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REGULAR ARTICLE

In vitro pseudobulb based micropropagation for mass development of *Cymbidium finlaysonianum* Lindl.

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ABSTRACT

An efficient protocol for micropropagation using the axenic pseudobulb derived from *in vitro* germinated seedlings of *Cymbidium finlaysonianum* Lindl was developed. The immature seeds were germinated on MS0 basal medium for seedling development. *In vitro* grown seedlings responded well and stimulated pseudobulb formation with benzylamino purine (BAP) 1.0 mg/L + α-naphthalene acetic acid (NAA) 0.5 mg/L on MS medium. For multiple shoot induction, the axenic pseudobulb segments excised from 8 month old seedlings were cultured on the medium containing different concentrations and combinations of growth regulators (NAA, BAP and Kinetin). Best response for shoot multiplication and elongation was obtained in medium with 0.75 mg/L NAA and 1.5 mg/L BAP. The individual shoots were transferred to half-strength MS medium supplemented with 0.5 to 2.0 mg/L of indole-3 acetic acid (IAA), indole-3 butyric acid (IBA) and α-naphthalene acetic acid (NAA) to observe root development. Half strength of MS + 1.0 mg/L IAA has proven the best for root induction. The well rooted plants were hardened successfully in the potting mixture containing coconut husk, charcoal pieces (5-7 mm size), decaying litter and very small brick pieces (5-7 mm size) in the ratio of 1:1:1:1 and eventually plants are established under natural condition. The present investigation can be used for rapid mass propagation of this highly important *Cymbidium finlaysonianum* orchid species.

Keywords: Axenic pseudobulb; *Cymbidium finlaysonianum*; Micropropagation; Multiple shoot; Plant growth regulators

INTRODUCTION

Orchids belong to the family Orchidaceae with 20,000 to 30,000 species and is the largest family of monocotyledonous that are undoubtedly recognized as economically important flowering plants in the floriculture industry (Antony et al., 2014; Godo et al., 2010). For continued reproduction in nature, orchids require a combination of multiplicity of factors. The seeds are special type and are poorly developed. Seeds lack endosperm and require a suitable fungal stimulant for germination in nature; the fungus is believed to augment the carbohydrate, auxin and vitamin transport in the orchid (Arditti et al., 1982). In nature, only 0.2 to 0.3% of seeds germinate (Bhattacharjee and Islam, 2014; Singh, 1992) even if they do so, the seeds take a long time for their germination and any disturbance in the habitat or physical environment destroys the whole population. Also the seedlings require a long period of up to 12 years to become an adult. They are highly heterozygous and their vegetative propagation through division is rather slow. This difficulty in natural population drives some of the indigenous species to extinction (Basker and Bai, 2006). So, *in vitro* cultural techniques are now adopted for quick propagation of commercially important orchid species.

Cymbidiums are one the most important and popular orchid in horticulture and they are versatile plants, marketed as cut-flowers, buttonholes and as pot plants, producing many large and long-lasting flowers (Puy and Cribb, 2007). They also reported that the genus of *Cymbidium* currently comprises some 52 species distributed throughout South and Eastern Asia, the Malay Archipelago and north and east Australia. The *C. finlaysonianum* Lindl is an epiphytic herbaceous species, up to about 90 cm tall, forming dense and wide tufts with close conical to ovoid pseudobulbs covered by the foliar bases, up to 6 cm long and 3 cm broad, with 5-7 lanceolate leaves with bilobed apex. Racemose inflorescence hanging from the base of the pseudobulb, 0.3 - 1.3 m long and carrying numerous flowers diameter is 5 - 6 cm.

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As horticultural important floriculture, *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 very important to conserve the germplasms of *Cymbidium* species.

Cymbidium finlaysonianum Lindl is a sympodial and pseudobulb orchid has long pendulous spikes carrying small yellow and red fragrant flowers (Opchat, 2000). It is well distributed in Vietnam, Cambodia, Thailand, Sumatra, Java, Borneo, Philippines and Malaysia (Davis and Steiner, 1952; Wood et al., 2011). However, due to lack of proper cultivation practices, destruction of plant habitats and illegal and indiscriminate collection of plants from natural habitats, many medicinal orchids are severely threatened and rapidly disappearing and it is now listed as vulnerable to extinction (Devendra et al., 2011). *Cymbidium finlaysonianum* is a commercially important orchid and owing to its commercial value in the floricultural industry, natural populations are under threat from over-exploitation (Davis and Steiner, 1952).

Normally orchids are propagated *in vitro* by using various explants such as meristematic shoot, leaves, roots, protocorms etc obtained from axenic cultures but the regenerative potential of pseudobulbs as explants has been less explored as compared to other explants (George and Ravishankar, 1997; Basker and Bai, 2006; Sunitibala and Kishor, 2009; Pant and Thapa, 2012). Recently, there are some reports came on the development of *in vitro* mass propagation with success using pseudobulb as explants of *Dendrobium* and *Coelogyne flaccida* Lindl (Kaur and Bhutani, 2013; Sharma, 2014). Hence, this study is one of the establishments of an efficient regeneration system on *C. finlaysonianum* using pseudobulbs as explants based on the suitable culture medium, the effect of growth regulators either single and in combination on regeneration and their subsequent development into plants.

MATERIALS AND METHODS

Plant materials

Seeds of *Cymbidium finlaysonianum* were collected from naturally pollinated plants growing in natural habitat from Universiti Sains Malaysia (USM) campus, Penang, Malaysia.

In vitro seed germination and seedling development

Under this study immature green pods/capsules were considered as seed source for *in vitro* culture. Due to non-endospermic nature of seed, the germination in nature is a unique phenomenon and requires fungal infection, which caused limiting germination. Immature pods of *Cymbidium finlaysonianum* Lindl washed under running tap water with few drops of liquid detergent and Tween-20. Then excised capsule treated with 0.2% (w/v) $HgCl_2$ solution for 7-8 minutes. After washing with sterile distilled water (4-5 times) finally dip the capsule in 70% ethanol for 10-12 seconds. Then the capsules kept at laminar air flow cabinet for 10 minutes for dried up ethanol and finally dissected longitudinally by a sterile surgical blade. For seed germination around 10-20 mg immature seeds were inoculate per petri dishes (90 cm) containing 10-12 mL of semi-solid medium such as MS (Murashige and Skoog, 1962), VW (Vacin and Went, 1949) and KC (Knudson, 1946). The pH of all media was adjusted 5.8. Then cultures were incubated at 25 ± 2°C under cool white fluorescent light with 16 hour photoperiod around 60 days. For seedlings development sub-culture were done in every second week on MS medium supplemented with different combination and concentration of NAA and BAP.

Cultures of pseudobulb segment for regeneration

The psuedobulb segments of about 1-1.5 cm were excised from eight months old plants as explants sources (Fig. 1a). Leaves or roots were removed from the segments prior to inoculation them. Single pseudobulb segment was cultured in a sterile glass vessel that contained with 25 ml semi-solid MS medium + different concentrations of BAP, NAA and Kinetin either single or in combination (Fig. 1b). The medium was solidified with 6 g/L of agar. After 45 days of culture initiation, the morphogenetic response was evaluated on the basis on shoot induction and elongation, as well as its subsequent development from the pseudobulb segments.

Developments of roots

For better induction of strong and stout root system, the elongated shoots around 5-7 cm long were considered. For efficient root induction three types of plant growth regulators *viz*. NAA, IAA and IBA (0.5, 1.0, 1.5 and 2.0 mg/L) were used with ½MS medium. Data were recorded after 45 days of culture initiation on the basis of length and number of roots. For root development the cultures were sub-cultured 2 to 3 times within 2-3 months of culture initiation on same medium described earlier. The pH for all media was adjusted 5.8 before autoclaving at 121°C, 15 lb pressure for 15 minutes.

Hardening of plants

Well rooted plants were transferred to pots after successful acclimatization. The culture vessels were kept open for one day in the culture room and then kept at room temperature for 6 hours and outside of rooms for 12 hours. Finally the seedlings were taken out from the culture vessels and rinsed with running tap water to remove agar which attached with the roots. *In vitro* regenerated rooted plantlets were successfully acclimatized into pot of 8.0 cm diameter

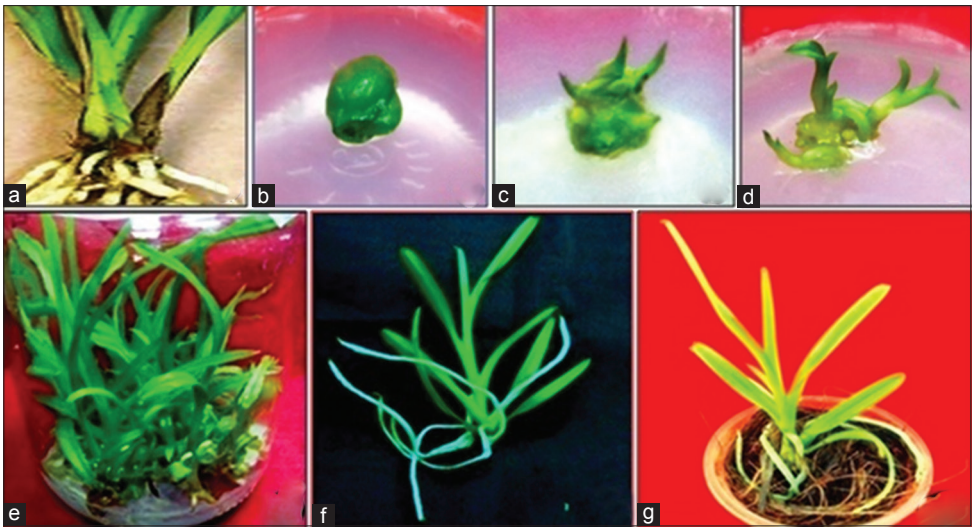

Fig 1. *In vitro* morphogenetic response of pseudobulb segments of *C. finlaysonianum*. a. Seedling with pseudobulb; b. Inoculated pseudobulb segment; c-d. Multiple shoot buds initiation and development from the cultured pseudobulb segment; e. Elongated multiple shoots; f. A well rooted hardened plant and g. Plant transferred to pot.

containing different matrix like charcoal pieces (5-7 mm size), small brick pieces (5-7 mm size each), coconut husk, decaying litter and sphagnum moss were used. The matrix were used following the mentioned combination *viz*., (i) small brick pieces 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) and (iv) brick, charcoal, decaying litter and coconut husk (1:1:1:1). All the substrate covered with a layer of 10 gm sphagnum moss. The plantlets were watered alternately in the every evening and fed with MS nutrient solutions (diluted 10 times) fortnightly for about a month and eventually established under natural condition.

Data recording and statistical analysis

The experiments were designed according to complete randomize design (CRD). For all cases ten (10) replicates were considered per treatment. The effects of different culture conditions on development and induction of multiple shoots and roots in the *in vitro* experiments was tested applying DMRT- Duncan's multiple range tests ($p \leq 0.05$) with one way analysis of variance (ANOVA). The statistical analyses were performed using SPSS programme (ver. 16, SPSS Inc., USA).

RESULTS

Under this study a comparative study on asymbiotic germination were tested with MS, ½MS, VW and KC media. Among the media tested, MS was the most suitable for seed germination where 100% germination was recorded followed by ½MS (90%), VW (85%) and KC (80%; Table 1). It might be due to the richness in micro-element regime in MS medium. For better growth, *in vitro* raised seedlings were transferred to MS medium supplemented with different concentration and combination of plant growth regulators. The pseudobulb (>3.0 cm long) segments obtained from eight months old *in vitro* cultures. Single pseudobulb were separated and cut into small segments having an average 1-2 cm length. It was observed that the frequency of multiple shoot formation differed with concentrations of BAP, NAA and Kinetin. The pseudobulb explants produced multiple shoot buds without any intervening callus or PLBs formation in culture (Fig. 1c-d). It was observed that the efficacy of multiple shoot formation differed with concentrations and combinations of BAP and NAA. For multiple shoot induction and shoot elongation when BAP and Kin were used alone with different concentration then showed varied responses. Higher concentration of BAP and Kin both at 1.5 mg/L favored shoot development. Shoot induction and elongation was significantly promoted when NAA (0.75 mg/L) added in medium with the same concentrations of BAP and Kin. After 45 days of culture initiation the combination of BAP (1.5 mg/L) and NAA (0.75 mg/L) showed highest number of shoots (10.80 ± 0.97) and lowest (1.70 ± 0.25) was recorded with lower concentration (0.5 mg/L) of kinetin (Fig. 1e and Table 2). On the other hand best shoot elongation (4.06 cm ± 0.25) was recorded on MS medium supplemented with BAP (1.5 mg/L) + NAA (0.75 mg/L) (Table 3).

In the regeneration medium, the plantlets showed poor root system (1-2/plants). Well developed roots were induced in all the regenerates treated with different auxins within 45 days of culture (Table 4). Whereas in control where half-strength of MS medium was used and not supplemented with any plant growth regulators, rooting was delayed and roots appeared after 45 days of culture. Analyzing the

Table 1: Effect of different media on *in vitro* seed germination of *Cymbidium finlaysonianum*

Media	Amount of seeds per culture vessel	Seed germination	
		DAC	% of germination
MS	200 mg	35-40	100.0
½ MS	200 mg	40-45	90.0
VW	200 mg	40-45	85.0
KC	200 mg	45-50	80.0

MS (Murashige and Skoog, 1962); VW (Vacin and Went, 1949), KC (Knudson, 1946). *DAC=Days after culture

Table 2: Effects of different PGRs with MS medium on multiple shoot buds development from pseudobulb segments

PGRs (mg/L)	Time required for sprouting	% of response	No. of shoot buds* (M±S.E)
BAP			
0.50	30-35	62.0	4.60±0.29^{c}
1.00	26-32	70.0	6.80±0.37^{e}
1.50	25-30	92.0	9.60±0.33^{f}
2.00	30-35	68.0	7.50±0.45^{e}
BAP+NAA			
0.50+0.25	26-32	62.0	6.30±0.30^{de}
1.00+0.50	26-30	70.0	7.50±0.89^{e}
1.50+0.75	25-30	90.0	10.80±0.97^{f}
2.00+1.00	25-30	84.0	9.70±0.66^{f}
KIN			
0.50	36-42	20.0	1.70±0.25^{a}
1.00	36-42	30.0	2.80±0.66^{ab}
1.50	30-35	60.0	4.70±0.34^{c}
2.00	35-40	30.0	2.80±0.73^{a,b}
KIN+NAA			
0.50+0.25	35-40	30.0	4.00±0.71^{bc}
1.0 0+0.50	35-40	36.0	4.50±0.45^{c}
1.50+0.75	30-35	48.0	4.80±0.37^{cd}
2.00+1.00	30-35	38.0	4.20±0.34^{bc}

*Based on observations from 20 segments and shoot buds. PGRs=Plant growth regulators (PGRs). Values represent M (Mean)±S.E (standard error). Values in a column with similar superscripts are not significantly different at $p \leq 0.05$ levels, Different letter(s) in a column indicate significant different at $p<0.05$ levels according to DMRT

Table 3: Effect of different concentrations and combinations of PGRs on the elongation of individual shoot

PGRs (mg/L)	Initial length (cm) Mean	Length (cm) of shoot after 45 days of culture (mean)	Increased length (cm) (M±S.E)
BAP			
0.50	2.50	5.38	2.88±0.69^{bcde}
1.00	2.80	5.95	3.15±0.33^{bcdef}
1.50	2.90	6.18	3.28±0.21^{cdef}
2.00	2.50	5.62	3.12±0.39^{bcdef}
BAP+NAA			
0.50+0.25	2.60	5.88	3.28±0.19^{cdef}
1.00+0.50	2.50	5.90	3.40±0.51^{def}
1.50+0.75	2.70	6.76	4.06±0.25^{ef}
2.00+1.00	2.50	6.30	3.80±0.37^{ef}
KIN			
0.50	2.60	4.02	1.42±0.18^{a}
1.00	2.30	4.57	2.27±0.32^{ab}
1.50	2.90	5.60	2.70±0.34^{bcd}
2.00	2.80	5.02	2.22±0.36^{ab}
KIN+NAA			
0.50+0.25	2.30	4.54	2.34±0.43^{abc}
1.00+0.50	2.50	4.78	2.28±0.31^{abc}
1.50+0.75	2.80	5.72	2.92±0.33^{bcde}
2.00+1.00	2.50	4.74	2.24±0.15^{ab}

*Values represent M (Mean)±S.E (standard error). Values in a column with similar superscripts are not significantly different at $p \leq 0.05$ levels, Different letter(s) in a column indicate significant different at $p<0.05$ levels according to DMRT

Table 4: Effects of different concentration of auxins with ½ MS medium on root development from individual shoot separated from multiple shoot cluster

PGRs (mg/L)	% of response	Initial length (cm) mean	Length (cm) of root after 45 days of culture (mean)	Increased length (cm) (M±S.E)
NAA				
0.50	82	1.40	4.35	2.95±0.32^{a}
1.00	88	1.70	4.90	3.20±0.37^{ab}
1.50	71	1.40	4.20	2.80±0.37^{a}
2.00	65	1.50	4.25	2.75±0.25^{a}
IBA				
0.50	75	1.80	5.32	3.52±0.22^{ab}
1.00	64	1.60	5.40	3.80±0.34^{b}
1.50	55	1.70	4.85	3.15±0.21^{ab}
2.00	45	1.50	4.30	2.80±0.25^{a}
IAA				
0.50	80	1.40	4.85	3.45±0.20^{ab}
1.00	72	1.60	5.35	3.75±0.16^{b}
1.50	60	1.80	4.70	2.90±0.19^{a}
2.00	54	1.70	4.45	2.75±0.29^{a}

*Values in a column with similar superscripts are not significantly different at $p \leq 0.05$ levels, Different letter(s) in a column indicate significant different at $p<0.05$ levels according to DMRT

rooting efficiency, 1.0 mg/L IAA singly in the medium showed significant response only on the highest number of roots per shoot (6.5) differing from other treatments (Fig. 2). However, development of maximum root length (3.8 cm) was recorded when 1.0 mg/L IBA added in the medium (Table 4). Well rooted plants were transferred in pot that was mixture by small brick pieces (5-7 mm size each), charcoal and coconut husk in 1:1:1 ratio and successfully hardened in a growth chamber (Fig. 1g and Table 5). Around 80% of the transplanted plants survived and they were shifted under the shade house for further development.

DISCUSSION

In the present investigation, regeneration capacity of pseudobulbs was tested for *C. finlaysonianum*. Our results showed that different PGRs and medium components affected shoot multiplication and elongation from pseudobulb segments. Exogenous application of cytokinins and auxins has been known to be important for shoot induction and elongation of many plant species *in vitro* (George, 1993); while BAP and NAA, respectively,

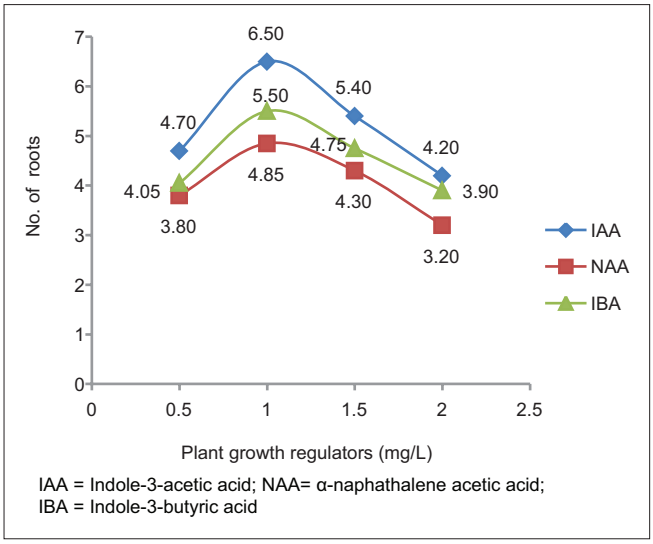

Fig 2. Effects of different auxins for developing roots per plantlet with ½MS medium.

Table 5: Establishment of plants in pots

Composition of substratum	No. of plants	Survival rate (%)
Brick+charcoal (1:1)+moss (10.0 gm/pot)	80	50.0
Brick+charcoal+decaying litter (1:1:1)+moss (10.0 gm/pot)	70	70.0
Brick+charcoal+decaying litter+saw dust (1:1:1:1)+moss (10.0 gm/pot)	50	52.0
Brick+charcoal+decaying litter+coconut husk (1:1:1:1)+moss (10.0 gm/pot)	60	80.0
Brick+charcoal+decaying litter+cow dung (1:1:1:1)+Moss (10.0 gm/pot)	40	60.0

Each pot diameter was 8.0 cm

are the two most commonly used cytokinin and auxin for shoot induction (Nasiruddin et al., 2003). In this study, we found that the development of multiple shoots from pseudobulb sections was significantly promoted with MS medium that supplemented with BAP (0.5-2.0 mg/L), NAA (0.25-1.0 mg/L) and Kinetin (0.5-2.0 mg/L) either single and combined uses. The results agreed well with the findings of *Coelogyne stricta* (Basker and Narmatha, 2006), *Malaxis acuminata* (Kaur and Bhutani, 2010), *Lycaste aromatic* (Mata-Rosas et al., 2010), *Coelogyne flaccid* (Kaur and Bhutani, 2013), *Cymbidium giganteum* (Ghosh et al., 2014). Present result showed that multiple shoot formation frequency and shoot length subsequently decreased when the plant growth regulators concentration was increased (Table 2 and 3). To generate a well developed root system facilitating adaptation is one of the major problems of *in vitro* grown plantlets to field conditions. Therefore, three plant growth regulators of IBA, IAA and NAA were evaluated for developing root system. Remarkably highest number of adventitious roots where found when shoots derived from pseudobulb exposed to half strength MS medium supplemented with 1.0 mg/L IAA that (Fig. 1f). The length of these roots was also highest in MS medium with 1.0 mg/L IBA (Table 4). The effect of IAA or IBA on induction of roots reported in other orchid species like *Vanda tessellata* (Bhattacharjee and Islam, 2014), *Rhynchostylis retusa* (Bhattacharjee and Islam, 2015) and *Dendrobium transparens* (Sunitibala and Kishor, 2009; Julkiflee et al., 2014), *Arundina graminifolia* (Bhadra and Bhowmik, 2005). All the substrate covered with a layer of 10.0 gm sphagnum moss (Table 5). The plantlets were watered alternately in the every evening and fed with MS nutrient salt solutions (diluted 10 times) fortnightly for about a month and eventually established under natural condition (Fig 1g). Further focus of the current study is on acclimatization of *in vitro* raised cultures of *C. finlaysonianum* and restoring them back in their natural habitat. Thus the simple protocol devised here will certainly contribute to the mass propagation and conservation of *C. finlaysonianum*. The importance of the acclimatization process has been highlighted in a number of reviews about orchid's growth (Das et al., 2007; Hossain et al., 2010; Panwar et al., 2012; Paudel and Pant, 2013; Shibu et al., 2014).

CONCLUSION

Pseudobulb segment culture is an efficient system for the production of large number of plantlets in a short time. Under this study a simple and an efficient protocol has been developed through *in vitro* culture for mass clonal propagation using pseudobulb segments of *Cymbidium finlaysonianum*. Using this protocol it is possible to clonally produce viable, uniform and healthy plants with maximum survival rate that can be used for large scale cultivation for orchid development. We used different concentration and combination of PGRs and found that medium components are very important and that affected shoot multiplication and elongation from pseudobulb segments. This system would be further established for an efficient system for ploidy enhancement scheme of orchids in the world. Furthermore, the protocol may facilitate conservation of this potential orchid from extinction in the natural population.

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Author contributions

S. M. S. I. conceived the idea, designed the experiments and contributed in writing. T. I., B. B., T. K. M. Conducted

experiments, sub-culturing, transferring plants to pots, data recording, maintaining plants up to maturity, and statistical analysis. S. S. providing *Cymbidium finlaysonianum* Lindl pods/capsules, wrote the manuscript, carry out statistical analysis and improvement of article.

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