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Protocorm Regeneration, Multiple Shoot Induction and *ex vitro* Establishment of *Cymbidium devonianum* Paxt.

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Abstract: *Cymbidium devonianum* Paxt., an epiphytic orchid of considerable ornamental and horticultural importance is an extremely rare and threatened orchid of Northeast India. An efficient method of propagation has been developed via protocorm regeneration from seeds. Seeds obtained from capsules of *C. devonianum* collected 9 months after artificial pollination developed into protocorms on basal media viz., Murashige and Skoog (MS), Gamborg *et al.* (B_5) and Mitra *et al.* Protocorm formation varied in different media. Maximum number (92.8%) of protocorms was observed in B_5 medium. The development of protocorm was also faster (48.0 days) in the same medium. However, in MS medium further development of protocorms into seedlings was faster. Seedlings obtained from protocorms showed optimal fresh weight (0.33 g) and shoot number (5.1 plantlet^{-1}) in MS + 1.0 mg L^{-1} of N^6-benzylaminopurine (BAP) + 1.0 mg L^{-1} indole-3-acetic acid (IAA); shoot length (2.9 cm plantlet^{-1}) and root number (3.9 plantlet^{-1}) in MS + 1.0 mg L^{-1} BAP + 0.5 mg L^{-1} IAA and root length (3.6 cm plantlet^{-1}) in MS + 1.0 mg L^{-1} of IAA. Ninety percent of hardened *C. devonianum* plantlets survived on substratum containing brick, charcoal, decaying litter (1:1:1) with a layer of moss on top. The given protocol of successful protocorm regeneration should permit rapid propagation and conservation of this rare and threatened *Cymbidium* species.

Key words: Conservation, *in vitro*, protocorm, regeneration, threatened

INTRODUCTION

Protocorm regeneration from seeds has become the favoured method for mass production of orchids. Most epiphytes are produced in this way. Though double fertilization in orchids is undertaken as in other angiosperms, however the endosperm fails to develop. The seeds of all orchids have to be nurtured by their specific mycorrhizal fungi in the initial stages of development because of which less than 5% of the orchid seeds germinate in nature (Rao, 1977). On the other hand, mass production of orchids could be achieved asymbiotically in flasks or test tubes. Seedling production *in vitro* and further development is also an effective means of saving many orchid species from extinction. *Cymbidium devonianum* is highly ornamental and has horticultural importance. In spite of its wide distribution earlier, it is now restricted to narrow pockets of Northeast India mainly because of its inability to withstand habitat destruction and overexploitation. This has resulted in its extremely rare and threatened status (Chowdhery, 2001). Hence, an effective strategy becomes essential to salvage and multiply this species for effective conservation as well as horticultural exploitation. Plant tissue culture offers opportunities to conserve and multiply threatened and overexploited plant species (Seeni and Latha, 2000; Decruse *et al.*, 2003). The present investigation on *C. devonianum* was carried out with the following objectives: to standardize conditions for protocorm regeneration from seeds; to accomplish seedling growth and development and to achieve *ex vitro* establishment of plantlets.

MATERIALS AND METHODS

The experiments were carried out in the Plant Biotechnology Laboratory, Department of Botany, North Eastern Hill University, Shillong, India, during November 2003-December 2005. Capsules collected from hand pollinated plants after 9 months of pollination were surface disinfected in 70% ethanol for 30 sec followed by surface flaming. This process was repeated three times and finally the capsules were rinsed five times with sterile distilled water. After sterilization the capsules were dried and dissected longitudinally with a surgical blade in laminar airflow cabinet. The seeds were scooped out from

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sterilized capsules and sown by spreading as thinly as possible over the surface of the culture medium in 25×150 mm glass test tubes (approximately 10 test tubes per capsule and one capsule for each treatment), each tube containing 10 mL of media viz., Murashige and Skoog (1962), Gamborg *et al.* (B_5, 1968) and Mitra *et al.* (1976). The pH of the media was adjusted to 5.8±0.1 with 1 N NaOH or HCl prior to autoclaving at 121°C at 1.06 kg cm^{-2} for 15 min. The culture tubes were incubated at 24±2°C under 16 h photoperiod of 150 μmol m^{-2} sec^{-1} light intensity. Seeds developed into protocorms on emergence of embryo from the testa. The inoculated seeds were examined daily. The number of protocorms was recorded after 60 days inoculation of seeds whereas other growth parameters (viz. number of protocorms with vegetative apex, number of protocorms with 2-3 leaves and number of seedlings with 1-2 roots) were recorded at 30 days of time interval from each stage. Averages of five microscopic fields were observed for recording the number of protocorms in each tube. Similarly, average of 10 test tubes was taken for each treatment.

To study the nutritional requirements of the developing seedlings 0.5-0.8 cm sized seedlings of *C. devonianum* devoid of roots (developed from protocorms on MS medium) were subcultured on MS medium supplemented with growth regulators. 6-benzyl amino purine (BAP) was used at concentrations of 0.0, 0.5, 1.0, 2.5 and 5.0 mg L^{-1} individually and in combination with indole-3-acetic acid (IAA) at 0.0. 0.5, 1.0, 2.5 and 5.0 mg L^{-1}. Five seedlings were cultured in each test tube. Observations of seedlings were made after 90 days of culture. Different growth parameters viz., fresh weight, shoot number and length and root number and length were recorded. Five replicates of each treatment were taken and the experiments were repeated thrice. Rooted plantlets measuring 2.5-3.0 cm in height were removed from the culture tubes/flasks and hardened in clean thermocol pots of 8 cm diameter containing different mixtures of compost viz., (i) brick and charcoal pieces (1:1), (ii) brick, charcoal and decaying litter (1:1:1), (iii) brick, charcoal, decaying litter and saw dust (1:1:1:1), (iv) brick, charcoal, decaying litter and cowdung (1:1:1:1:1), (v) brick, charcoal, decaying litter and coconut husks (1:1:1:1). All the substrata used were covered with a layer of sphagnum moss. The plantlets were watered alternately in the evening and fed with MS nutrient salt solutions (diluted 10 times) fortnightly for about a month. Readings were recorded after 90 days of hardening. Twenty plantlets were raised for each treatment, which were replicated thrice. Data were analyzed using one way Analysis of Variance (ANOVA) and comparisons between the mean values of treatments were made by the Least Significant Difference (LSD) test (Snecodor and Cochran, 1989).

RESULTS AND DISCUSSION

In orchids, protocorm development takes place in a sequence. Initially the immature embryo of *C. devonianum* was elongated and transparent white in colour with covered testa (Fig. 1a). On being cultured onto the media the seeds turned green and formed swollen structures called protocorms within 5-9 weeks time (Fig. 1b). Protocorm development of *C. devonianum* was observed on all media devoid of plant growth regulators. The maximum germination percentage (92.8%) of protocorms recorded was significantly high on B_5 medium followed by MS medium (67.3%) and least (28.8%) in Mitra medium (Table 1). However, further growth and development of protocorms was optimal in MS medium (Table 1 and Fig. 1c). The protocorms on Mitra medium did not develop beyond vegetative apex stage. These protocorms turned brown after 130 days of culture and died subsequently. The initial morphogenetic response of embryo into swollen structure was faster on B_5 medium (36.6 days). Further development of seeds into protocorms was also rapid in B_5 medium (11.4 days) but was delayed in MS and Mitra media by 24 and 27 days respectively. On the other hand, the time taken for initiation of vegetative apex in the protocorms was significantly rapid in MS medium (14.3 days) as compared to B_5 medium which was delayed by 36.6 days. Subsequent development of seedlings with 2-3 leaves and roots was also faster in MS medium (Fig. 1d and e). That is, the developments of different stages in *C. devonianum* cultures were dependent on the media used. The variation in nutritional requirements of germinating orchid seeds has been reported by Yam and Weatherhead (1988).

Table 1: Effect of culture medium on protocorm regeneration of *Cymbidium devonianum*

Media	No. of protocorms (%)	Protocorms with vegetative apex (%)	Protocorms with 2-3 leaves (%)	Seedlings with 1-2 roots (%)	Days taken for stages				
					I	II	III	IV	V
MS	67.3	63.7	62.7	61.3	50.0	74.0	88.3	104.3	119.3
B_5	92.8	52.7	43.5	41.8	36.6	48.0	84.6	118.0	144.0
Mitra	28.8	7.8	-	-	63.3	83.3	124.3	-	-
$LSD_{(0.05)}$	15.56	5.58	9.75	9.29	6.06	7.59	8.96	7.74	14.6

Stage I-embryo slightly swollen, covered with testa; Stage II-embryo swollen forming protocorms without testa; Stage III-young protocorms with pointed vegetative apex; Stage IV-protocorms with 2-3 leaves; Stage V-seedlings with 1-2 roots

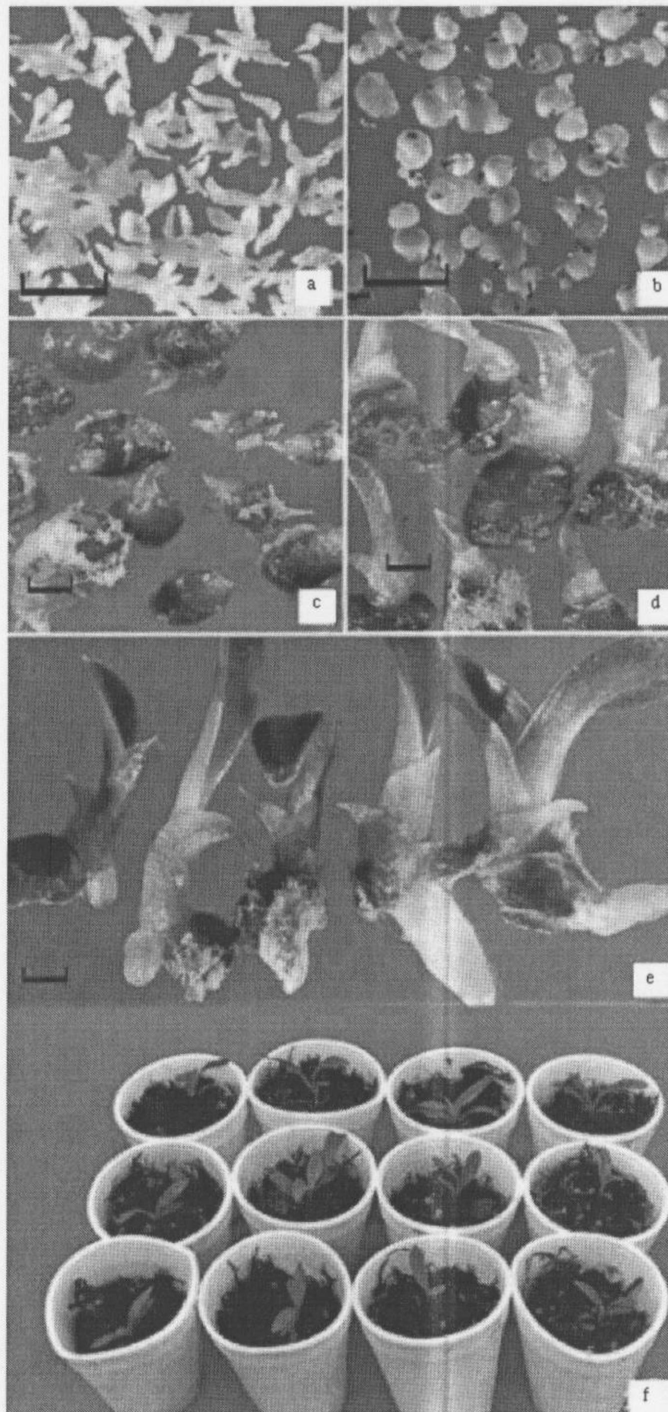

Fig. 1: Different stages of development of *C. devonianum* seeds *in vitro* (a-e) Seeds covered with testa at 0 day (bar = 1.2 cm) (a). Protocorm development after 50 days of culture on B_5 medium (bar = 1.2 cm) (b). Protocorms with pointed vegetative apex after 90 days on MS medium (bar = 0.8 cm) (c). with 2-3 leaves at 110 days on MS medium (bar = 0.8 cm) (d). and 1-2 roots at 125 days on MS medium (bar = 0.8 cm) (e). Well established hardened plantlets of *C. devonianum* after 90 days of hardening in brick + charcoal + decaying litter (1:1:1) + moss (f)

The morphogenetic response of the cultured seedlings of *C. devonianum* to cytokinin and/or auxin in MS medium is shown in Table 2. Incorporation of auxin (IAA) and cytokinin (BAP) singly and in combination in the medium showed varying responses in terms of fresh weight, shoot number and length, root number and length. The present study showed significant influence of cytokinins on number of shoots. Of the various concentrations of BAP tested in MS medium, maximum number of shoots (4.4 plantlet^{-1}) was recorded on 1.0 mg L^{-1} BAP. Latha (1999) has also reported that addition of 1.0 mg L^{-1} BAP in medium is effective in inducing higher frequency of shoot formation in *Habenaria crinifera*. Besides, induction of maximum number of shoots in response to low concentration of BAP in the medium is also reported in some species of *Cymbidium* and *Cattleya* (Nagaraju *et al*., 2003). Higher concentration of BAP in the medium led to a decrease in the number of shoots. The development of roots was completely suppressed at higher concentrations of BAP in the medium. Shimura and Koda (2004) also reported the level of BAP to be crucial for vegetative growth in *Cymbidium* species. Addition of 1.0 mg L^{-1} IAA singly in the medium showed significant response only on root number. Auxins have been reported to enhance root formation in plants (Bhojwani and Razdan, 1983). Statistical analysis showed that the other growth parameters studied had no significant effect on different concentrations of IAA. The interacting effects of cytokinin and auxin on shoot/root balance in *C. devonianum* were varying. The increase in fresh weight (0.33 g) and root number (3.9 plantlets^{-1}) recorded in MS medium supplemented with 1.0 mg L^{-1} BAP + 1.0 mg L^{-1} IAA and 1.0 mg L^{-1} BAP + 0.5 mg L^{-1} IAA, respectively were not significant. However, seedling length and shoot number were significantly influenced by different combinations of BAP and NAA in the medium. Maximum shoot length (2.9 cm plantlets^{-1}) and shoot number (5.1 plantlets^{-1}) of the seedlings were recorded in medium supplemented with 1.0 mg L^{-1} BAP + 0.5 mg L^{-1} IAA and 1.0 mg L^{-1} BAP + 1.0 mg L^{-1} IAA, respectively. Combinations of all concentrations of BAP and IAA had no effect on root length and significantly decreased with increase in concentration of either BAP or IAA in the medium.

The different composts used for hardening of *in vitro* grown plantlets of *C. devonianum* were found to be satisfactory for survivability and normal growth of the plantlets. However, highest percentage survivability (90%) with maximum length (5.8 cm) of *C. devonianum* hardened plants was obtained on substratum containing brick + charcoal + decaying litter (1:1:1) with a layer of moss (Table 3, Fig. 1f). The layer of moss on top proved to be beneficial due to higher retention of moisture content. As reported earlier by Kumaria and Tandon (1994) in *D. fimbriatum var. oculatum*, feeding the plantlets with dilute MS nutrient salt solution was found beneficial to the developing hardened plantlets of *C. devonianum*.

Table 2: Influence of growth hormones in MS medium on seedlings of *C. devonianum* Hormones (mg L^{-1})

BAP		Fresh weight (g)	Shoot length (cm)	No. of shoots plantlet^{-1}	No. of roots plantlet^{-1}	Root length (cm)
0.0		0.28	2.1	1.8	2.6	2.2
0.5		0.24	1.1	2.3	2.4	1.4
1.0		0.21	1.1	4.4	1.3	1.2
2.5		0.18	1.0	3.4	0	0
5.0		0.07	0.9	1.2	0	0
$LSD_{(0.05)}$		0.18	0.99	0.837	1.34	1.23
ANOVA		$F_{4,10} = 1.89$ NS	$F_{4,10} = 3.35$ NS	$F_{4,10} = 23.13$**	$F_{2,6} = 3.33$ NS	$F_{2,6} = 2.21$ NS
IAA						
0.0		0.23	2.5	1.5	2.3	2.1
0.5		0.26	2.1	1.6	3.2	2.4
1.0		0.24	1.9	3.2	3.4	3.6
2.5		0.21	1.8	1.2	2.2	2.9
5.0		1.70	1.1	1.8	2.5	1.9
$LSD_{(0.05)}$		0.12	0.73	1.05	0.99	0.88
ANOVA		$F_{4,10} = 1.06$ NS	$F_{4,10} = 3.35$ NS	$F_{4,10} = 0.39$ NS	$F_{4,10} = 4.76$*	$F_{4,10} = 3.05$ NS
BAP	IAA					
0.0	0.0	0.25	2.3	1.8	2.4	2.2
1.0	0.5	0.28	2.9	2.6	3.9	1.9
1.0	1.0	0.33	2.4	5.1	3.2	1.6
1.0	2.5	0.24	2.2	3.2	2.9	1.1
2.5	0.5	0.26	1.7	2.2	2.4	0.8
2.5	1.0	0.32	1.3	3.7	2.1	0.6
5.0	0.5	0.21	1.1	1.7	2.0	0.5
5.0	1.0	0.19	1.0	1.3	1.8	0.3
$LSD_{(0.05)}$		0.18	0.76	1.66	0.71	0.05
ANOVA		$F_{7,16} = 0.66$ NS	$F_{7,16} = 7.42$**	$F_{7,16} = 4.59$**	$F_{7,16} = 2.14$ NS	$F_{7,16} = 27.69$**

*Significant at $p<0.05$, **Significant at $p<0.01$

Table 3: *Ex vitro* establishment of *Cymbidium devonianum* plantlets after 90 days

Treatments	Survival (%)	Height (cm)
brick+charcoal (1:1)+moss	70	2.9
Brick+charcoal+decaying litter (1:1:1)+moss	90	5.8
brick+charcoal+decaying litter+saw dust (1:1:1:1)+moss	55	4.6
brick+charcoal+decaying litter+cow dung (1:1:1:1)+moss	72	3.6
brick+charcoal+decaying litter+coconut husk (1:1:1:1)+moss	45	3.7
$LSD_{(0.05)}$	14.15	2.96

This protocol of protocorm regeneration, multiple shoot formation and *ex vitro* establishment of *C. devonianum* can be effectively used for its rapid propagation and hence conservation of this rare and threatened epiphytic orchid.

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