J. Orchid Soc. India, 34: 131-136, 2020 *ISSN 0971-5371*

MICROPROPAGATION OF *COELOGYNE FIMBRIATA* LINDL. USING PSEUDOBULB EXPLANTS

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Abstract

The regeneration competence of the *Coelogyne fimbriata* pseudobulb explants, obtained from *in vitro* raised cultures, was evaluated in various combinations on Mitra *et al*., 1976 (M) medium alone and with PGRs [auxin (2,4-D, 1-3mgl^{-1}) and cytokinin (BAP 1-3mgl^{-1})]. The explants failed to respond in the basal medium; these turned brown and perished within 60 days of culture. Inclusion of 2,4-D (1-3mgl^{-1}) in the medium invariably induced regeneration in the explants through callus formation, while the presence of BAP favoured regeneration through the formation of shoot buds. Incidentally, a combination containing 2,4-D and BAP (2 and 3mgl^{-1} each) revealed regeneration in the explants via protocorm like bodies (PLBs). Plantlets were transferred to clay pots containing potting mixture (brick-bats, charcoal, moss) of 1:1:1 ratio. Nearly 70% survival of plantlets was recorded.

Introduction

COELOGYNE FIMBRIATA Lindl. is a small to medium cool growing plant found on limestone hills (lithophytic)/ or on trees (epiphytic) in evergreen forests. It is a floriculturally important species bearing long lasting, fragrant, and buff-yellow coloured flowers with a fringed lip; it inhabits tropical and sub-tropical zone at an elevation of 800-2200 m in NorthEast India, Sikkim and West Bengal. Besides being a victim of its own beauty and utility, it is progressively loosing its natural habitats and figures in Appendix II of the Convention on International Trade in Endangered species of Wild Fauna and Flora (CITES, 2017). As this species is endangered due to habitat destruction and heavy collection pressures, presently studies were planned to assess the regeneration competence of its pseudobulbs, with a view to developing efficient and mass propagation protocol for the micropropagation of the species.

Materials and Methods

Plant Material

Pseudobulbs (0.5-0.8 cm long) procured from 38 wks old *in vitro* raised seedlings were used for assessing the regeneration competence.

Culture Media and Incubation Conditions

Mitra *et al.* (1976, M) medium and its different combinations of growth regulators, auxin [2,4-dichlorophenoxyacetic acid (2,4-D; 1-3mgl^{-1}) and cytokinin [6-Benzylaminopurine (BAP; 1-3mgl^{-1})] were used. The cultures were maintained at 25±2°C temperature and exposed to 12 hrs illumination of 3500 lux intensity. These were subcultured at regular intervals.

Acclimatization

Well-developed plantlets (about 4 cm long) with 2-3 leaves and 1-2 roots were gradually hardended *in vitro*, by sequential elimination of growth additives, vitamins, sucrose, and minor salts from the nutrient matrix at 15 days interval. In order to eliminate agar, the hardened plantlets were thoroughly washed with lukewarm water and potted in clay pots, using charcoal, moss, brick-bats (1:1:1) as the potting media.

Statistical Analysis

One way variance analysis was performed with respect to each response (average ± standard error against each additive is mentioned in Fig. 1). As ANOVA results showed the non-significant difference of additives at 5% level of significance, various groups of additives showing identical/similar response were formed statistically. To this end, Tukey Test was performed at 5% level with respect to each response.

Results and Discussion

The regeneration competence of the pseudobulbs seems to be markedly influenced by physiological age of the mother plant, position of donor explants, and growth stimulus in nutrient pool (Vajrabhaya, 1978). In the present investigation, pseudobulbs (0.5-0.8 cm long) procured from 38 wks old *in vitro* raised seedlings were sliced into apical and basal segments and their

Received: September 20, 2020; Accepted: December 24, 2020

regenerative competence were assessed on M medium, with and without growth additives; it was markedly influenced by position and the growth stimulus in the nutrient pool. The explants invariably regenerated by bud break; however, PLBs and callusing were also observed in selective combination in the nutrient mix (Table 1; Fig. 1). Regeneration potential of pseudobulb explants has been successfully tested in a few orchids including *Coelogyne spicata* (Basker and NarmathaBai, 2006), *Coelogyne flaccida* (Kaur and Bhutani, 2013), *Coelogyne ovalis* (Sharma, 2017), *Cymbidium finlaysonianum* (Islam *et al*., 2015), *Dendrobium* (Sunitibala and Kishor, 2009; Vij and Pathak, 1989), *Dendrobium nobile* (*Anuprabha et al*., 2017*), Dendrobium palpebrae* (Bhowmik and Rahman, 2020), *Eulophia epidendraea* (Maridass *et al*., 2012), *Malaxis acuminata* (Deb and Arenmongla, 2014; Vij and Kaur, 1998).

In the basal medium, the explants failed to respond; these turned brown and perished within 60 days of culture. A treatment with PGRs in general, proved obligatory for inducing the regeneration response in these. The regeneration pathway varied with the quality of the growth regulator(s) used in the medium. Incorporation of 2,4-D (1-3mgl^{-1}) in the medium invariably induced regeneration via callus formation in the explants, whereas presence of BAP favoured the regeneration via shoot bud formation. Incidentally, combination containing 2,4-D and BAP (2 and 3mgl^{-1} each) showed the regeneration via PLBs in the explants. 2,4-D at 1 mgl^{-1} initiated the regeneration response in 56.25±6.25% explants; pale green callus was observed within 36.00±0.82 days (Fig. 2A). Callus was organogenetic and it differentiated nearly 21 plantlets within 256.50±0.65 days. 2,4-D when used at 2 and 3mgl^{-1} concentration, also induced regeneration via organogenetic callus formation within 40.50±0.50 and 41.25±0.48 days respectively. 2,4-D at 2mgl^{-1} not only enhanced the regeneration response but also proved beneficial for producing 25.25±0.48 regenerants per explant (Fig. 2E). The benign role of auxins during *in vitro* propagation has also been reported earlier in *Bletilla* (Vij and Dhiman, 1997), *Bulbophyllum* (Vij *et al*., 2000), *Coelogyne cristata* (Kaur *et al.,* 2017), *Crepidium acuminatum* (Vasundhra *et al*., 2019), *Dendrobium ovatum* (Gurudeva, 2019), *Rhynchostylis retusa* (Pathak *et al.,* 2017), *Smithsonia maculata* (Decruse and Gangaprasad, 2018), *Spathoglottis* (Bapat and Narayanaswamy, 1977), *Vanda tessellata* (Madhavi and Shankar, 2019). BAP at 1 mgl^{-1} initiated regeneration response in 50.00±0.00% explants via shoot bud formation and complete healthy plantlets were obtained within 190.00±0.82 days. The per cent regeneration response was more pronounced in combination containing BAP. BAP at 2mgl^{-1} induced early regeneration (30.00±0.82 days) and plantlet formation (181.00±0.58 days) (Fig. 2I) and its concentration at 3 mgl^{-1} proved beneficial in enhancing and advancing, the regeneration response in the explants. Plantlets were obtained in 190.75±0.48 days. All the above combinations proved beneficial for multiple shoot production.Vij *et al*. (2000) has also reported a multifold increase in the proliferation potential of the shoot buds on addition of BAP, in cultures. Roy and Benerjee (2003) reported that the concentration of BAP higher than 2mgl^{-1} was effective

Table 1. Regeneration potential of *in vitro* sourced pseudobulb explants on Mitra *et al*. (1976) medium, in *Coelogyne fimbriata.*

Growth additives	Regeneration pathway	Remarks
M	-	-
2,4-D_1	Callus	Organogenetic callus
2,4-D_2	Callus	Organogenetic callus
2,4-D_3	Callus	Organogenetic callus
BAP_1	Sb	Multiple shoot production; Healthy plantlets
BAP_2	Sb	Multiple shoot production; Early plantlet formation
BAP_3	Sb	Multiple shoot production
2,4-D_1+BAP_1	-	-
2,4-D_2+BAP_2	PLBs	Multiplication of PLBs
2,4-D_3+BAP_3	PLBs	Multiplication of PLBs

Figures as subscripts indicate concentration of growth regulators (mgl^{-1}).

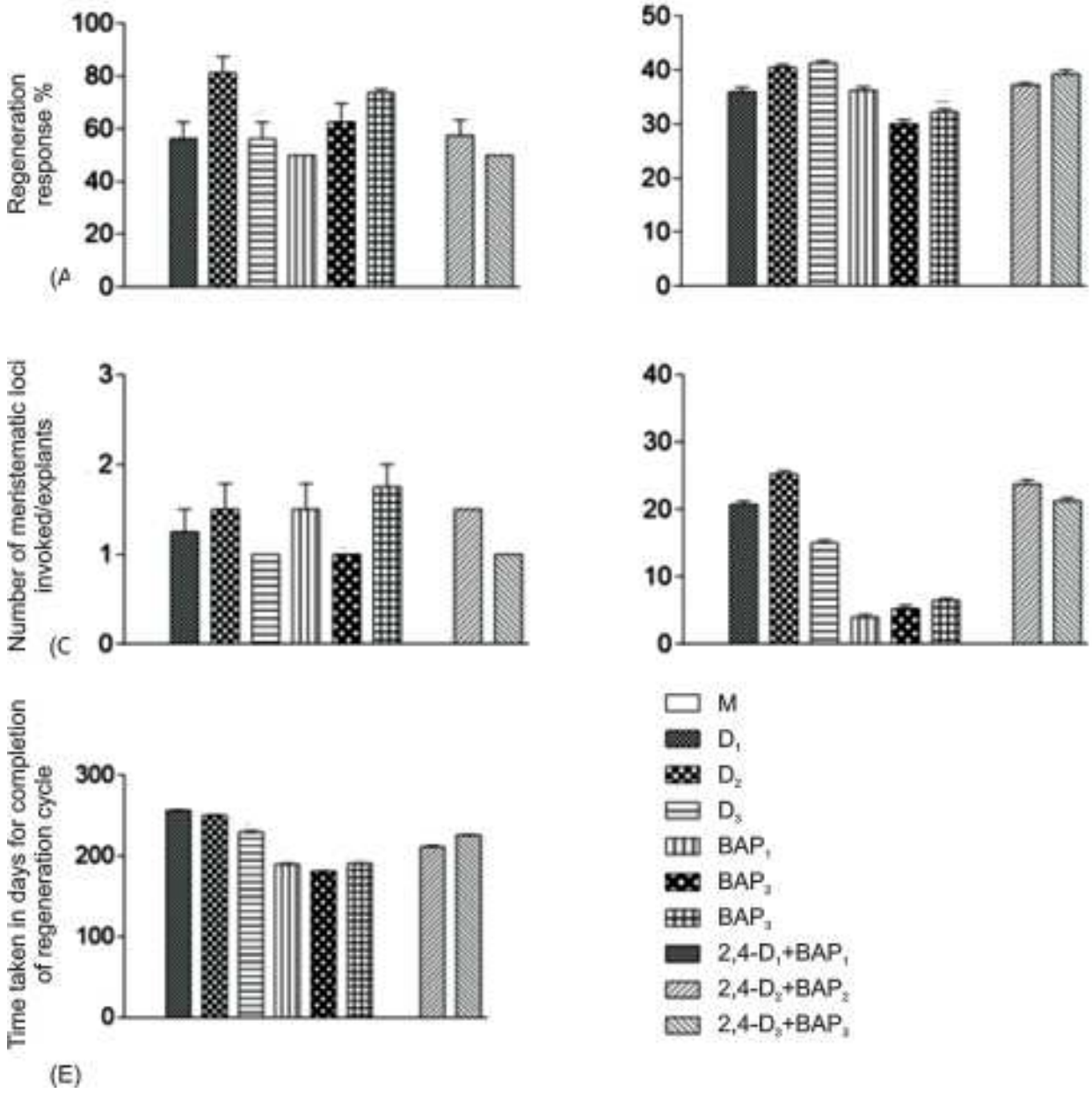

Fig. 1. Effect of different nutritional combinations with growth additives on the regeneration response in *Coelogyne fimbriata* pseudobulb explants: A, Per cent regeneration response; B, Time taken for initiation of regeneration response; C, Number of meristematic loci invoked per explant; D, Number of regenerants obtained per explant; E, Time taken in days for completion of regeneration cycle. Statistical analysis: Mean ± S.E.; same alphabetical letter in the superscript denotes that the corresponding means are in the same group using Tukey test at 5%. M, Mitra *et al.* (1976) medium; 2,4-D, 2,4-dichlorophenoxyacetic acid; BAP, 6-Benzylaminopurine.

in production of multiple shoots in *Dendrobium oculatum.*

A nutritional combination containing 2,4-D (1mgl^{-1}) and BAP (1mgl^{-1}) in the medium proved inhibitory as the explants failed to respond despite repeated subculturing. However, nutrient medium containing 2,4-D and BAP at 2 and 3mgl^{-1} each successfully induced regeneration response in the explants via PLB formation. Synergistic action was apparent in both these combinations for enhanced number of regenerants obtained per explants and PLB formation and their multiplication thereof. In the combination containing 2,4-D and BAP (2mgl^{-1} each), as many as 24 plantlets were obtained within 211.50±1.71 days (Fig. 2J), however, its concentration at 3mgl^{-1} each generated 21.25±0.48 regenerants per explants (Fig. 2G); complete plantlets were obtained in 226.00±0.41 days. The synergistic action of the combination of auxin and cytokinin in inducing regeneration in explants is in compliance with earlier

Fig. 2. A-K. Regeneration competence of *in vitro* pseudobulb explant culture in *Coelogyne fimbriata*: A, Callus formation [M+2,4-D (3mgl^{-1})]; B-C, Regeneration via shoot bud formation [M+BAP (1mgl^{-1})], [M+BAP (3mgl^{-1})]; D, PLBs formation [M+2,4-D (2mgl^{-1}) + BAP (2mgl^{-1})]; E-F, Callus mediated profuse multiplication and further plantlets formation [M+2,4-D (2mgl^{-1})], [M+2,4-D (3mgl^{-1})]; G, PLBs regenerated plantlets [M+2,4-D (3mgl^{-1})+BAP (3mgl^{-1})]; H-J, Plantlets with roots development [M+2,4-D (3mgl^{-1})], [M+BAP (2mgl^{-1}), [M+2,4-D (2mgl^{-1})+BAP (2mgl^{-1})]; K, Complete plantlets transferred to a clay pot.

reports (Anuprabha and Pathak, 2019; Deb and Temjensangba, 2006; Ghosh *et al*., 2014; Jiang *et al*., 2011; Kaur, 2017; Kaur and Bhutani, 2013; Pant and Thapa, 2012; RajKarnikar, 2011; Sunitibala and Kishor, 2009).

Conclusion

These data indicate that the 2,4-D (2mgl^{-1}) containing M medium was optimal for enhanced meristematic loci activation; and 2,4-D and BAP (2mgl^{-1}each) containing

combination was proved an optimal nutritional combination not only for numeral meristematic loci activation but also for the development of PLBs and multiplication of cultures in *Coelogyne fimbriata.*

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