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Study of *in vitro* seed germination and micropropagation of an orchid *Cymbidium cyperifolium* Wall. of Bangladesh

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Abstract
A protocol for *in vitro* germination and micropropagation of *Cymbidium cyperifolium* Wall. is an ornamental orchid species of Bangladesh, has been developed and PM basal medium was best for seeds germination than MS, KC, MVW media correspondingly. Sucrose was better for germination as source of carbohydrate than glucose and lactose. Maximum elongation of seedlings was recorded on liquid MS medium with 0.5 mg/l IAA + 1.0 mg/l BAP (4.48 ± 0.13cm) followed by agar solidified MS + 0.5 mg/l NAA + 1.0 mg/l BAP (3.31 ± 0.14cm) medium. Nodal segments directly produced highest average number of multiple shoot buds *via* organogenesis sprouted on MS + 1.0 mg/l IBA + 2.0 mg/l BAP (5.89 ± 0.35/segment) medium followed by MS + 1.0 mg/l NAA + 2.0 mg/l BAP (5.43 ± 0.36/segment) medium. Leaf segments underwent proliferation *via* embryogenesis and produced maximum percent of greenish PLBs on MS medium + 1.0 mg/l IBA + 2.0 mg/l Kn followed by MS + 1.0 mg/l Pic + 2.0 mg/l Kn. Highest mean increased individual shoot bud length was achieved on liquid MS with 0.5 mg/l IAA + 1.0 mg/l BAP (3.57 ± 0.15cm) followed by agar solidified MS with 1.0 mg/l Pic + 1.0 mg/l BAP (3.33 ± 0.12cm) medium. Full strength MS media supplemented with 0.5 mg/l NAA was the ideal condition for root formation followed by half strength MS0 medium. The highest number of SPSs formation took place in liquid MS + 0.5 mg/l IAA + 1.0 mg/l BAP followed by agar solidified PM + 1.0 mg/l Pic + 1.0 mg/l BAP medium. Rooted plantlets were able to grow in *ex vitro* condition after a short period of acclimatization.

Keywords: *Cymbidium cyperifolium*; *in vitro* germination; MSBs; PLBs; SPSs

1. Introduction

Orchids are economically important group of plants for their various uses in floriculture, medicine and food industries. The rich diversity and population of orchids in the world is decreasing due to habitat destruction, indiscriminate collections by orchid lovers and over harvesting of selected orchids for commercial trade. Tissue culture technique has been widely used for *in vitro* mass propagation of commercially important orchids [1, 2]. Highly exploited species immediately require *ex situ* conservation [3].

The genus *Cymbidium* comprises 70 species [4] world wide of which 5 species are reported in Bangladesh [5]. *Cymbidium cyperifolium* is a terrestrial herb species flowered at October to January [6, 7] and inflorescence of this orchid looks very attractive, so it has good horticultural value. It is found in Nepal, North East India, Bhutan, China, Myanmar, Thailand, Cambodia and Philippines [8].

In nature, orchid seed germination is very slow due to lack of endosperms and the seeds do not germinate in adequate percentage whereas, a single orchid capsule/pod contains millions of seeds. This problem may be overcome by adopting *in vitro* tissue culture technique [9, 10]. The *Cymbidiums* are conventionally propagated through separation of pseudobulbs, but the proliferation rate is very low. More efficient approach is *in vitro* seed culture and different media compositions has significant role to initiate protocorms that develop into plantlets. For mass multiplication, regeneration from tissue cultured explants is superior to seed culture due to year-round availability of plant materials and an exponential propagation rate. During the last few years, tissue culture technique has been extensively exploited for the large-scale propagation as well as *ex situ* conservation of *Cymbidiums* [3, 11-16].

Micropropagation of orchid could be done with the use of aseptically grown *in vitro* seedlings. In fact, the discovery of tissue culture techniques added a new dimension to the production of quality plants in large quantities and propagation of exquisite and rare orchids [17-23]. However, very little work has been done of this species. Therefore, in the present study was undertaken to develop effective *in vitro* germination, micropropagation based nodal and leaf culture technique and establishment of SPSs development protocol for the conservation of *Cymbidium cyperifolium* orchid.

2. Materials and Methods

The green mature capsules of *Cymbidium cyperifolium* were collected from National Botanic Garden, Mirpur, Dhaka, Bangladesh. The capsules were washed with tap water to remove the external dust particles. Then added few drops of teepol solution for few min and washed under running tap water for 10 min. The capsule was surface sterilized by immersing it in the solution of 0.1 % (w/v) $HgCl_2$ for 10 min, 70% (v/v) ethanol for 30 sec and finally by rinsing three times with sterile distilled water.

Full strength KC [24], MS [25], MVW [26] and PM [27] basal media with different carbohydrates sources sucrose, lactose, glucose were used for *in vitro* seed germination. Eighteen types of MS and PM based solid and liquid condition with different concentrations and combinations of PGRs elongation media were prepared. In solid media 0.8% (w/v) agar was used as gelling agent but no agar was added in liquid media. Sixteen types of micropropagation media were prepared using MS basal media with different

concentrations and combinations of PGRs. Half strength MS and nine types of full-strength auxin supplemented MS media were prepared for well-developed rooting. Plant Growth Regulators (PGRs) *viz*. Kn, BAP, Pic, NAA, IAA and IBA are freshly prepared. The pH of all media was adjusted to 5.8 with 0.1N NaOH or HCl before autoclaving. Agar was dissolved by boiling the mixture and about 50-100 ml media was dispensed into each culture vessel and autoclaved at 121ºC for 20 min/15 lb. Culture was maintained in the growth room at 14/10h continuous light and dark conditions at 25±2 ºC. The cultures were monitored regularly and the data was recorded.

For the inoculation of seeds, green capsule was cut opened longitudinally and the seeds were scooped out with the help of forceps and spread over the surface of the germination media. The seeds were allowed to germinate and differentiate into protocorms and seedling development. Nodal and leaf were excised from *in vitro* grown seedlings and cultured on the PGRs supplemented agar solidified medium. Nodal segments produced directly multiple shoot buds (MSBs) *via* organogenesis but leaf segments proliferated and produced Protocorm Like Bodies (PLBs). MSBs and PLBs were subcultured on the elongation media and thereafter in rooting media for induction of well-developed root system. Huge quantities of SPSs were produced at the basal zone of some culture vessels. These SPSs were then subcultured on elongation and rooting media respectively. These SPSs were taken as test materials for conservation. 0.8% (w/v) agar solidified half strength MS0 and auxin supplemented (IAA, IBA, NAA) nine different MS media were used for induction of strong and stout root system. The efficiency of the media in terms of enhancing the development of root system was assessed based on the increase in number and length of roots that developed within 30d of culture in rooting media.

Rooted *in vitro* developed seedlings were taken out of the culture vessel and transferred to outside the culture room by successive phases of acclimatization. Then the seedlings of *C. cyperifolium* were transferred to pots containing soil, sand, pit moss, saw dust and charcoal. Transplanted seedlings were watered regularly for about 2-3 months.

3. Results and Discussions

The seeds of *Cymbidium cyperifolium* were aseptically grown on 0.8% (w/v) agar solidified KC, MS, MVW and PM media with three different sources of carbohydrates *viz.* sucrose, glucose and lactose (Table-1). The overall results show that PM (80%) was superior to MS, KC, MVW media in respect of required time and the percentage of germination (Fig. 1a). The germination was significantly impaired on lactose containing MVW (20%) medium. Each plant species has specific nutritional requirements, for its seed germination and plant regeneration. Similar result was also found in *Cymbidium bicolor* [28]; *Cymbidium aloifolium* [29] and *Calanthe densiflora* [23] orchid species. The germination behaviour of different species of *Cymbidiums* was investigated using various basal media [30-31]. PM media is enriched with vitamins and organic additives which are enhanced for seed germination and seedling development of many orchids [32]. Peptones has significant role as excellent natural sources of amino acids, peptides and proteins in growth media. Peptone in media enhances the germination rate and also favours the healthy protocorm development. Carbon source has also great role for *in vitro* orchid seed germination. Sugar is an important component used in tissue culture studies. Among three carbon sources, the percentage of seed germination was higher in sucrose containing medium than glucose and lactose containing media respectively.

For continued further growth, germinated seedlings were transferred to elongation media (Table-2). 0.8% (w/v) agar solidified & liquid MS and PM elongation media supplemented with different concentrations and combinations of PGRs (BAP, Kn, NAA, IAA, IBA and Pic.) were used for enhancing elongation of seedlings. The efficiency of a medium in terms of enhancing shoot elongation was determined based on the increase in length of shoot system within 30d of culture. The highest rate of elongation, mean increased seedlings length took place in liquid MS medium with 0.5 mg/l IAA + 1.0 mg/l BAP (4.48 ± 0.13 cm; Fig. 1b) followed by 0.8% (w/v) agar solidified MS medium supplemented with 0.5 mg/l NAA + 1.0 mg/l BAP (3.31 ± 0.14 cm; Fig. 1c). Comparison of the results of liquid and solid media revealed that liquid culture was better than agar solidified condition and MS better than PM for enhancing elongation of shoot system of the seedlings [33-35].

Table 1: *In vitro* germination of seeds of *Cymbidium cyperifolium*.

Nutrient medium	Carbohydrate source with concentration (w/v)	Number of culture vessels used	Number of culture vessels in which seeds germinated		Time (d) required for germination	Remarks
			No.	%		
KC	2% glucose	10	03	30	35 - 40	Yellowish green PLBs
	2% lactose	10	02	20	42 - 45	Whitish Green PLBs
	2% sucrose	10	05	50	34 - 38	Deep green PLBs
MS	3% glucose	10	05	50	32 - 36	Greenish PLBs
	3% lactose	10	03	30	35 - 40	Whitish green PLBs
	3% sucrose	10	06	60	35 - 38	Green PLBs
PM	2% glucose	10	06	60	36 - 40	Yellowish green PLBs
	2% lactose	10	04	40	38 - 42	Brownish green PLBs
	2% sucrose	10	08	80	35 - 38	Green PLBs
MVW	2% glucose	10	03	30	35 - 40	Yellowish green PLBs
	2% lactose	10	02	20	45 - 48	Whitish Green PLBs
	2% sucrose	10	05	50	36 - 40	Green PLBs

In vitro derived nodal and leaf explants were used for rapid micropropagation [36-37]. The nodal explants were cultured on 0.8% (w/v) agar solidified MS media supplemented with different combinations and concentrations of PGRs and produced multiple shoot buds *via* direct organogenesis

(Table-3). The nodal segments of *C. cyperifolium* underwent direct organogenesis and maximum number of shoot buds were produced on 0.8% (w/v) agar solidified MS medium supplemented with 3% (w/v) sucrose + 1.0 mg/l IBA + 2.0 mg/l BAP (5.89 ± 0.35/segment; Fig. 1d) followed by that on MS + 3% (w/v) sucrose + 1.0 mg/l NAA + 2.0 mg/l BAP (5.43 ± 0.36/segment). Leaf segments underwent proliferation and produced seedlings *via* PLBs and maximum percent of greenish PLBs were produced on MS medium fortified with 3% (w/v) sucrose + 1.0 mg/l IBA + 2.0 mg/l Kn (Fig. 1e) followed by MS + 3% (w/v) sucrose + 1.0 mg/l Pic + 2.0 mg/l Kn. Similarly, different explants have been investigated for the clonal mass propagation of *Cymbidium* species [12, 14].

With combinations of auxins and cytokinins was more effective for SPSs development at the base of the seedlings and liquid media more effective than agar solidified condition. The highest number of SPSs formation took place in liquid MS + 0.5 mg/l Pic + 1.0 mg/l BAP

Table 2: Mean increased in length (cm) and SPSs production per seed originated and shoot bud originated seedlings of *C. cyperifolium* on 0.8% (w/v) agar solidified and liquid media with different kinds of PGRs.

Culture medium with different combinations and concentrations of PGRs	Solid media			Liquid media		
	Mean increased seedlings length (cm) after 30d of culture ± SE	Time required (days) for SPSs production and SPSs colour	Mean increased individual shoot bud length (cm) after 30d of culture ± SE	Mean increased seedlings length (cm) after 30d of culture ± SE	Time required (days) for SPSs production and SPSs colour	Mean increased individual shoot bud length (cm) after 30d of culture ± SE
MS + 3% (w/v) sucrose + 1.0 mg/l IAA + 0.5 mg/l BAP	2.56±0.21	—	2.42±0.17	3.20±0.14	—	2.84± 0.12
MS + 3% (w/v) sucrose + 0.5 mg/l IAA + 1.0 mg/l BAP	3.08±0.18	GS	3.04±0.14	4.48±0.13	GS	3.28±0.16
MS + 3% (w/v) sucrose + 1.0 mg/l IAA + 1.0 mg/l BAP	3.12±0.13	—	3.21±0.20	4.36±0.20	—	3.47±0.13
MS + 3% (w/v) sucrose + 1.0 mg/l NAA + 0.5 mg/l BAP	2.75±0.17	—	2.80±0.18	3.08±0.21	—	2.88±0.18
MS + 3% (w/v) sucrose + 0.5 mg/l NAA + 1.0 mg/l BAP	3.31±0.14	GS	3.33±0.12	4.41±0.17	GS	3.39±0.11
MS + 3% (w/v) sucrose + 1.0 mg/l NAA + 1.0 mg/l BAP	3.25±0.16	—	3.18±0.15	4.13±0.19	GS	3.57±0.15
MS + 3% (w/v) sucrose + 1.0 mg/l Pic + 0.5 mg/l BAP	2.88±0.11	—	2.72±0.13	3.72±0.11	—	2.72±0.10
MS + 3% (w/v) sucrose + 0.5 mg/l Pic + 1.0 mg/l BAP	3.14±0.15	GS	3.04±0.19	4.18±0.13	GS	3.17±0.19
MS + 3% (w/v) sucrose + 1.0 mg/l Pic + 1.0 mg/l BAP	3.26±0.12	—	3.26±0.21	4.23±0.15	GS	3.35±0.17
PM + 2% (w/v) sucrose + 1.0 mg/l IAA + 0.5 mg/l BAP	3.01±0.18	—	3.01±0.14	3.87±0.18	—	2.87±0.13
PM + 2% (w/v) sucrose + 0.5 mg/l IAA + 1.0 mg/l BAP	3.18±0.13	YS	3.27±0.13	4.41±0.12	YS	3.45±0.15
PM + 2% (w/v) sucrose + 1.0 mg/l IAA + 1.0 mg/l BAP	3.12±0.20	—	3.14±0.10	4.35±0.14	—	3.31±0.12
PM + 2% (w/v) sucrose + 1.0 mg/l NAA + 0.5 mg/l BAP	2.96±0.21	—	2.91±0.12	3.82±0.17	—	2.87±0.11
PM + 2% (w/v) sucrose + 0.5 mg/l NAA + 1.0 mg/l BAP	3.28±0.19	GS	3.24±0.17	4.11±0.16	GS	3.18±0.10
PM + 2% (w/v) sucrose + 1.0 mg/l NAA + 1.0 mg/l BAP	3.12±0.14	—	3.11±0.15	4.32±0.10	GS	3.42±0.19
PM + 2% (w/v) sucrose + 1.0 mg/l Pic + 0.5 mg/l BAP	2.81±0.12	—	2.85±0.11	3.94±0.14	—	2.97±0.18
PM + 2% (w/v) sucrose + 0.5 mg/l Pic + 1.0 mg/l BAP	3.24±0.18	GS	3.31±0.18	4.41±0.12	GS	3.24±0.13
PM + 2% (w/v) sucrose + 1.0 mg/l Pic + 1.0 mg/l BAP	3.12±0.15	—	3.17±0.13	4.25±0.15	GS	3.51±0.12

Shoot length recorded from 50 seedlings/shoot bud taking 5 at random from each of 10 culture vessels; GS=Greenish SPSs; YS=Yellowish SPSs; '—' Indicates no response.

Table 3: Development of multiple shoot buds/ PLBs from nodal and leaf explants of *C. cyperifolium* when grown on 0.8% (w/v) agar solidified MS media supplemented with different PGRs.

Combinations and concentrations of PGRs	Explants	% of induced multiple shoot buds/ PLBs per segment	Time (d) required for sprouting of multiple shoot buds/ PLBs	Number of multiple shoot buds/ PLBs produced per segment (Mean ± S.E.)
0.5 mg/l IAA + 1.0 mg/l BAP	NS	40	34 - 38	3.06 ± 0.21
	LS	25	65 - 70	Green PLBs

0.5 mg/l IAA + 1.0 mg/l Kn	NS	35	35 - 40	2.88 ± 0.22
	LS	35	55 - 60	Green PLBs
1.0 mg/l IAA + 2.0 mg/l BAP	NS	50	32 - 35	4.12 ± 0.29
	LS	30	60 - 65	Green PLBs
1.0 mg/l IAA + 2.0 mg/l Kn	NS	45	30 - 35	3.78 ± 0.25
	LS	45	52 -56	Green PLBs
0.5 mg/l IBA + 1.0 mg/l BAP	NS	50	33 - 36	4.19 ± 0.26
	LS	20	65 - 70	Yellowish PLBs
0.5 mg/l IBA + 1.0 mg/l Kn	NS	45	30 - 34	3.66 ± 0.21
	LS	40	55 - 60	Yellowish PLBs
1.0 mg/l IBA + 2.0 mg/l BAP	NS	70	28 - 32	5.89 ± 0.35
	LS	25	60 - 65	Yellowish PLBs
1.0 mg/l IBA + 2.0 mg/l Kn	NS	60	28 - 32	5.24 ± 0.29
	LS	55	48 - 52	Green PLBs
0.5 mg/l NAA + 1.0 mg/l BAP	NS	45	30 - 35	3.83 ± 0.22
	LS	25	65 - 70	Green PLBs
0.5 mg/l NAA + 1.0 mg/l Kn	NS	40	34 - 38	3.16 ± 0.19
	LS	25	65 - 70	Green PLBs
1.0 mg/l NAA + 2.0 mg/l BAP	NS	65	30 - 34	5.43 ± 0.36
	LS	30	60 - 65	Green PLBs
1.0 mg/l NAA + 2.0 mg/l Kn	NS	50	32 -36	4.25 ± 0.28
	LS	30	60 - 65	Green PLBs
0.5 mg/l Pic + 1.0 mg/l BAP	NS	45	30 - 35	3.76 ± 0.26
	LS	25	65 - 70	Green PLBs
0.5 mg/l Pic + 1.0 mg/l Kn	NS	45	32 - 36	3.82 ± 0.29
	LS	40	53 - 56	Green PLBs
1.0 mg/l Pic + 2.0 mg/l BAP	NS	55	30 - 32	4.93 ± 0.35
	LS	40	55 - 60	Yellowish PLBs
1.0 mg/l Pic + 2.0 mg/l Kn	NS	50	32 - 34	4.17 ± 0.27
	LS	50	50 - 55	Green PLBs

Based on observations recorded from 10 cultured segments in each medium; NS= Nodal Segment; LS= Leaf Segment.

Fig 1: *In vitro* seed germination, elongation, production, micropropagation, SPSs development, rooting and acclimatization of *Cymbidium cyperifolium*.

(Fig. 1f) and agar solidified PM + 0.5 mg/l NAA + 1.0 mg/l BAP. Most of the SPSs were greenish and few were yellowish. MS based media was better than PM based media for induction of SPSs [38, 39]. The MSBs and PLBs were further grown individually on elongation media (Table-2). The highest mean increased individual shoot bud length was achieved on liquid MS + 1.0 mg/l NAA + 1.0 mg/l BAP (3.57 ± 0.15 cm) followed by agar solidified MS + 0.5 mg/l NAA + 1.0 mg/l BAP medium (3.33 ± 0.12 cm; Fig. 1g).

The elongated Shoot buds and seed originated seedlings produced roots in elongation media but those were weak and few in number. Half strength MS0 and nine different types of PGRs (IAA, IBA, NAA) supplemented MS media were used for induction of strong and stout root system (Table-4). Increased in length as well as the number of roots developed seed originated and shoot bud derived seedlings were more on MS medium supplemented with 3% (w/v) sucrose + 0.5 mg/l NAA containing rooting medium (Fig. 1h) followed by half strength MS0 medium with 1.5% (w/v) sucrose. Similar result was found in *Esmeralda clarkei* and *Coelogyne fuscescens* respectively [40, 41]. IBA was effective for rooting in *Dendrobium* orchid and *Ilex khasiana* respectively [42, 43]. The opposite result was also noted that IAA was most appropriate in inducing roots in *Dendrobium* hybrid and *Dendrobium thyrsiflorum* [44, 45]. Combine effect of IAA, IBA or NAA induced excellent rooting response in *Vanda tessellata* and *Aerides ringens* [46, 47] orchid species.

Table 4: Mean increased in length (cm) and number of roots per seed originated and shoot bud derived seedlings of *C. cyperifolium* in ½ MS0 and auxin supplemented MS rooting media.

Culture medium			Average increased length and number of roots per seed derived seedling		Average increased length and number of roots per shoot bud	
			Mean length (cm) ± S.E.	Mean no. of roots/ seedling ± S.E.	Mean length (cm) ± S.E.	Mean no. of roots/ shoot bud ± S.E.
½ MS0			3.86 ± 0.18	2.35 ± 0.16	4.17 ± 0.20	2.48 ± 0.16
Auxin (mg/l)	IAA	0.5	3.12 ± 0.20	1.71 ± 0.11	3.02 ± 0.21	1.24 ± 0.14
		1.0	3.47 ± 0.24	2.03 ± 0.15	3.38 ± 0.20	1.91 ± 0.11
		1.5	3.83 ± 0.21	2.28 ± 0.12	4.01 ± 0.23	2.37 ± 0.15
	IBA	0.5	2.04 ± 0.12	1.16 ± 0.12	2.37 ± 0.19	1.18 ± 0.09
		1.0	3.21 ± 0.19	1.65 ± 0.11	3.86 ± 0.22	1.92 ± 0.10
		1.5	3.72 ± 0.22	2.08 ± 0.13	4.13 ± 0.24	2.25 ± 0.14
	NAA	0.5	4.32 ± 0.21	2.48 ± 0.14	4.69 ± 0.22	2.63 ± 0.13
		1.0	3.27 ± 0.20	2.15 ± 0.15	4.11 ± 0.24	2.34 ± 0.14
		1.5	2.46 ± 0.14	1.68 ± 0.17	3.14 ± 0.17	1.96 ± 0.11

Based on observations from 50 seedlings/ shoot buds taking five at random from each of ten culture vessels.

The *in vitro* derived seedlings and micropropagated plantlets were acclimatized at room temperature. The seedlings of *C. cyperifolium* were transferred to pots containing soil, sand, pit moss, saw dust and charcoal. Rooted plantlets were able to grow into normal plantlets in *ex vitro* condition after a short period of acclimatization (Fig. 1i) and transplanted seedlings were watered regularly for about 2-3 months.

4. Conclusions

PM media was found superior than MS, KC, MVW media respectively and sucrose supplemented media was better than glucose & lactose for promoting germination of this orchid seeds. 2% lactose supplemented MVW medium was found to be ineffective for germination. For comparing the efficiency in terms of enhancing seedling, liquid culture was better in terms of promoting shoot elongation than solidify condition. Increased in root length and number of roots is higher in NAA supplemented MS medium than IAA, IBA respectively for induction of well-developed root system. *Ex situ* conservation by tissue culture technique of this species is highly recommended. Mass propagation using nodal and leaf culture can be started in commercial scale to conserve this species in their natural habitat.

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