^{bcdef}
	–	1.0	–	8–9	11–12	75.55 ± 12.37^{efg}
	1.0	–	–	6–7	9–10	100.00 ± 0.00^{a}
	2.0	2.0	–	6–7	9–10	73.54 ± 8.51^{efg}
	–	–	P	7–8	7–8	100.00 ± 4.17^{a}
	–	–	AC	7–8	7–8	89.17 ± 5.83^{bcd}
M	–	–	–	6–7	9–10	80.00 ± 5.77^{bcdef}
	–	1.0	–	7–8	10–11	75.55 ± 12.37^{efg}
	1.0	–	–	6–7	9–10	100.00 ± 0.00^{a}
	2.0	2.0	–	6–7	9–10	76.14 ± 4.85^{bcdefg}
	–	–	P	6–7	6–7	100.00 ± 0.00^{a}
	–	–	AC	6–7	6–7	100.00 ± 0.00^{a}
KC	–	–	–	9–10	12–13	50.00 ± 2.62^{hi}
	–	1.0	–	9–10	13–14	30.00 ± 5.77^{i}
	1.0	–	–	8–9	11–12	50.00 ± 5.77^{hi}
	2.0	2.0	–	8–9	11–12	56.11 ± 1.14^{gh}
	–	–	P	9–10	11–12	88.89 ± 11.11^{bcdef}
	–	–	AC	9–10	11–12	77.97 ± 11.31^{bcdefg}

a) AC = Activated charcoal; P = Peptone

b) Value within a column followed by the same letters are not significantly different at $p = 0.05$

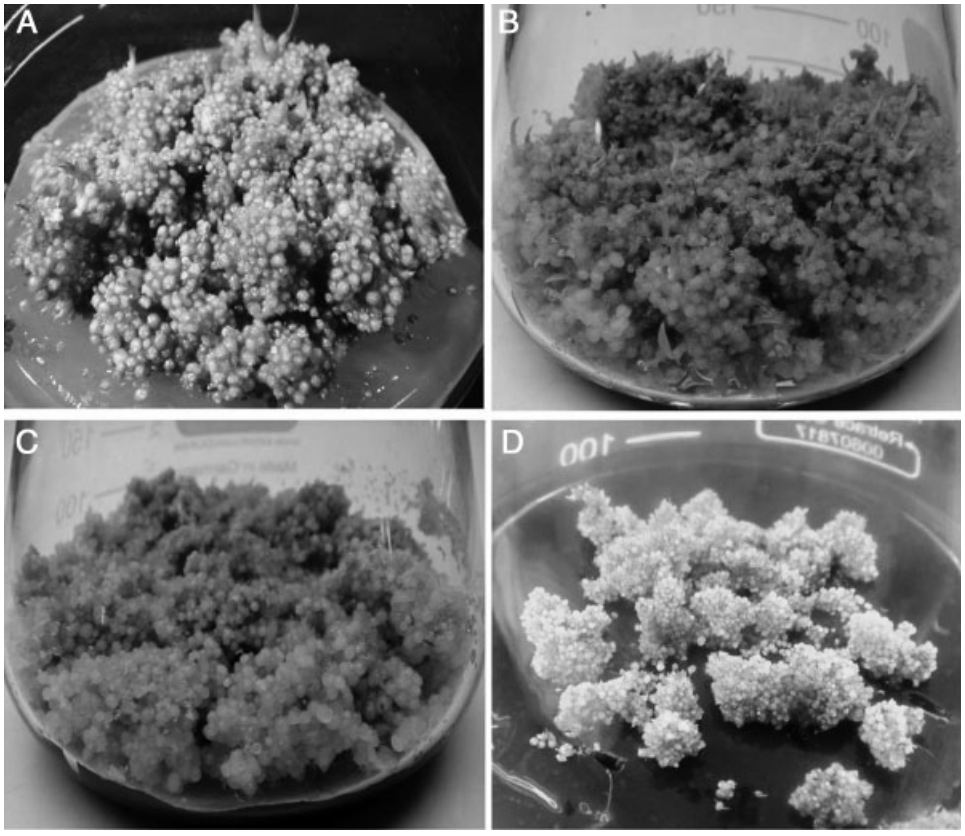

Figure 1. Asymbiotic seed germination of *Cymbidium aloifolium* on M medium. (A) Basal medium; (B) 1.0 mg/L BAP supplemented medium; (C) 2.0g/L activated charcoal supplemented medium; (D) Seeds germinating in dark condition.

multiplication as well as shoot formation in *Cymbidium pendulum* [26], and *Cattleya aurantiaca* [27]. Medium supplemented with 2,4-D showed comparatively low seed germination percentage and lingering germination period as compared to other media (Table 2). Inhibitory effects of 2,4-D on seed germination and protocorm development are recorded in orchid literature [28–31].

3.2 Influence of AC and P on seed germination and protocorm development

Both M and PM media supplemented with 2.0 g/L activated charcoal was found to be effective for enhancing germination rate (Table 2) but M was better than PM in terms of vigorous growth, production of significantly large protocorms (1.64 ± 0.19 mm in dia.) (Fig. 1C) and comparatively lesser time requirement. High adsorption affinity of activated charcoal to excessive and inhibitory compounds in the culture media may be responsible for maximum germination of seed and production of significantly large protocorms in this species. Curtis [32] first used charcoal as darkening agent for asymbiotic germination of orchid seeds. Later on a number of investigators favoured charcoal in asymbiotic medium [33, 34]. The beneficial effects of charcoal in culture media are: i) adsorption of unidentified morphogeneticaly active or toxic substances [35], ii) adsorption of 5-hydroxymethylfurfural which is produced by the dehydration of sucrose during autoclaving [36], iii) adsorption of inhibitory phenolics and carboxylic compounds

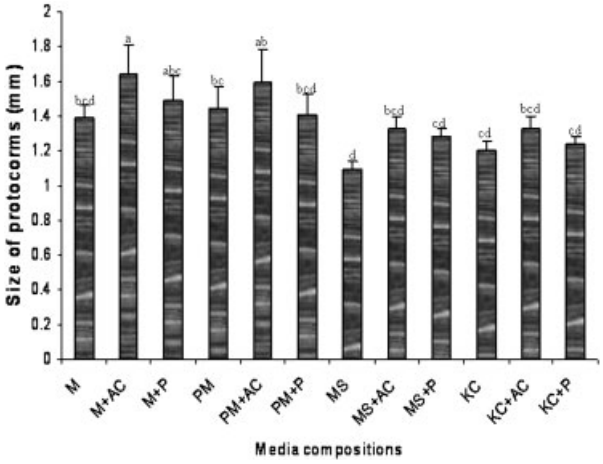

Figure 2. Protocorm size (mm in dia.) of *C. aloifolium* after 30 days of germination.

produced by the tissues [37], and iv) adsorption of excessive hormones and vitamins in the media [38].

Peptone also improved germination percentage i.e. 100% in both PM and M medium but size of protocorms was smaller (1.49 ± 0.14 mm in dia.) than those produced in AC supplemented medium. Lami [39] reported that peptone enhanced seed germination in *Vanda* and *Phalaenopsis*. Oliva and Arditti [40] regarded amino acids, amides and vitamin contents of peptone to be responsible for enhancing germination of seeds. In case of

Spiranthes cernua and *Kingidium taenialis* seed germination it was found to be obligatory [41]. The germination of seeds and growth of protocorms invariably required peptone in the medium [26, 42]. It is pertinent to mention here that, in both AC and P supplemented medium, the protocorms were larger in size as compared to control (Fig. 2). Therefore, evaluating the above findings, M medium fortified with AC was most suitable for seed germination and protocorm development in this species.

3.3 Influence of dark condition on seed germination

Dark condition did not play any adverse role on germination of seeds except formation of chlorotic protocorms (Fig. 1D). These protocorms quickly became green upon transfer from dark to light conditions. However, under dark condition etiolated and very weak seedlings were developed.

The effect of light and dark conditions on germination of orchid seeds is still a debatable issue. Epiphytic orchid species are generally thought to germinate in either light or dark [6, 43, 44], but light improves germination and normal seedling development [45]). However, species specific light and dark requirements for germination of orchid seeds are scarce. Dutra *et al.* [46] stated that highest germination of *Cyrtopodium punctatum* seeds occurred under continual darkness (0/24 h L/D) while in *Cattleya loddigesii*, 90% seeds were germinated in continuous light and only 30% in the dark [47]. Similar inconsistency was found in terrestrial orchids also. Takahashi *et al.* [48] reported that no significant difference was observed on seed germination percentages of *Habenaria radiata* when cultured under continual darkness (0/24 h L/D) and 24/0 h L/D conditions. Reduction of seed germination percentage due to dark pretreatment of seed before light exposure in temperate terrestrial orchid species have been reported [49–51]. However, initial light exposure followed by dark resulted inhibition of seed germination in many temperate terrestrial orchid species [11, 52–54]. Thibul and Jantasilp [55] reported that light caused higher percentage of seed germination in *Vandopsis gigantean* while reverse findings observed in *Platanthera integrilabia* [49] and *Paphiopedilum spp.* [56]. These conflicting results warranted further research on germination of seeds in different light conditions of both terrestrial and epiphytic orchids. The genus *Cymbidium* is mostly comprised of epiphytic species, however, a few species in the genus are terrestrial. *C. aloifolium*, investigated in the present studies is an epiphytic orchid which corroborated insensitive on light/dark conditions for germination of seeds.

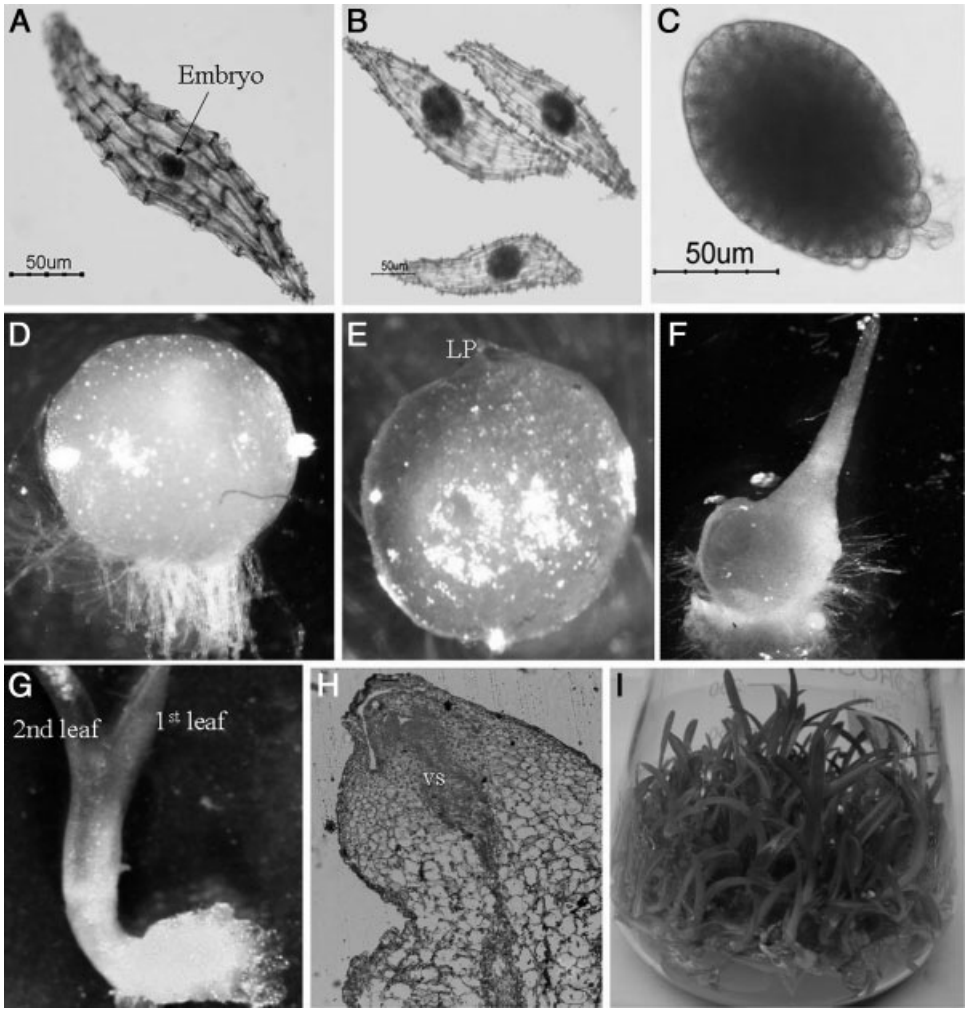

Figure 3. Different stages of seed germination and protocorm development of *Cymbidium aloifolium*. (A) Mature seed; (B) Seeds after 15 days of culture; (C) Embryo after 30d of culture; (D) Spherule with hairs; (E) Young protocorm with a leaf primordium (LP); (F) Protocorm; (G) Young seedling; (H) LS of protocorm showing shoot apex and development of vascular strand (VS); (I) Complete seedlings.

3.4 Morphogenesis of the embryo and development of protocorm

Orchid seeds are unique due to presence of only a few celled unorganized embryo and devoid of functional endosperm. Exogenous water and nutrients are essential for seed germination. Within two weeks of culture, cell number of undifferentiated embryos increased through repeated divisions and irregular shaped parenchymatous cell mass emerged out due to cracking of the testa. Thereafter, these turned to globular structures called spherules, with hairy growth at the basal part which turned into green protocorms within 3-4 weeks (Figs. 3A-D). The upper part of the protocorm formed the shoot apex with a leaf primordium. A second foliaceous primordium appeared afterwards, thus delimiting the vegetative point of the stem (Figs. 3E-G). Large pyramidal cells in the apical part of the protocorm developed shoot, whereas central elongated cells developed vascular strand (Fig. 3H). Protocorms develop further to form complete seedlings (Fig. 3I). Formation of protocorm is considered to be a peculiarity of postseminal development in orchids and shape of protocorms is taxon-specific. These are round, oval, elongated, disk-shaped, branched, thorn-shaped, spherical or spindle-shaped [57, 58]. In *C. aloifolium*, early protocom was round, green and radially symmetrical that gradually turned to oval shaped. As the shoot organs are formed it became asymmetrical. The apical part of the protocorm, consisting of small number of cells formed a 'tubercle' which turns into shoot apex. In the early development of protocorm, a leaf primordium appeared at upper part that looked like a closed ridge (Fig. 3E). As the primordium keeps growing, an opening formed at the edges of the ridge. Interpretations of the first foliar organ of orchids differ in different species. It is referred to as either a cotyledon [57] or as a leaf proper [58]. Teryokhin and Nikiticheva [59] stated that not only the first but also the second appendicular organ formed on the protocorm are cotyledons. The term "cotyledon" is still debatable. The majority of researchers [58, 60] consider cotyledons to be modified leaves initiated at the embryo stage of sporophyte development in flowering plants. In view of the fact that the foliar organs in the majority of orchids appear during postseminal development, that is, at the protocorm stage, it is inexpedient to refer them as cotyledons or leaf-like organs [60].

Table 3. Rooting response of *in vitro* seedlings of *Cymbidium aloifolium*.

Culture medium	No. of roots/seedling* (mean ± SEm)		Length of roots (cm) (mean ± SEm)	
	Initial	After 30d of culture	Initial	After 30d of culture
PM	1.70 ± 0.21^{a}	3.40 ± 0.27^{c}	1.56 ± 0.11^{a}	2.52 ± 0.14^{d}
1/2PM	1.70 ± 0.26^{a}	4.40 ± 0.22^{b}	1.51 ± 0.09^{a}	2.76 ± 0.13^{cd}
PM+0.5 mg/L IAA	1.80 ± 0.20^{a}	4.30 ± 0.33^{b}	1.81 ± 0.19^{a}	3.42 ± 0.17^{bc}
PM+1.0 mg/L IAA	1.80 ± 0.25^{a}	4.50 ± 0.27^{b}	1.79 ± 0.12^{a}	3.12 ± 0.16^{c}
1/2PM+ 0.5 mg/L IAA	1.70 ± 0.26^{a}	5.40 ± 0.31^{a}	1.51 ± 0.13^{a}	4.32 ± 0.15^{ab}
1/2PM+ 1.0 mg/L IAA	1.60 ± 0.16^{a}	5.00 ± 0.26^{ab}	1.74 ± 0.11^{a}	4.74 ± 0.22^{a}
M	1.70 ± 0.21^{a}	3.40 ± 0.27^{c}	1.78 ± 0.16^{a}	2.50 ± 0.12^{d}
1/2M	1.60 ± 0.22^{a}	4.20 ± 0.20^{b}	1.78 ± 0.16^{a}	2.78 ± 0.12^{cd}
M+ 0.5 mg/L IAA	1.60 ± 0.22^{a}	4.40 ± 0.22^{b}	1.68 ± 0.15^{a}	3.32 ± 0.15^{bc}
M+ 1.0 mg/L IAA	1.60 ± 0.22^{a}	4.90 ± 0.23^{ab}	1.59 ± 0.15^{a}	3.12 ± 0.16^{bc}
1/2M+ 0.5 mg/L IAA	1.80 ± 0.25^{a}	5.30 ± 0.30^{a}	1.74 ± 0.12^{a}	4.33 ± 0.10^{ab}
1/2M+ 1.0 mg/L IAA	1.80 ± 0.20^{a}	4.70 ± 0.30^{b}	1.67 ± 0.13^{a}	4.71 ± 0.19^{a}

* Mean number were scored from 10 plants for each treatment. Mean value within a column followed by the same letters are not significantly different at $p = 0.05$

3.5 Influence of nutritional stress and IAA on the induction of roots

Half strength of both PM and M media supplemented with 0.5 mg/L IAA proved effective for induction of stout root system (>5roots/seedling, Table 3) but the seedlings were comparatively dark green in PM medium (Figs. 4A,B). Half strength PM and M fortified with 1.0 mg/L IAA also showed rooting but those were very thin and long. Full strength PM or

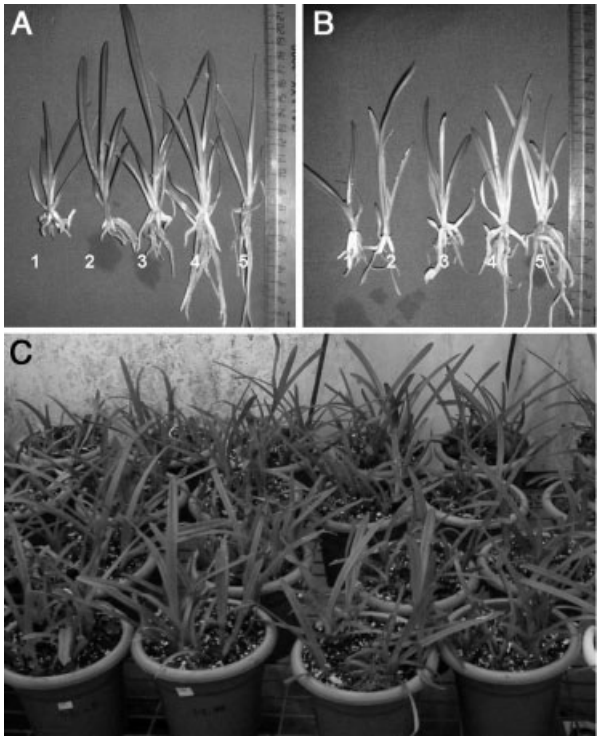

Figure 4. (A) Seedlings rooted in PM medium (1 = PM, 2 = PM +0.5 mg/L IAA, 3 = PM+1.0 mg/L IAA, 4 = 1/2PM+0.5 mg/L IAA and 5 = 1/2PM+1.0 mg/L IAA); (B) Seedlings rooted in M medium (1 = M, 2 = M+0.5 mg/L IAA, 3 = M+1.0 mg/L IAA, 4 = 1/2M+0.5 mg/L IAA and 5 = 1/2M+1.0 mg/L IAA); (C) Hardened *in vitro* raised seedlings of *Cymbidium aloifolium* growing in pots in greenhouse.

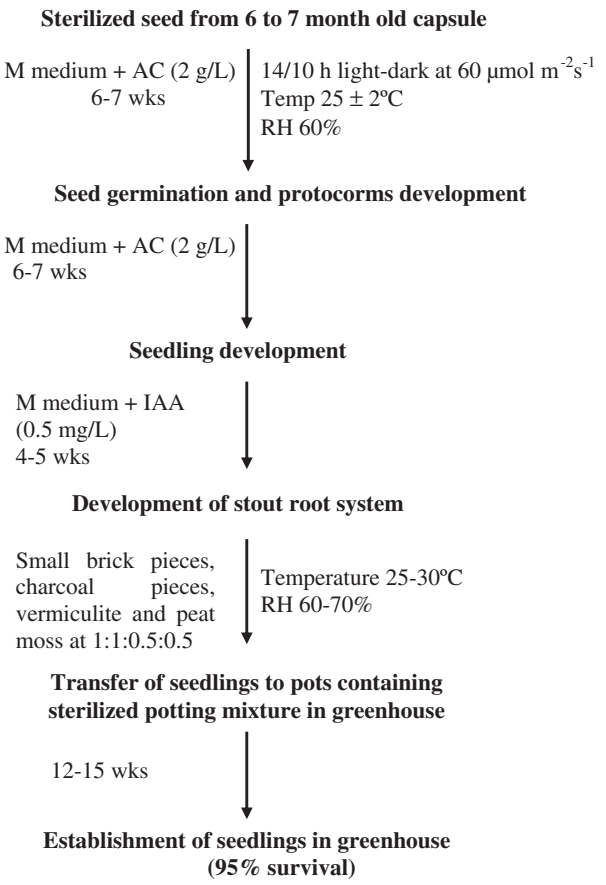

Figure 5. Protocol for mass propagation of *C. aloifolium*.

Table 4. Comparative cost of seedling production of *C. aloifolium*.[a)]

Components	Cost (in Indian Rupees)	
	M medium	PM medium
Culture medium	0.067	0.067
AC	0.001	–
P	–	0.066
IAA (for rooting)	0.003	0.003
Electricity	0.237	0.260
Manpower	0.250	0.250
Instrumentations (10% of total cost)	0.030	0.041
Total	**0.585**	**0.687**

a) cost per seedling

M medium fortified with 0.5 or 1.0 mg/L IAA produced a few stunted roots per seedling. Considering all these features 1/2 PM or 1/2 M medium fortified with 0.5 mg/L IAA was convenient for induction of stout root system in this species. Well-rooted seedlings were then transferred to greenhouse where 95% seedlings survived (Fig. 4C).

Induction of healthy root system in *in vitro* seedlings is very important for their survival in outside environment. Bhadra *et al.* [61] reported that deficiency of nutrition ions in the culture medium could enhance root induction in some orchid species, most probably to explore nutrient ions and water from its surroundings. Development of roots is an innate nature of plants which is controlled by endogenous level of hormones [62]. In *in vitro* conditions, supplementation of exogenous hormone (auxins) to the medium enhances rooting response. Sivakumar *et al.* [63] reported that shoot buds of *Centella asiatica* (a medicinal herb) failed to root in half strength MS medium unless supplemented with auxins. Stimulatory effects of IAA on rooting were reported in *Cymbidium devonianum* [64], *Vanda coerulea* [65], *Encyclia mariae* [66]. The present study recommended that combined effects of nutritional ion deficiency with IAA enhanced the development of stout root system in *C. aloifolium*.

4 Conclusions

Although M+AC and PM+P showed 100% seed germination, the size of protocorms was superior and incubation period was shorter in M medium. However, no significant difference was observed in development of stout root system in both 1/2 M and 1/2 PM medium fortified with 0.5 mg/L IAA. Furthermore, the total cost of production of seedlings (Table 4) including medium, manpower, electricity and instrumentation cost was also less in M Re (0.585/seedling) than PM Re (0.687/seedling). Thus the results clearly demonstrated that M medium was the most effective and economic for high frequency seedling production of *C. aloifolium* and the protocol (Fig. 5) offers an opportunity to commercial nurseries for large-scale propagation as well as for *ex situ* conservation of this orchid.

Abbreviations: BAP, 6-benzylaminopurine**; 2,4-D,** 2,4-dichlorophenoxy acetic acid**; IAA,** indole acetic acid**; P,** peptone**; AC,** activated charcoal

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Conflict of interest

The authors have declared no conflict of interest.

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