Pak. J. Bot., 47(6): 2361-2367, 2015.

AXENIC SEED CULTURE AND *IN VITRO* MASS PROPAGATION OF MALAYSIAN WILD ORCHID *CYMBIDIUM FINLAYSONIANUM* LINDL.

TOUHIDUL ISLAM[1], BAKUL BHATTACHARJEE[1], S.M. SHAHINUL ISLAM[1*], JASIM UDDAIN[2] AND SREERAMANAN SUBRAMANIAM[2]

[1]*Plant Genetic Engineering Lab., Institute of Biological Sciences, University of Rajshahi, Rajshahi-6205, Bangladesh*
[2]*School of Biological Sciences, University Sains Malaysia (USM), 11800 Gelugor, Penang, Malaysia*
[*]*Correspondence author: shahin_ibsc@ru.ac.bd*

Abstract

Under this study an efficient protocol on mass propagation of *Cymbidium finlaysonianum* an epiphytic Malaysian wild orchid has been established using axenic culture. To obtain an axenic seed culture, it is important to perform an adequate a disinfection procedure in tissue culture. Four nutrient media *viz.* MS, ½MS, KC and VW were evaluated on *In vitro* seed germination with callus initiation. The maximum seed germination with callus initiation (100%) was recorded in MS basal medium with a short span of time (40 days after culture). After 45 days of callus initiation the effect of eight different treatments (T_1-T_8) on callus size and nature were also studied. The experiment revealed that in T_3 (MS + 2.0 mgl^{-1} BAP + 0.5 mgl^{-1} NAA) was found to be the best for callus development (1.98 cm length and 1.01 cm breadth). The effect of different concentration of BAP was evaluated on protocorm formation and its proliferation. Maximum number (7.75) and percentage (81.40) of PLBs was recorded in MS medium supplemented with 1.5 mgl^{-1} BAP. Very good PLBs development was recorded also in MS + BAP 1.0 mgl^{-1} + NAA 0.5 mgl^{-1}. The highest elongation of shoot (3.80 cm) was observed in MS + 1.0 mgl^{-1} BAP + 0.50 mgl^{-1} NAA. For root induction 1.0 mgl^{-1} NAA has proven to the best in ½MS medium. The *In vitro* developed seedlings were finally transferred to pots by successive phases of acclimatization.

Key words: Axenic culture; *Cymbidium finlaysonianum*; Protocorm like bodies (PLBs); Mass propagation, Media.

Introduction

Orchid family Orchidaceae is a morphologically diverse and widespread family and contains about 1,250 species of monocots and it is second largest family of flowering plants (Siripipatthana *et al*., 2014). Orchids are one of the most striking decorative plants all over the world. The great majority are to be found in the tropics, mostly in Asia, South America and Central America (Chowdhery, 2001). Many of them have floricultural importance because of their beautiful flowers and persist for a long time. In recent years many of the commercially important orchids are artificially grown for its high price in the national and international markets. However, almost 7% of total flowering plant species which represent one of the most expensive ornamental known today and dominate the international cut flower market (Rahman *et al*., 2005). It is not only important for floricultural value but also some of them are used as medicine and food (Bose & Bhattacharjee, 1980; Bhattacharjee *et al*., 2015). They have rich contents of alkaloids, glycerides and other useful phytochemicals (Gutierrez, 2010; Pant, 2011).

The genus *Cymbidium* known as boat orchid, comprises approximately 52 species native to regions of Asia ranging from the northwestern Himalayas to Japan, Indochina and Malaysia to northern and eastern Australia and has been one of the most commercially successful orchid as well as cut flowers around the world (Puy & Cribb, 2007). It is quite diversified ecologically and they exhibit terrestrial, epiphytic and lithophytic life-forms. *Cymbidium macrorhizon* lacks foliage leaves and has a strongly mycoparasitic nature (Yukawa & Stern, 2002). However, as horticultural importance many *Cymbidium* species are in danger of extinction because of the extensive disturbance of their natural habitat and indiscriminate harvesting of naturally growing plants. Therefore, it is extremely important to conserve the germplasms of these orchids. *Cymbidium finlaysonianum* Lindl is a sympodial and pseudobulb orchid has long pendulous spikes carrying small yellow and red fragrant flowers (Opchat, 2000). Distributed in Vietnam, Cambodia, Thailand, Sumatra, Java and Borneo, The Philippines and Sulawesi; in Malaya found throughout the country in the lowland, as an epiphyte in rather exposed places, but especially near the sea and most abundantly in the north, where it sometimes grow into very long masses (Davis & Steiner, 1952). Due to lack of proper cultivation practices, destruction of plant habitats and illegal and indiscriminate collection of orchids from natural habitats, many medicinal plants are severely threatened (Devendra *et al*., 2011). *Cymbidium finlaysonianum* Lindl is a commercially important orchid (Davis & Steiner, 1952) and is one that rapidly disappearing and is now listed as vulnerable to extinction (Vaddhanaphuti & Seidenfaden, 2005). Owing to its commercial value in the floricultural industry, natural populations are under threat from over-exploitation. Mass propagation provides an alternative means of satisfying the demand. For successful micropropagation an axenic culture is required, otherwise serious losses happen due to deficiency in the growth or death of the tissue explants might occur (Curvetto *et al.*, 2006). To obtain an axenic culture, it is important to have an adequate a disinfection procedure (Snow, 1985). Conventional propagation is slow and sometimes it is very difficult, suggesting *In vitro* methods for mass multiplication may be more appropriate. With the aim of developing a low cost protocol to mass micropropagation of *Cymbidium finlaysonianum* using *In vitro* axenic seed culture has been successfully established.

Materials and Methods

Plant materials: Seeds of *Cymbidium finlaysonianum* were collected from naturally pollinated plants growing in natural habitat from University Sains Malaysia (USM) campus, Penang, Malaysia. Immature pods of *Cymbidium finlaysonianum* were used as explants for the present study (Fig. 1a).

Sterilization: The seed pod was washed with running tap water along with detergent and Tween 20. Then pods were surface sterilized sequentially with 70% ethyl alcohol for 1 minute, 10% clorex (sodium hypochlorite) solution for 10 minutes and finally rinsed thoroughly 5-6 times with sterile distilled water. Sterilized capsules were cut longitudinally by a sterile surgical blade (Fig. 1b). Around 200 mg seeds were inoculated per culture vessel.

Media, axenic culture of seed germination and protocorm development: Four (04) nutrient media *viz.* MS and ½MS (Murashige & Skoog, 1962), KC (Knudson, 1946), VW (Vacin & Went, 1949) were evaluated to compare their effectiveness on seed germination and initial callus induction efficiency. As carbon sources 3% sucrose and solidifying agent 0.8% agar was used for all media. The pH of all media was adjusted 5.6 - 5.8 prior to autoclaving at 121ºC with 15 psi for 15 minutes. The cultures were maintained at 25±2ºC. Seed germination and callus initiation were recorded after five days of interval. The initiated callus were inoculated for better proliferation and development in MS media with 9 different concentration and combination of auxin and cytokinin *viz.* Cont. = without PGRs; T_1 = BAP (1.0 mgl^{-1}) + NAA (0.5 mgl^{-1}), T_2 = BAP (1.5 mgl^{-1}) + NAA (0.5 mgl^{-1}),T_3 = BAP (2.0 mgl^{-1}) + NAA (0.5 mgl^{-1}), T_4 = BAP (2.5 mgl^{-1}) + NAA (0.5 mgl^{-1}), T_5 = BAP (1.0 mgl^{-1}) + NAA (1.0 mgl^{-1}), T_6 = BAP (1.5 mgl^{-1}) + NAA (1.0 mgl^{-1}), T_7 = BAP (2.0 mgl^{-1}) + NAA (1.0 mgl^{-1}), T_8 = BAP (2.5 mgl^{-1}) + NAA (1.0 mgl^{-1}). Data were recorded on the basis of spread, nature and colour of callus after 45 days of inoculation. The calli were transferred to MS media containing six (0.0, 0.5, 1.0, 1.5, 2.0 and 2.5 mgl^{-1}) different concentrations of BAP for protocorm formation. Developmental stages of PLBs were recorded and cultured them in MS medium containing 13 different treatments *viz.* Cont. = without PGRs; T_1 = BAP (0.5 mgl^{-1}), T_2 = BAP (1.0 mgl^{-1}), T_3 = BAP (1.5 mgl^{-1}), T_4 = BAP (2.0 mgl^{-1}), T_5 = BAP (0.5 mgl^{-1}) + NAA (0.5 mgl^{-1}), T_6 = BAP (1.0 mgl^{-1}) + NAA (0.5 mgl^{-1}), T_7 = BAP (1.5 mgl^{-1}) + NAA (0.5 mgl^{-1}), T_8 = BAP (2.0 mgl^{-1}) + NAA (0.5 mgl^{-1}), T_9 = BAP (0.5 mgl^{-1}) + NAA (1.0 mgl^{-1}), T_{10} = BAP (1.0 mgl^{-1}) + NAA (1.0 mgl^{-1}), T_{11} = BAP (1.5 mgl^{-1}) + NAA (1.0 mgl^{-1}), T_{12} = BAP (2.0 mgl^{-1}) + NAA (1.0 mgl^{-1}). Developmental stages of PLBs (i.e. percentage of protocorms with vegetative apex, plantlets with 2-3 leaves and 1-2 roots) were recorded in every 30 days of culture initiation.

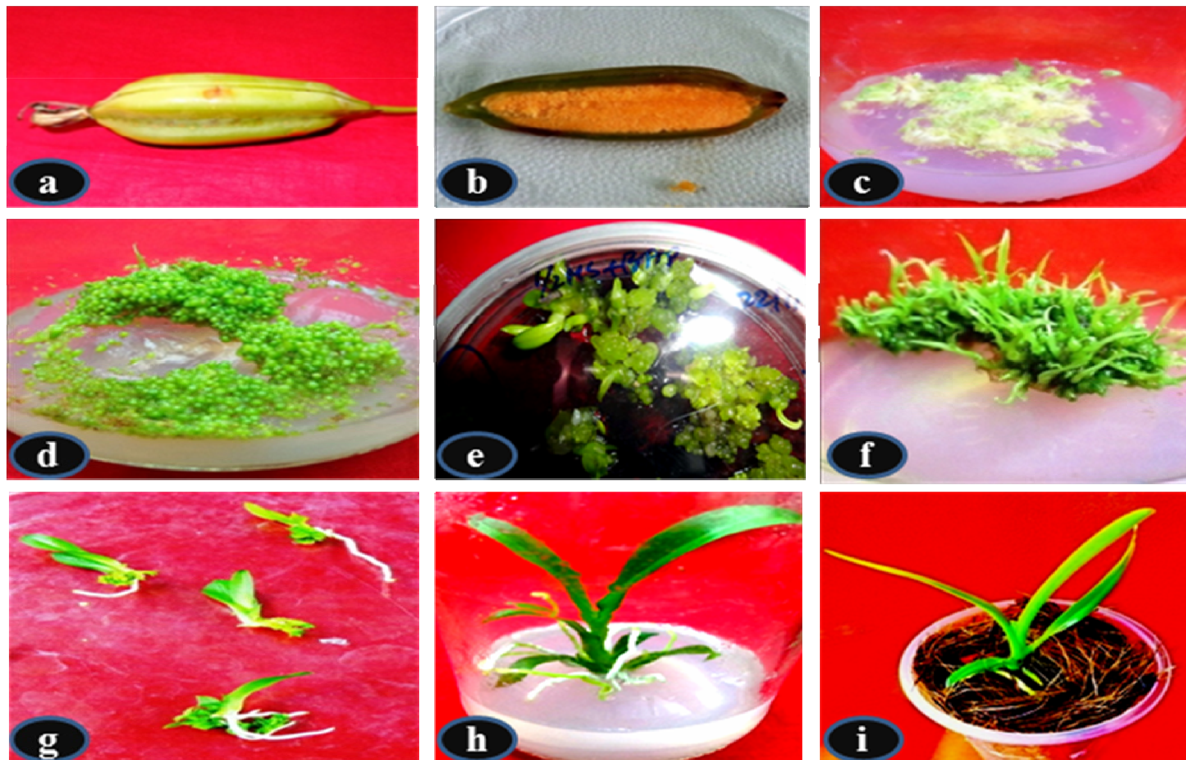

Fig. 1. Axenic seed germination and protocorm development of *C. finlaysonianum*; a) Seed pod of *C. finlaysonianum*, b) Dissection of seed pod, c) Green spots indicates germination initiation with callus, d) Mass development of PLBs, e) Protocorm with vegetative apex, f) 2-3 leaves stage of protocorms, g) Root and shoots initiation from PLBs, h) Well developed plants and i) Acclimatized plants transferred in pot.

Shoot elongation: Individual shoots were sub-cultured on MS basal medium supplemented with BAP, NAA and Kinetin alone or in combination for elongation. After 30 days of incubation data were recorded on the basis of shoot length.

Rooting and acclimatization: When the individual shoot reached 3-4 cm height was transferred into rooting medium. For efficient root induction three plant growth regulators *viz.* NAA, IAA and IBA (0.5, 1.0, 1.5 and 2.0 mgl^{-1}) were used in ½MS medium. *In vitro* rooted plantlets were taken out of the culture vessels and transferred to outside the culture room following successive phases of acclimatization. The plantlets were watered alternately in the every 2nd days and fed with MS nutrient solutions (diluted 10 times) fortnightly for about a month and eventually established under natural condition following the standard protocols of Das *et al*. (2007).

Experimental design and statistical analysis: Experiments were designed following complete randomize design (CRD). For each experiment five replicates and for rooting 20 replicates were considered. The effects of different culture conditions on germination of seeds, callus & protocorm size, PLBs developmental stages, shoot and root elongation in the *In vitro* experiments were tested applying DMRT- Duncan's multiple range test (P = 0.05) in one way analysis of variance (ANOVA). The statistical analyses were performed using the programme package using SPSS (ver. 16, SPSS Inc., USA).

Results

Seed culture and callus development: We established the axenic culture of *Cymbidium finlaysonianum* from seed using four basal media *viz.* MS, ½MS, KC and VW. In most of the tested media, within 6 to 8 weeks all the culture vessels showed light green indicating the completion of germination process and initiation of callus (Fig. 1c). The highest percentage of seed germination with callus induction (100%) was observed in MS medium with the shortest span of time (40 DAC) (Table 1). The maximum proliferation of callus (1.98 cm length and 1.01 cm breadth) was obtained in MS medium fortified with 2.0 mgl^{-1} BAP and 0.5 mgl^{-1} NAA (T_3) which produced a compact dark greenish callus mass followed by the combination of 2.5 mgl^{-1} BAP and 0.5 mgl^{-1} NAA (1.70 cm length and 0.74 cm breadth) (Fig. 1d). The minimum proliferation of callus (0.70 cm length and 0.30 cm breadth) was obtained in Control without plant growth regulators in MS medium (Table 2).

Table 1. Effect of different media on *In vitro* seed germination with callus induction of *Cymbidium finlaysonianum* at different days after culture.

Media	Seed germination with callus induction (%)						
	DAC						
	25	30	35	40	45	50	55
MS	35	50	80	100	100	100	100
½MS	15	35	55	80	90	100	100
VW	30	45	75	85	100	100	100
KC	-	20	30	55	80	90	100

MS (Murashige & Skoog, 1962), VW (Vacin & Went, 1949), KC (Knudson, 1946). Days after culture (DAC)

Table 2. Effect of plant growth regulators (PGRs) on size and nature of callus after 45 days of culture initiation on MS medium.

Treatments	PGRs (mgl^{-1})		Size of callus (cm)		Nature of the callus	
	BAP	NAA	Length M ± S.E	Breadth M ± S.E	Texture	Colour
Control	0.0	0.0	0.70 ± 0.02^{a}	0.30 ± 0.02^{a}	CP	GR
T_1	1.0	0.5	1.10 ± 0.07^{bc}	0.50 ± 0.03^{b}	CP	GR
T_2	1.5	0.5	1.50 ± 0.07^{d}	0.76 ± 0.05^{c}	CP	GR
T_3	2.0	0.5	1.98 ± 0.06^{f}	1.01 ± 0.03^{d}	CP	DGR
T_4	2.5	0.5	1.70 ± 0.07^{e}	0.74 ± 0.04^{c}	CP	DGR
T_5	1.0	1.0	1.25 ± 0.08^{c}	0.53 ± 0.02^{b}	CP	DGR
T_6	1.5	1.0	1.18 ± 0.07^{bc}	0.55 ± 0.03^{b}	FB	DGR
T_7	2.0	1.0	1.14 ± 0.04^{bc}	0.50 ± 0.02^{b}	FB	DGR
T_8	2.5	1.0	1.08 ± 0.02^{b}	0.49 ± 0.03^{b}	FB	DGR

Cont. = without PGRs ; T_1 = BAP (1.0 mgl^{-1}) + NAA (0.5 mgl^{-1}), T_2 = BAP (1.5 mgl^{-1}) + NAA (0.5 mgl^{-1}), T_3 = BAP (2.0 mgl^{-1}) + NAA (0.5 mgl^{-1}), T_4 = BAP (2.5 mgl^{-1}) + NAA (0.5 mgl^{-1}), T_5 = BAP (1.0 mgl^{-1}) + NAA (1.0 mgl^{-1}), T_6 = BAP (1.5 mgl^{-1}) + NAA (1.0 mgl^{-1}), T_7 = BAP (2.0 mgl^{-1}) + NAA (1.0 mgl^{-1}), T_8 = BAP (2.5 mgl^{-1}) + NAA (1.0 mgl^{-1}). M = Mean, S.R = Standard error, CP = Compact; FB = Friable; GR = Green; DGR = Dark Green. Values in a column with similar superscripts are not significantly different at $p \leq 0.05$ levels

Protocorm multiplication and development: The effect of various concentrations of BAP was observed on the basis of number of protocorms per vessel, proliferation of protocorms and nature (Fig. 1c-e). The observation was recorded after 30 and 45 days of culture initiation. The result reveals that after 45 days of inoculation the highest number of protocorms per culture vessel was highest (7.75) in MS medium fortified with 1.5 mgl^{-1} BAP (Table 3). On the other hand, the proliferation of protocorm was maximum (length 1.20 cm and breadth 0.64 cm after 30 days of culture) in BAP 1.5 mgl^{-1}. At the same concentration of BAP, the protocorms showed the highest swelling (1.90 cm length and 1.01 cm breadth) after 45 days of culture followed by 1.0 mgl^{-1} BAP. The protocorms are compact and green in BAP 1.5 mgl^{-1}. After 9 weeks of culture initiation the highest percentage of PLB's formation (81.40 %) was recorded in T_3 (Table 4) which is fortified with MS + BAP (1.5 mgl^{-1}).

Shoot elongation and Root development from seedlings: The effect of different combination and concentrations of PGRs with MS medium on elongation of individual shoots were evaluated. Incubation period of 30 days showed that 1.0 mgl^{-1} BAP and 0.5 mgl^{-1} NAA was responded better with a shoot length of 3.80 cm (Table 5). The elongated seedlings produced weak root system on the germination and elongation media. In order to induce strong and stout root system the elongated seedlings of 2-3 cm length were transferred to rooting media. The efficiency of the rooting media was evaluated based on the increase in length and number of roots developed per seedling. Highest root development was observed both in number (2.60) and length (2.58 cm) in ½MS fortified with 1.0 mgl^{-1} NAA (Figs. 2 & 3). *In vitro* regenerated rooted plantlets were successfully acclimatized in the pot in shed house conditions (Fig. 1i).

Table 3. Effect of different concentration of BAP on number, spread and nature of protocorms on MS medium (M ± S.E).

PGRs	Number of protocorms per vessel		Proliferation of protocorms (cm)				Nature of protocorms			
			Days (DAC)							
			30	30	45	45	Texure		Colour	
BAP (mgl^{-1})	30	45	Length	Breadth	Length	Breadth	30	45	30	45
0.0	$3.00{\pm}0.22^{a}$	$3.90{\pm}0.24^{a}$	$0.90{\pm}0.03^{a}$	$0.45{\pm}0.02^{a}$	$1.02{\pm}0.02^{a}$	$0.55{\pm}0.02^{a}$	CP	CP	GR	GR
0.5	$4.98{\pm}0.15^{b}$	$5.50{\pm}0.22^{b}$	$0.93{\pm}0.02^{ab}$	$0.51{\pm}0.02^{b}$	$1.15{\pm}0.01^{ab}$	$0.68{\pm}0.01^{b}$	FB	FB	GR	GR
1.0	$6.30{\pm}0.10^{c}$	$7.50{\pm}0.27^{c}$	$1.02{\pm}0.06^{b}$	$0.59{\pm}0.01^{d}$	$1.60{\pm}0.07^{c}$	$0.82{\pm}0.02^{c}$	FB	FB	GR	GR
1.5	$6.83{\pm}0.23^{c}$	$7.75{\pm}0.37^{c}$	$1.20{\pm}0.03^{c}$	$0.64{\pm}0.01^{e}$	$1.90{\pm}0.07^{d}$	$1.01{\pm}0.02^{d}$	CP	CP	GR	GR
2.0	$5.05{\pm}0.28^{b}$	$5.65{\pm}0.40^{b}$	$0.98{\pm}0.04^{ab}$	$0.55{\pm}0.01^{c}$	$1.28{\pm}0.06^{b}$	$0.70{\pm}0.02^{b}$	FB	FB	GR	GR
2.5	$4.45{\pm}0.23^{b}$	$4.80{\pm}0.49^{ab}$	$0.89{\pm}0.01^{a}$	$0.52{\pm}0.01^{b}$	$1.05{\pm}0.07^{a}$	$0.66{\pm}0.02^{b}$	FB	FB	GR	GR

DAC = Days after culture; CP = Compact; FB = Friable; GR = Green. M = Mean, S.R = Standard error, Values in a column with similar superscripts are not significantly different at $p \leq 0.05$ levels

Table 4. Effects of PGRs on the protocorm like bodies (PLBs) formation and developments of *Cymbidium finlaysonianum* after nine weeks of culture initiation.

Treatment (T)	PGRs (mgl^{-1})		PLBs formation (%)	Stages of PLBs development (% of response)		
				VAS	LS	RS
	BAP	NAA	M ± S.E	M ± S.E	M ± S.E	M ± S.E
Cont.	0.0	0.0	58.20 ± 0.86^{c}	60.40 ± 0.68^{c}	52.00 ± 0.71^{b}	42.00 ± 0.55^{b}
T_1	0.5	0.0	68.60 ± 0.93^{f}	64.60 ± 0.51^{d}	55.60 ± 0.51^{cd}	45.20 ± 0.58^{c}
T_2	1.0	0.0	72.80 ± 0.73^{g}	65.00 ± 0.71^{d}	56.00 ± 0.55^{c}	45.60 ± 0.51^{cd}
T_3	1.5	0.0	81.40 ± 0.51^{h}	70.20 ± 0.80^{f}	60.00 ± 0.84^{fg}	55.20 ± 0.86^{g}
T_4	2.0	0.0	62.20 ± 0.58^{d}	55.20 ± 0.86^{b}	45.40 ± 0.81^{a}	35.00 ± 0.45^{a}
T_5	0.5	0.5	53.40 ± 0.93^{ab}	68.40 ± 0.68^{ef}	61.40 ± 0.93^{g}	52.00 ± 0.95^{f}
T_6	1.0	0.5	64.40 ± 0.51^{e}	73.60 ± 0.81^{g}	64.60 ± 0.51^{h}	59.00 ± 0.89^{h}
T_7	1.5	0.5	61.80 ± 0.58^{d}	67.20 ± 0.58^{e}	58.00 ± 0.89^{ef}	56.40 ± 0.68^{g}
T_8	2.0	0.5	57.20 ± 0.97^{c}	65.00 ± 0.84^{d}	57.60 ± 0.93^{de}	48.00 ± 0.71^{e}
T_9	0.5	1.0	52.40 ± 0.81^{a}	64.20 ± 0.66^{d}	56.20 ± 0.58^{cde}	46.20 ± 0.37^{de}
T_{10}	1.0	1.0	56.20 ± 0.73^{bc}	61.40 ± 0.93^{c}	55.00 ± 0.71^{c}	44.60 ± 0.51^{c}
T_{11}	1.5	1.0	54.60 ± 0.60^{b}	56.20 ± 0.73^{b}	50.60 ± 0.93^{b}	41.00 ± 0.45^{b}
T_{12}	2.0	1.0	51.80 ± 0.37^{a}	51.00 ± 0.32^{a}	44.00 ± 0.71^{a}	35.20 ± 0.66^{a}

Cont. = without PGRs; T_1 = BAP (0.5 mgl^{-1}), T_2 = BAP (1.0 mgl^{-1}), T_3 = BAP (1.5 mgl^{-1}), T_4 = BAP (2.0 mgl^{-1}), T_5 = BAP (0.5 mgl^{-1}) + NAA (0.5 mgl^{-1}), T_6 = BAP (1.0 mgl^{-1}) + NAA (0.5 mgl^{-1}), T_7 = BAP (1.5 mgl^{-1}) + NAA (0.5 mgl^{-1}), T_8 = BAP (2.0 mgl^{-1}) + NAA (0.5 mgl^{-1}), T_9 = BAP (0.5 mgl^{-1}) + NAA (1.0 mgl^{-1}), T_{10} = BAP (1.0 mgl^{-1}) + NAA (1.0 mgl^{-1}), T_{11} = BAP (1.5 mgl^{-1}) + NAA (1.0 mgl^{-1}) and T_{12} = BAP (2.0 mgl^{-1}) + NAA (1.0 mgl^{-1}). M = Mean, S.E = Standard error, VAS =Vegetative apex stage; LS = 2–3 Leaves stage; RS = 1–2 Roots stage. Values in a column with similar superscripts are not significantly different at $p \leq 0.05$ levels

Table 5. Effect of different concentrations and combinations of PGRs with MS medium on the elongation of individual shoot.

PGRs (mgl^{-1})	Initial length (cm) Mean	Length (cm) of shoot after 30 days of culture (Mean)	Increased length (cm) (M ± S.E)
BAP			
0.50	3.00	4.05	1.05 ± 0.22^{cd}
1.00	3.10	5.78	2.68 ± 0.09^{bc}
1.50	3.00	4.50	1.50 ± 0.22^{cd}
2.00	3.20	4.19	0.99 ± 0.15^{d}
BAP + NAA			
0.50 + 0.25	2.80	4.80	2.00 ± 0.27^{bc}
1.00 + 0.50	3.10	4.90	3.80 ± 0.37^{a}
1.50 + 0.75	3.00	5.26	2.26 ± 0.39^{bc}
2.00 + 1.00	2.90	4.45	1.55 ± 0.20^{cd}
KIN			
0.50	2.85	3.89	1.04 ± 0.22^{cd}
1.00	3.10	5.38	2.28 ± 0.18^{bc}
1.50	2.90	4.92	2.02 ± 0.32^{bc}
2.00	2.80	3.55	0.75 ± 0.07^{d}
KIN + NAA			
0.50 + 0.25	3.30	5.17	1.87 ± 0.09^{bcd}
1.0 0 + 0.50	3.10	5.92	2.82 ± 0.33^{bc}
1.50 + 0.75	2.90	4.00	2.10 ± 0.31^{bc}
2.00 + 1.00	3.00	4.30	1.30 ± 0.15^{cd}

PGRs = Plant growth regulators (PGRs), values represent mean ± S.E (standard error). Values in a column with similar superscripts are not significantly different at $p\leq 0.05$ levels

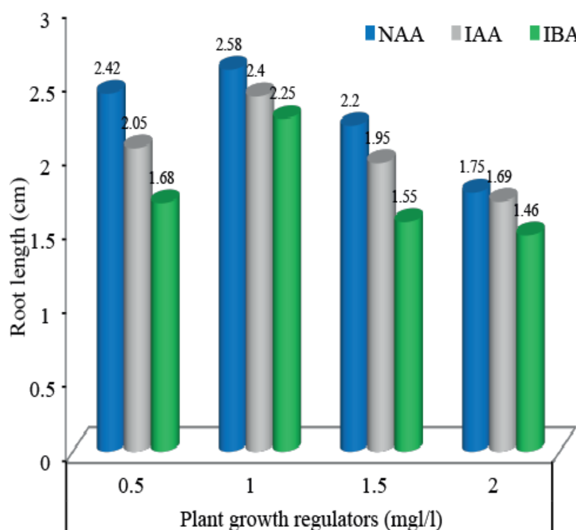

Fig. 2. Effects of different auxins on root development with ½MS medium.

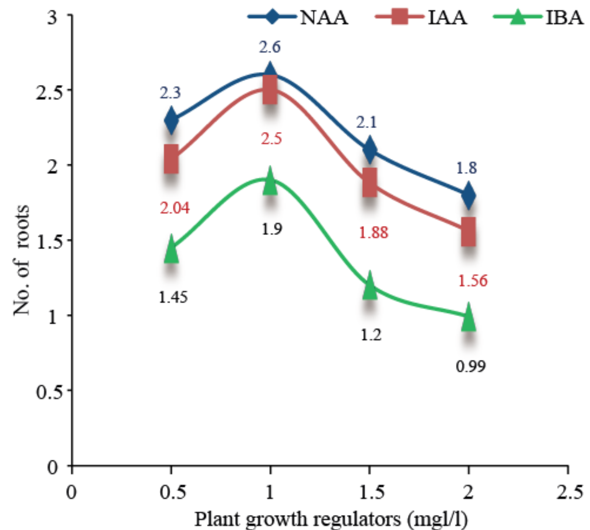

Fig. 3. Effects of different auxins with ½MS on number of root per plant.

Discussion

Our results showed that various additives and medium components affected germination, callus induction, protocorm development, shoot elongation and root formation in *C. finlaysonianum*. In the experiment, axenic seed culture was successfully established, which is similar to the previous report in *Spathoglottis plicata* (Sebastinraj & Muhirkuzhali, 2014), *Esmeralda clarkei* (Paudel & Pant, 2013) and *Arundina graminifolia* (Bhadra & Bhowmik, 2005). Arditti & Ernst *et al.* (1984) reported that orchid seeds have neither endosperm nor cotyledons in the embryo as primary storage materials. Thus, nutrients in the culture medium are required for orchid seed germination. In our study, all media were devoid of synthesis plant growth regulators (PGRs). Thus, organic additives had a profound effect on the subsequent germination and callus development. This could be due to the presence of sufficient endogenous growth regulators required for the initial stages of germination. Similar results were also found in *Dendrobium tosaense* that seed germination without plant growth regulators in medium (Lo *et al.*, 2004). The *In vitro* cultured seeds developed into

protocorms within 6-7 weeks of culture in BAP (1.5 mgl^{-1}) containing in MS medium and differentiated into shoots after 9 weeks. The previous studies on seed germination of *Aerides odorata* (Pant & Gurung 2005); *Cymbidium* sp., *Dendrobium noblile*; *Cymbidium findlaysonianum* (Tawaro *et al.,* 2008); *Epidendrum ibaguense* (Hossain, 2008); *Phaius tancarvilleae* (Pant *et al.,* 2011), *Dendrobium* cv. Banyat pink (Salam *et al.,* 2013) and *Vanda tessellata* (Bhattacharjee & Islam, 2014) were showed similarity with our finding. We found that 1.0 mgl^{-1} BAP and 0.5 mgl^{-1} NAA was best for shoot elongation. Furthermore, alteration in media composition (Malik & Saxena 1992; Shiva *et al.* 1994), substitution of plant growth regulators had a significant effect on shoot elongation are reported by Mohamed *et al.* (1991) and Nagi *et al.* (1997). Finally *In vitro* regenerated well rooted plants were successfully acclimatized and transferred them to field conditions. The importance of the acclimatization process has been highlighted in a number of reviews about orchid growth (Lauzer *et al*., 2007; Hossain *et al*., 2010; Rittirat *et al*., 2011; Panwar *et al*., 2012; Paudel & Pant, 2013 and Shibu *et al.,* 2014). It is expected that this system would be further established as an efficient mutation and ploidy level enhancement scheme for this orchid species for its further improvement.

Conclusion

For rapid production of *Cymbidium finlaysonianum* from using axenic seed culture protocol has been standardized. The results obtained in the experiment indicated that the exogenous plant growth regulators are important for callus induction in orchids. The concentration of 1.5 mgl^{-1} BAP is the optimum for effective protocorm development in *C. finlaysonianum*. Plantlets could be hardened under shade house conditions on coconut peat. Using this protocol, it is possible to produce viable, uniform and healthy plants that can be used for large scale cultivation which could significantly contribute to meeting the commercial demand for this beautiful orchid. Furthermore, the protocol may facilitate conservation of this orchid from extinction in the natural and also proven to be valuable for polyploidy and mutation breeding of the desired characters of *C. finlaysonianum*.

Acknowledgements

The authors are gratefully acknowledges to Plant Genetic Engineering Lab., Institute of Biological Sciences, University of Rajshahi, Bangladesh for research facilities and the University Grant Commission (UGC) of Bangladesh for providing fellowships for this study.

References

Arditti, J. and R. Ernst. 1984. Physiology of germinating orchid seeds. In: Orchid biology: reviews and perspectives III. (Ed.): Arditti J., Ithaca, New York: Cornell University Press; pp.177-222.

Bhadra, S.K. and T.K. Bhowmik. 2005. Axenic germination of seeds and rhizome-based micropropagation of an orchid *Arundina graminifolia* (D. Don.) Hochr. *Bangladesh J. Bot.,* 34: 59-64.

Bhattacharjee, B. and S.M.S. Islam. 2014. Effects of plant growth regulators on multiple shoot induction in *Vanda tessellata* (Roxb.) Ex. G. Don an endangered medicinal orchid. *Int. J. Sci. Nat*., 5(4): 707-712.

Bhattacharjee, B., T. Islam, Z. Rahman and S.M.S. Islam. 2015. Antimicrobial activity and phytochemical screening of whole plant extracts of *Vanda tessellata* (Roxb.) Hook. Ex.G.Don. *World J. Phar. Pharmace. Sci*., 4(1): 72-83.

Bose, T.K. and S.K. Bhattacharjee. 1980. Orchids of India. Calcutta, India: *Naya Prakash*, p. 188.

Chowdhery, H.J. 2001. Orchid diversity in North-East India. *J. Orchid Soc. India*, 15: 1-17.

Curvetto, N., M. Pablo and M. Gabriela. 2006. Hydrogen peroxide in micropropagation of *Lilium*. A comparison with a traditional methodology. *Biocell*., 30(3): 497-500.

Das, M.C., S. Kumaria and P. Tandon. 2007. Protocorm regeneration, Multiple shoot induction and *ex vitro* establishment of *Cymbidium devonianum* Paxt. *Asian J. Plant Sci*., 6(2): 349-353.

Davis, R.S. and M.L. Steiner. 1952. Philippine Orchids; Published by "The William Frederick Press" New York. pp. 1-270.

Devendra, B.N., N. Srinivas and G.R. Naik. 2011. Direct somatic embryogenesis and synthetic seed production from *Tylophora indica* (Burm.f.) Merrill. An endangered, medicinally important plant. *Int. J. Bot*., 7: 216-222.

Gutierrez, P.M.P. 2010. Orchids: A review of uses in traditional medicine, its photochemistry and pharmacology. *J. Med. Plants Res.* 4: 592-638.

Hossain, M.M. 2008. Asymbiotic seed germination and *In vitro* seedling development of *Epidendrum ibaguense* Kunth. (Orchidaceae). *Afr. J. Biotech*., **7**(20): 3614-3619.

Hossain, M.M., M. Sharma, J.A. Teixeira da Silva and P. Pathak. 2010. Seed germination and tissue culture of *Cymbidium giganteum* Wall. ex Lindl. *Scientia Hort.,* 123: 479-487.

Knudson, L. 1946. A new nutrient solution for germination of orchid seed. *Am. Orchid Soc. Bull*., 15: 214-217.

Lauzer, D., S. Renaut, M.S. Arnaud and D. Barabe. 2007. *In vitro* asymbiotic germination, protocorm development, and plantlet acclimatization of *Aplectrum hyemale* (Muhl. Ex. Willd.) Torr. (Orchidaceae), *J. Torrey Bot. Soc.,* 134(3): 344-348.

Lo, S.F., S.M. Nalawade, C.L. Kuo, C.L. Chen and H.S. Tsay. 2004. Asymbiotic germination of immature seeds, plantlet development and *ex vitro* establishment of plants of *Dendrobium tosaense* Makino - a medicinally important orchid. *In Vitro Cell. Dev. Biol- Plant.,* 40: 528-535.

Malik, K.A. and P.K Saxena. 1992. Thidiazuron induces high frequency shoot regeneration in intact seedlings of pea (*Pisum sativum*), chickpea (*Cicer arietinum*) and lentil (*Lens culinaris*). *Aus. J. Plant Physiol.,* 19:731-740.

Mohamed, M.F., P.E. Read and D.P. Coyne .1991. Plant regeneration *In vitro* from the embryonic axes of common and tepary beans. *Bean Improv. Coop Bull*., 34: 149-153.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassay with tobacco tissue culture. *Physiol. Plantarum*. 15: 473-497.

Nagi, W., S. Ignacimuthu and J. Becker. 1997. Genetic engineering and regeneration of Phaseolus and Vigna. State the art and new attempts. *J. Plant Physiol*., 150: 625-644.

Opchat, T. 2000. *Orchids in Thailand*. Home and Garden Publishing (in Thai.), Bangkok, pp. 2.

Pant, B. 2011. Orchids of Nepal with their medicinal properties. *Orch. J.,* 18(3): 92-98.

Pant, B. and R. Gurung. 2005. *In vitro* seed germination and seedling development in *Aerides odorata* Lour. *The J. Orchid Soc., India,* 19(1&2): 51-55.

Pant, B., S. Shrestha and S. Pradhan. 2011. *In vitro* seed germination and seedling development of *Phaius tankervilleae* (L'Her.) Blume. *Scientific World,* 9(9): 50-52.

Panwar, D., R. Kheta and H.N.S. Shekhawat. 2012. *In vitro* propagation of *Eulophia nuda* Lindl., an endangered orchid. *Scientia Hort.,* 139: 46-52.

Paudel, M.R. and B. Pant. 2013. A reliable protocol for micropropagation of *Esmeralda clarkei* Rchb.f. (Orchidaceae), *As. Pac J. Mol. Biol. Biotech.,* 21 (3): 114-120.

Puy, D. and P. Cribb. 2007. Genus *Cymbidium*. 2nd ed. Kew: Royal Botanic Gardens, Kew. p. 369.

Rahman, A.R.M., M.O. Islam, A.K.M.A. Prodhan and I. Syoichi. 2005. Effects of carbohydrates on callus growth and callus derived plantlet regeneration in *Doritaenopsis* orchid. *Biotechnology,* 4: 126-131.

Rittirat, S., S. Te-chato, N. Kerdsuwan and S. Kongruk. 2011. Micropropagation of Chang Daeng (*Rhynchostylis gigantean var. Sagarik*) by embryogenic callus. *Songklanakarin J. Sci. Tech.,* 33: 659-663.

Salam, P., J.S. Salam and C.R. Mohanty. 2013. Effect of benzylamino purine and naphthalene acetic acid on callus and protocorm formation of *Dendrobium* cv. Banyat pink. *J. Cell Tiss. Res.,* 13(3): 3977-3981.

Sebastinraj, J. and S. Muhirkuzhali. 2014. Asymbiotic seed germination and micropropagation of *Spathoglottis plicata* Blume. *Int. J. Adv. Phar. Biol. Chem*., 3(2): 495-501.

Shibu, B.S., P.S. Wesley, S. Moin and B.C. Devi. 2014. *In vitro* regeneration of *Coelogyne nervosa* A. Rich. and *Eria pseudoclavicaulis* Blatt. Threatened orchids of Westarn Ghats, India. *Ind. J. Exp. Biol*., 52: 658-663.

Shiva P.N., D. Pental and N. Bhalla-Sarin. 1994. Regeneration of pigeon pea (*Cajanus cajan*) from cotyledonary node via multiple shoot formation. *Plant Cell Rep*., 13:623-627.

Siripipatthana, P., N. Phaonakrop, S. Roytrakul, G. Senawong, W. Bunyatratchata, R.G. Mudalige-Jayawickrama and N. Sattayasai. 2014. Genetic differences between *Dendrobium chrysotoxum* native to North eastern and Northern regions of Thailand based on *Galanthus nivalis* agglutinin-related lectins and internal transcribed spacer regions of ribosomal DNA. *Pak. J. Bot.,* 46(5): 1561-1572.

Snow, R. 1985. Improvements in methods for the germination of orchid seeds. *Am. Orchid Soc. Bull*. 54: 178-181.

Tawaro, S., P. Suraninpong and S. Chanprame. 2008. Germination and regeneration of *Cymbidium findlaysonianum* Lindl. on a medium supplemented with some organic sources, *Walailak J. Sci. Tech*. 5(2): 125-135.

Vacin, E. and F. Went . 1949. Some pH change in nutrient solution. *Bot. Gar. Conserv. News*, 110: 605-613.

Vaddhanaphuti, N. and G. Seidenfaden. 2005. A field guide to the wild orchids of Thailand**,** Published by Silkworm Books, Chiang Mai, p. 1-436.

Yukawa, T. and W.L. Stern. 2002. Comparative vegetative anatomy and systematics of *Cymbidium* (Cymbidieae: Orchidaceae). *Bot. J. Linn. Soc*., 138: 383-419.

(Received for publication 24 December 2014)