Bioscene

Bioscene
Volume- 21 Number- 03
ISSN: 1539-2422 (P) 2055-1583 (O)
www.explorebioscene.com

In Vitro Multiplication of *Cymbidium Finlaysonianum* (Lindl.) through Protocorm Segment Culture

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Abstract: *Cymbidium* orchids are highly valued for their exotic flowers and medicinal values. However, its propagation through conventional methods is challenging due to its slow growth. *In vitro* multiplication offers a promising solution. In this study, we developed an efficient *in vitro* multiplication protocol for *Cymbidium finlaysonianum* using the protocorm segment culture. Protocorm segments were cultured on Murashige and Skoog medium supplemented by various plant growth adjuncts such as Naphthalene acetic acid (NAA) and 6-Benzyl amino purine (BAP), their effect on shoot regeneration, root formation and plantlet growth were investigated. The results showed high frequency of shoot regeneration and root formation in MS + BAP ($1mgl^{-1}$) within 7.6 weeks. Whereas, in case of MS medium and MS + NAA ($1mgl^{-1}$) complete plantlet formation was observed within 10.2 and 8.8 weeks respectively. BAP favoured early regeneration response in the explants and profuse multiplication in the segments.

The protocol offers a rapid and efficient method for large-scale multiplication *in vitro* of *C. finlaysonianum* which can support the conservation and commercial propagation of this valuable species of Orchid.

Keywords: *Cymbidium finlaysonianum*, protocorm segment culture, *in vitro* multiplication, orchid propagation, tissue culture

1. Introduction

Orchid flowers belong to the largest monocot family of flowering plants known as Orchidaceae. The flowers of this family show great genetic

diversity, varying in shape, size, color, structure and the number of flowers. It accounts for around 7.2% of flowering species of the Earth (Antony *et al*., 2014). Cymbidiums are also known as Boat orchid, which account for about 75–80 species (Balilashaki *et al*., 2023). These are evergreen flowers which blooms during winters and spring. They grow as epiphytes, lithophytes, terrestrials or saprophytic herbs, in the tropical and sub-tropical regions of northeast-India, northern-Australia and eastern-Asia (Zotz, 2013). These are rarely leafless and usually with pseudo-bulbs*. Cymbidiums* rank 1st in floricultural crops and accounts for about 2.7% of the total cut-flower production (Arditti & Ghani, 2000). *Cymbidium finlaysonianum* is an epiphytic orchid found in South Asia (Zotz, 2013). It is native species of Borneo, Java, Cambodia, Thailand, Philippines, Vietnam and Peninsular Malaysia (Arditti & Ghani, 2000).
Nowadays, the demand for orchids has increased significantly due to their vibrant colors, fragrances and the long shelf life. Cymbidiumsare widely used in pharmaceutical industry due to their varied medicinal properties. Some species of orchids have been found to be utilized by Indians since the Vedic era for its healing and aphrodisiac properties (Hossain & Sharma, 2019).As a result, they are being stealthily collected from nature. They have become rare and endangered due to over-exploitation, habitat degradation and fragmentation of habitat, clearing of land (Cribb *et al*., 2003; Coats & Dixon, 2007). Apart from it due to their sensitivity to microclimate and unique habitat needs, their population is declining dramatically. The entire family is included in Appendix - I and II of Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), where international trade is strictly monitored and controlled. The need of the hour is to save their population through sound scientific methodology to meet the ever-increasing demand, since traditional orchid propagation methods are very slow (Paudel *et al*., 2012). To replenish the orchid reserves of *Cymbidium finlaysonianum,* currently, it is propagated using protocorm segment culture *in vitro*; successive morphological events and multiplication of cultures under *in vitro* conditions were assessed.

2. Materials and Methods

2.1 Plant material and culture medium

Cymbidium finlaysonianum (Lindl.) protocorms (2mm in length) were taken (36 weeks old) from *in vitro* cultures. Murashige and Skoog (1962) medium (Hi, media, Mumbai, India) was used as a nutrient source to initiate the cultures. $CaCl_2$ ($0.04gl^{-1}$) and agar 0.9% (wv^{-1}) was added to it. The PLB's were maintained for about 2-3 weeks on Basal MS Medium. The effect of plant growth regulators (PGRs) such as auxins [NAA (α-Naphthalene acetic acid)] and cytokinin [BAP (6-Benzyl-amino-purine)] at 1 mgl^{-1} were added to the medium. The pH of the medium was set at 5.8 using 1N HCl / 1N NaOH before autoclaving. Medium was autoclaved at (121° C) at a pressure of 1.06kg cm^{-2} for about 15 minutes. Autoclaved medium was stored at room temperature for 2-3 days to check any contamination.

2.2 Inoculation and Culture Condition

After 3-4 days, inoculation was done inside the Laminar Air Flow. The cultured vessels were maintained at about 25 ± 2°C temperature, under a photoperiod of 12 hr illuminations of 3,500 lux intensity (Philips India Ltd., Bombay).

2.3 Observations and Result Analysis

The cultures were observed regularly at an interval of 1 week and data were recorded accordingly. The experiment was set in a completely randomized manner. Eight replicates were used per treatment. The results were tested as mean ± standard deviation. The cultures were observed regularly at an interval of 1 week and data were recorded accordingly.

2.4 Histological studies

To know the origin of *neo*-formations, hand sections of regenerating protocorm segments were cut using sharp razor blade. Those sections which were floating on the surface of water were selected to prepare slide for observation in order to trace the origin point of daughter *neo*-formations (PLBs).

3. Results and Discussion

In vitro culture and multiplication techniques are applied to obtain healthy plantlets over a short period of time, that too without relying upon the mycorrhizal associations. In recent years, the technique of tissue culture has been widely utilized for the large-scale proliferation and *ex-situ* conservation of *Cymbidiums*(Fonnesbech, 1972; Bannerjee & Mandal, 1999; Chang & Chang, 2000; Jamir *et al*., 2002; Pant & Pradhan, 2010). *In vitro* multiplication by using Protocorm-Like bodies (PLB's) is an excellent method of micro propagation. In order to save the germplasm of *Cymbidium finlaysonianum* from getting extinct, protocorm segments, from 36 weeks old culture were selected and inoculated in MS medium and its supplementation with growth adjuncts. The results are summarized (Table 1) and illustrated (Figure 1-4).

In earlier studies protocorm segments of *Cymbidium finlaysonianum* were regenerated in Vacin and Went (VW) medium (Rittirat *et al*., 2019). Whereas, currently, they could regenerate in MS medium suggesting there by, that the species has wide nutritional amplitudes. The protocorm segments regenerated even in basal MS medium also (Table 1) indicating that the species has simple nutritional requirements. In MS medium (control) eighty per cent explants responded to regeneration after 4.9 weeks of culture (Table 1). The explants followed callus mediated PLB pathway of regeneration; phenotypically, the callus was compact, brown and organogenetic in nature (Figure 1a). Complete plantlet containing 4-3 leaves and 2-3 roots were formed after 10.2 weeks (Figure 1b,1c).

While MS medium supplemented by auxin showed 80 per cent regeneration response and the initiation of response was observed after 4.2 weeks, 6-8 days earlier than MS medium. Complete plantlets were formed in about 8.8 weeks (Figure 2).When auxin (NAA) was replaced with cytokinin (BAP), it showed 100 per cent regeneration rate, i.e. 20 per cent more than individual treatment with auxin and basal medium(MS). Initiation of response was seen within 3.6 weeks.

Complete plantlets with 2-3 leaves and 1-2 roots were formed within 7.6 weeks of culture (Table 1; Figure 3).

It is clearly seen that incorporation of PGRs in the basal MS medium proved to be highly beneficial as they enhanced the regeneration response in the cultures. Best results were seen in MS medium supplemented by BAP followed by NAA. BAP favored early initiation of regeneration response and plantlet development in the explants. Previous studies in orchid genus supported our present study where the PLBs in *Dendrobium chrysotoxum* also followed PLB mediated pathway of regeneration (Kaur & Bhutani, 2011).

Interestingly, the PLBs multiplied at the base of the explant through budding through repeated cycles of regeneration (Figure 4 a, b, c). Histological hand sections, revealed that the PLBs multiplied through budding at the surface of the mother PLBs (Figure 4 d-f). Similar kind of response was earlier obtained in *Cymbidium aloifolium*where the protocorms followed direct PLB formation (Regmi *et al*., 2017). A survey of literature also points to the fact that somatic embryogenesis is the initial stage of PLB regeneration in orchid species (Huang *et al*., 2004). The invocation of small clusters of meristematic cells converted into *neo*-meristemoids in the epidermal layer of the protocorm. Induction of such meristemoids on the outer surface of the explants is earlier also indicated in the literature (Young *et al*., 2000; Ziv *et al*., 1998; Kaur, 2017). Protocorms can be effectively utilized to mass propagate the germplasm of *Cymbidium finlaysonianum invitro*.

4. Conclusion

Cymbidium finlaysonianum (Lindl.)is an epiphytic orchid which is commercially very important. Since this orchid rarely available in market, whose flowers are in high demand. This study highlights an effective protocol for *in vitro* mass-propagation through PLBs segment cultures. The current investigation proves that the protocorms could be effectively utilized for initiation of *in vitro* cultures and PGRs such as BAP is more efficient in mass propagation of *Cymbidium finlaysonianum* (Lindl.) The results of this study has important implications for the orchid industry, conservation efforts, and research applications, and

also demonstrates the potential of tissue culture for the sustainable development of valuable orchids and plant species.

Table 1*In vitro* regeneration response of PLBs of *Cymbidium finlaysonianum* in Murashige and Skoog's (1962) medium and its combination with growth adjuncts.

Medium	Time taken for initiation of response (in weeks)	Pathway followed	Time taken for complete plantlet formation (in weeks)
MS	4.9 ± 0.57	C→PLB	10.2 ± 0.50
MS + NAA	4.1 ± 0.95	PLB	8.8 ± 0.50
MS + BAP	3.6 ± 0.44	PLB	7.6 ± 0.44

Results are represented as Mean ± Standard deviation; BAP- 1mgl^{-1}; NAA-1mgl^{-1}; MS medium (basal); PLB- Protocorm like bodies

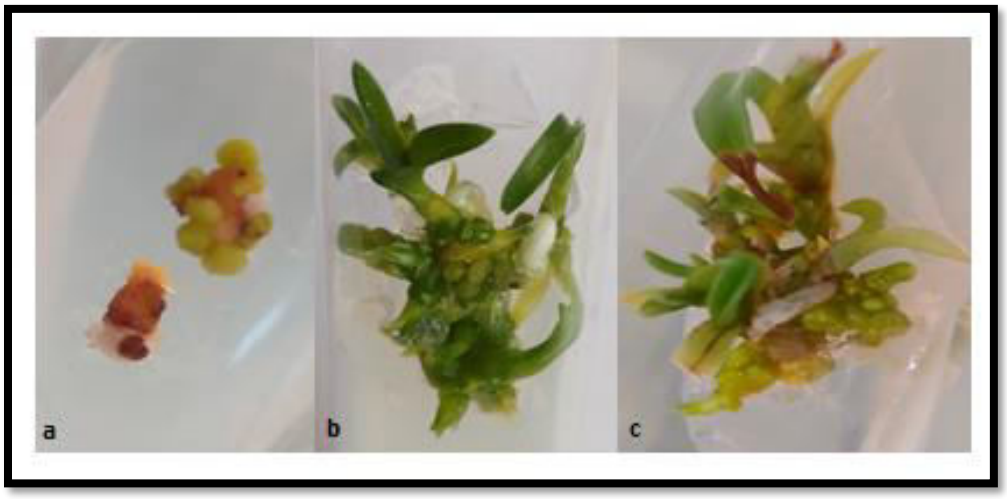

Figure 1. *In vitro* regeneration response of PLBs in *Cymbidium finlaysonianum* (Lindl.) in MS medium,**a-c)** Callus mediated PLB mediated regeneration response and complete plantlet formation with 4-5 leaves and 2-3 roots.

Figure 2.*In vitro* regeneration response of PLBs in *Cymbidium finlaysonianum* (Lindl.) in MS + NAA (1 mgl^{-1}). **a)** PLB mediated regeneration response **c)** Complete plantlet formation with 4-5 leaves and 2-3 roots.

Figures 3. *In vitro* regeneration response of PLBs in *Cymbidium finlaysonianum* (Lindl.) in MS + BAP **a)** PLB mediated regeneration response in MS + BAP 1 mgl^{-1}, **b)** Shoot formation in MS + BAP (1 mgl^{-1}), **c)** Complete plantlet formation with 2-3 leaves and 1-2 roots in MS + NAA (1 mgl^{-1})

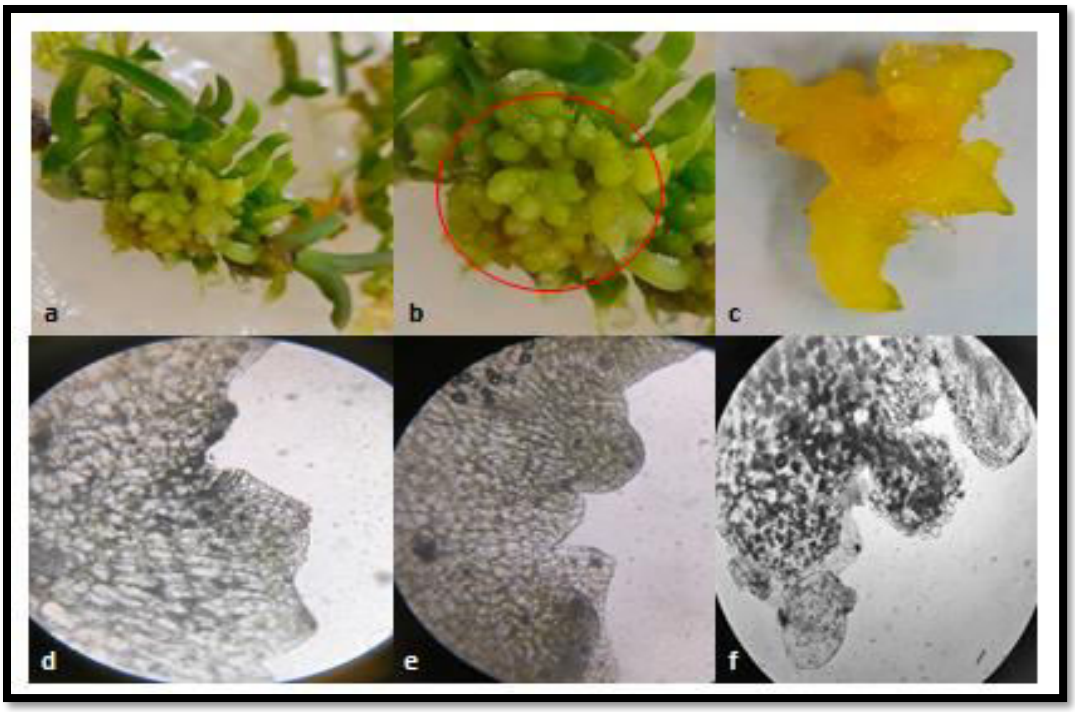

Figure 4.a-b) *In vitro* multiplication of *Cymbidium finlaysonianum* protocorms in MS medium**)** Single mother PLB of *Cymbidium finlaysonianum* showing profuse budding at its surface, **d -f)** Hand section of PLB of *Cymbidium finlaysonianum* showing budding (10X).

5. Acknowledgement

The authors are thankful to the Department of Biosciences, University Institute of Biotechnology (UIBT), Chandigarh University for providing all the infrastructure and chemicals for this research.

6. Conflict of Interest

The authors declare that they have no conflict of interest.

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