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Micropropagation of therapeutically important swarna Jibanti (*Coelogyne cristata* Lindl.) through Pseudobulb Segments-a study *in vitro*

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ABSTRACT

Presently, an efficient protocol for micropropagation using the pseudobulb derived from *in vivo* plants of *Coelogyne cristata* Lindl was developed. The regeneration competence of the pseudobulb segments was markedly influenced by physiological age of the mother plant, position on donor axis and the nutrient regime. Juvenility of the tissues emerged as the important factor in inducing cell proliferations, as the explants from well developed pseudobulb remained recalcitrant to regeneration, whereas regeneration in the freshly formed pseudobulbs was obligatory to the exogenous application of cytokinin in dose double to auxin (10.25±0.25), which multiplied through budding. The pure-line stability of regenerants was confirmed at chromosome level (2n=38). The well rooted regenerated plantlets were acclimatized successfully in the potting mixture containing moss, pinebark, brick pieces(5-7mm size) and charcoal pieces (1:1:1:1) mixture with 90% survival success.

Key words : *Orchid, Flowering plants, Pseudobulb, Tissue culture, Protocorm-like-bodies (Plbs)*

Introduction

The flowering plants satisfy man's requirements for food, shelter, clothing and drugs, besides adding his aesthetic values. Their latter utility accounts for a highly lucrative international trade in floriculture. Till very recently, the trade primarily revolved around roses, lilies, carnations, chrysanthemums, tulips, but the trend now, fast shifting in favour of orchids for their exquisite and long lasting flowers of myriad shapes, sizes, fragrances and colors. Orchids (Orchidaceae), represents a diverse group of geologically young plants, comprising of 35000 species in 700-800 genera, have outsmarted their counterparts by evolving higher level of specialization in their vegetative and reproductive traits (Christenhusz and Byng, 2016; Zhang *et al*., 2018). Heavy pressures of commercial collection which far exceeds their natural regeneration and habit destruction have detrimentally affected the size and frequency of orchids population and the family orchidaceae figures prominently in the Red Data Book prepared by International Union for Conservation of Nature and Natural Resources (IUCN, 1991).

Tissue culture technique has added new dimensions and opened new avenues in conservation and clonal propagation of particularly out breeders like orchids which generate a great deal of heterozygosity in the progenies, a disadvantageous proposition in pureline preferred cut flower industry. Morel (1964) pioneered the utility of shoot meristem to

raise virus free plants, but the technique was detrimental to the monopodial orchids, as it requires the sacrifice of the entire growth, hence it was desirable to develop an alternate and equally effective multiplication system by activating adventitious meristems. Normally, orchids were propagated *in vitro* by using different explants leaves, roots, Infloresecence and pseudobulbs, but there were fewer reports obtained on *in vivo and in vitro* culture of pseudobulb (Goswami *et al*., 2015; Islam *et al*., 2015; Singh *et al*., 2016; Bhowmik and Rahman, 2020) as compared to the other explants.

Coelogyne cristata (crested *Coelogyne)*, an ethnomedicinally important foliar herb endemic to Himalayan ranges from Garhwal eastwards to Arunachal Pradesh (1700-2300 m), commonly known as 'Jibanti', mostly used in folk medicines for treatment of fractured bones, epilepsy, nerve disorders, antiageing and stress problems (Mohammad, 2011; Pramanick, 2016; Tsering *et al*., 2017). The latter property makes *C.cristata*, a good alternative of health tonic and rejuvenator. Besides this, due to unregulated commercial collections, the natural population of *C. cristata* was getting alarmingly rare with passage of time and genus included in Appendix II of the Convention on International Trade in Endangered species of Wild fauna and flora (CITES, 2017). The present study was a pioneer attempt which aims to prepare reproducible and efficient micropropagation blue prints using *C. cristata* pseudobulb segments.

Materials and Methods

Plant Material

C. cristata plants are collected from Darjeeling, West Bengal (26°31-27°13 N; 87°59 -88°53 E) and grown under greenhouse conditions in the Post Graduate Government College for Girls, Sector 11, Chandigarh. About 2 cm long freshly sprouted pseudo bulb (6 wk after emergence) from green house grown plants were used as explants.

Sterilization Method

The explants were sequentially surface sterilized with solutions of streptomycin (0.1%, 15 min), sodium hypochlorite (4%, 15 min) and ethanol (70%, 3sec). The explants were subsequently rinsed repeatedly thrice with double sterilized distilled water after every treatment. The cut ends of the explants exposed to sterilizing agents were sliced off and the sterilized pseudo bulbs were cut horizontally into distal and proximal segments and inoculated on the media.

Media and culture conditions

The pseudo bulb segments (0.5-1cm), were inoculated on BM medium (Mitra *et al*., 1976) with BAP(6-Benzyl amino urine; 1-10 mg/L), KN (Kinetin;1-10 mg/L) alone and in combination with NAA (α-Naphthalene acetic acid; 1-10 mg/L). The pre-inoculation medium pH was adjusted at 5.6. In parallel set of experiments 0.2% activated charcoal (AC) was used in the medium. Sixteen replicates for each treatment and the experiments were repeated four times. All experimental manipulations were done under aseptic conditions and the cultures incubated at 25±2 °C under 12 hr photoperiod of 3500 lx light intensity, were regularly observed

Data recording

The cultures were observed fortnightly and their response to different chemical regime was recorded. In order to study the effect of chemical stimulus on regeneration, the different parameters observed were: regeneration frequency, number of proliferative loci/explants and plantlet formation.

Acclimatization of the Plantlet

The plantlets (3 cm tall) were subjected to semi-solid medium with half concentration macro and micro salts of BM (Mitra *et al*., 1976 medium) and after a week time, macro, micro nutrients, sucrose and vitamins were eliminated sequentially. The plantlets were kept in nutrient and carbohydrate bereft medium till they are 4-5 cm tall. Finally the plantlets were taken out and rinsed with warm water before transferring to moss, pine bark, brick and charcoal pieces (1:1:1:1) mixture in poly bags under greenhouse conditions. The humidity level was maintained by increasing the number of holes in poly bags with passage of time. The poly bags were removed after 4 weeks and the acclimatized plants were fortnightly sprayed with Bavistin (1%) with 90% survival rate. Figure 5 shows an acclimatized plantlet

Results and Discussion

The regeneration competence of the pseudo bulbs was markedly influenced by physiological age of

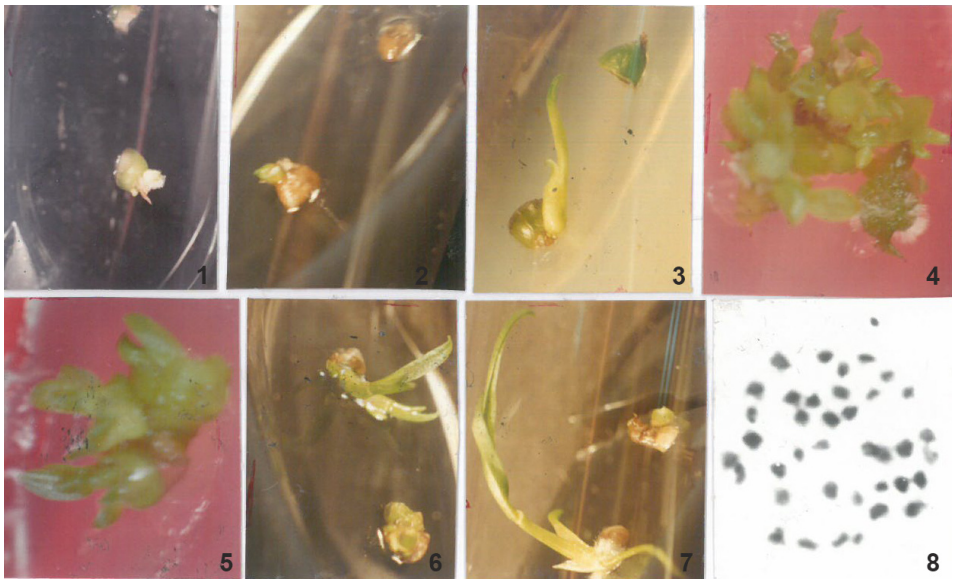
Table 1. Regeneration response to PGRs in *C.cristata* pseudobulb explants :

PGRs in BM medium	PGRs Quantity (mgL^{-1})	Regeneration (%)	No. of plantlet obtained/explant	Regeneration Pathway
BA	1	-	-	-
	5	-	-	-
	10	-	-	-
Kn	1	-	-	-
	5	-	-	-
	10	-	-	-
BAP+NAA	1:1	35.94±1.56	3	Callus
BAP+NAA*	1:1	35.94±1.56	3	Callus
BAP+NA**A**	5:5	51.56±1.56	5	Callus
BAP+NAA*	5:5	45.31±1.56	5	Callus
BAP+NAA	10:5	60.94±1.56	10.25±0.25	Callus
BAP+NA**A***	10:5	59.38±1.56	9.5±0.25	Callus
KN+NAA	1:1	35.94±1.56	3	PLBs
KN+NAA*	1:1	35.94±1.56	3	PLBs
KN+NA**A**	5:5	51.56±1.56	1.75±0.25	PLBs
KN+NAA*	5:5	45.31±1.56	1.50±0.25	Shoot Primordia
KN+NA**A**	10:5	51.56±1.56	2.75±0.25	PLBs
KN +NA**A***	10:5	45.31±1.56	1.75±0.25	Shoot Primodia

*Medium supplemented with activated charcoal (0.2%)

the mother plant, position on donor axis and medium composition. Juvenility of the tissues emerged as the important factor in inducing regeneration and cell proliferations since the explants from well developed pseudo bulbs (>3 cm long) remained recalcitrant to regeneration and those from freshly formed pseudo bulbs (<3 cm long) reacted positively, where the lower part of pseudo bulb segments showed better response than upper part (Fig.1),in compliance with earlier studies in *Bulbophyllum careyanum*, *Malaxis acuminata*, *Coelogyne flaccida*, *Coelogyne ovalis* ,*Coelogyne stricta*, *Dendrobum nobile*, due to the fact that the younger tissues with less rigid cell walls were physiologically and biochemically more active and show better morphogenetic potential in harmony to earlier reports (Sharma and Vij, 1997; Basker and Bai, 2006; Sungkumlong and Deb, 2009; Sunita Bala and Kishor, 2009; Kaur and Bhutani, 2010; 2013; Deb and Arenmongla, 2014; Ghosh *et al.*,2014; Islam *et al*., 2015; Singh *et al*., 2016; Sharma, 2017; Bhowmik and Rahman, 2020). The perusal of literature reveals that the positive results prominently restricted to the *in vitro* sourced explants, however, presently, the regeneration was successfully achieved in the *in vivo* procured explants.

The explants showed small projections initially at cut ends after 8 days of culture and subsequently along the entire surface and within 4 weeks of culture, the swollen area was proliferated eventually into plbs. The morphogenetic response and regeneration pathway was markedly influenced with type of cytokinins and auxin used in the nutrient pool and showed variation in initiating the meristematic activity of the explants. It was observed that the lower part of pseudo bulb yielded better result over the segments (Fig. 3). The conversion frequency in BAP (10 mg/L) + NAA (5 mg/L) was through indirect somatic embryogenesis (callus) in 60.94±1.56 pseudo bulb segments producing 10.25±0.25 plantlets in 8 wks (Figs.1-2). However, when BAP (10 mg/L) was substituted with KN (10mg/L) in the latter medium, the 51.56±1.56 pseudo bulb segments showed direct shoot development with plbs formed at base producing 2.75±0.25 plantlets (Fig. 4). Presently, synergistic organogenesis responses were observed in segments when BAP is used in dose double than that of NAA in compliance to earlier reports in *Coelogyne flaccid*, *Coelogyne stricta,Coelogyne ovalis, Changnienia amoena, Cymbidium aloifolium*, *Cymbidium finlaysoniacum, Cymbidium giganteum, Dendrobium fimbriatum, D.palpebrae , Dendrobium transparens, Eulophia andamanensis, Lycaste aromatic,Taenia latifolia* (Basker and Narmatha, 2006; Sunitibala and Kishor, 2009; Mota-Rosas *et al.*, 2010; Jiang *et al*., 2011;



Figs. 1-2. Direct Somatic embryogenesis in both distal and proximal segments in BM+BAP (10 mg/L)+NAA (5 mg/L); **Fig. 3.** Direct Shoot primordia in distal segment in BM+KN (5 mg/L) +NAA (5 mg/L)+AC; **Fig. 4.** Multiplication of Plbs through budding in BM+BAP (10 mg/L)+NAA (5 mg/L); **Fig. 5.** Callusing at the base of plantlet in BM+BAP (10 mg/L)+NAA (5 mg/L) (5 mg/L); **Figs. 6-7.** Direct Shoot primordial in both proximal and distal segment in BM+KN (5 mg/L) +NAA (5 mg/L)+AC; **Fig. 8.** Cytological study of *C. cristata* (2n=38).

Hossain, 2014; Deband Arenmongla, 2014; Ghosh *et al.*, 2014; Islam *et al*., 2015; Singh *et al*., 2016; Regmi *et al*., 2017; Sharma, 2017; Ramesh *et al*., 2019; Bhowmik and Rahman, 2020. Addition use of activated charcoal (0.2%), hampered the regeneration response and favoured direct shoot primordial formation (present study; Sunitibal and Kishor, 2009). This differential response was attributed to adsorbing quality of activated charcoal (AC), which markedly influenced the morphogenesis by alternation in the endogenous level of auxin/cytokinin ratio (Philip and Nainar,1988; Taji *et al*., 2002). The significance of acclimatization in survival of *in vitro* raised plantlets observed presently was in accord with the earlier findings (Paudel and Pant, 2013; Shibu *et al*., 2014; Islam *et al*., 2015).

Conclusion

The present study aimed to develop reproducible micropropagation blue print in *C. cristata* pseudobulb segments for mass clonal production of viable, cytologically stable and healthy plants with maximum survival. The studies reveals that the regeneration reponse was influenced with juvenility, source of explants and the nutrient environment. The regeneration in BAP enriched combinations in invariably indirect somatic embryogenesis mediated. The efficacy of this cytokinins (BAP) was obligatory to use of NAA and was more pronounced at dose double than that of NAA in lower part of pseudobulb segments. However, substitution of BAP with KN in the latter medium favoured direct shoot devolopment. The protocol will facilitate conservation of endangered orchid from eminent extinction.

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References

Basker, S. and Bai, V.N. 2006. Micropropagation of *Coelogyne stricta* (D. Don) Schltr. via pseudobulb segment cultures, *Trop. Subtrop. Agroeco.* 6 : 31-35.

Bhattacharjee, B. and Islam, S.M.S. 2014. Development of an efficient protocol for *in vitro* germination and enhancing protocorm-like body development in three indigenous orchid species in Bangladesh, *2As. Pac. J. Mol. Biol. Biotech.* 22 (3) : 209-218.

Bhowmik, T.K. and Rahman, M.M. 2020.

Micropropagation of commercially important orchid *Dendrobium palpebrae* Lindl through *in vitro* developed pseudobulb culture. *J. Adv Biotechnol Exp Ther.* 3 : 225-232.

CITES, 2017. Convention on International Trade in Endangered Species of Wild Fauna and Flora CITES Appendices I, II and III,http://www.cites.org.

Chauhan, S. and Sharma, S. 2017.Conservation of rare and threatened therapeutically important orchid *Pleione maculate* (Lindl.) & Paxton through pseudobulb culture *in vitro*, *Indian J. Sci. Res.* 15 (1) : 22-26.

Christenhusz, M.J.M. and Byng J.W.2016. The number of known plant species in the world and its annual increase. *Phytotaxa*. 261 : 201-217.

Deb, C.R. and Arenmongla, T. 2014. Development of cost effective in vitro regeneration protocol of *Malaxis acuminate* D. Don, a therapeutically important orchid using pseudobulbs as explants source. *J. of Pl. Studies*. 392 : 13-22.

George E.S. 1993. *Plant propagation by tissue culture. Part 1:The Technology, 2nded*. Exegetics Ltd. Edington, Wilts. BA13 4QG, England.

Ghosh, A., Hossain, M.M. and Sharma, M. 2014. Mass propagation of *Cymbidium giganteum* Wall. ex Lindl using *in vitro* seedlings. *Indian J. Exp. Biol*. 52 : 905-911.

Goswami, K.S., Yasmin, Nasiruddin, K.M., Khatun, F. and Akte, J. 2015. *In vitro* regeneration of *Dendrobium* sp of orchid using leaf tip as explants.*J.Environ.Sci. & Natural Res*. 8 (2) : 75-78.

Hossain, M.M.2014 *In vitro* Embryo Morphogenesis and Micropropagation of *Dendrobium* aggregatum Roxb. *Plant Tissue Cult Biotechnol.* 23 (2) : 241-249.

Islam, S.M.S., Islam, T., Bhattacharjee, B., Mondal, T. K. and Subramaniam, S. 2015. *In vitro* pseudobulb based micro-propagation for mass development of *Cymbidium finlaysonianum* Lindl.*Emirates Journal of Food and Agriculture*. 27(6) : 469-474.

IUCN, 1991. IUCN Directory of Protected Areas in Oceania prepared by the World Conservation Monitoring Centre. IUCN, Gland, Switzerland and Cambridge, U.K. 447 pages.

Jiang, W.M., Shui, Z.M., Cheng Xin, F.U. 2011. Studies on *in vitro* regeneration competence of pseudobulb cultures in *Changnienia amoena* Chien. *Chinese Science Bull.* 56 (24) : 2580-2585.

Kaur, S. and Bhutani, K.K.2010.Micropropagation of *Malaxis acuminate* D.Don:A rare orchid of high therapeutic value. *J. Med. Arom. Plant.* 1 : 29-33.

Kaur, S. and Bhutani, K.K. 2013. *In vitro* Mass Propagation of Ornamentally and Medicinally Important *Coelogyne flaccida* Lindl. through pseudobulb Segments. *Plant Tissue Cult. & Biot*ech. 23(1) : 39-47.

Malabadi, R.B., Mulgund, G.S. and Kallapa, N. 2004. Efficient regeneration of *Vanda coerulea*,an endangered orchid using thidiazuron. *Pl. Cell Tissue Org. Cul*. 76: 289-293.

Martin, K.P. and Madssery, J. 2006. Rapid *in vitro* propagation of *Dendrobium* hybrid through direct shoot formation from foliar explants and protocorm like bodies. *Scientia Horticulturae.* 108(1) : 95-99.

Mata-Rosas, M., Baltazar-Garcia, R. J., Moon, P., Heitz, P. and Luna-Monterrojo, V. E. 2010. *In vitro* regeneration of *Lycaste aromatic* (Graham ex Hook.) Lindl (Orchidaceae) from pseudobulb sections. *Plant Biotech. Rep*. 4 : 57-163.

Mohammad, M.H. 2011. Therapeutic Orchid: Traditional Uses and Recent Advances-An Overview. *Fitoterapia*. 82 (2) : 102-140.

Mitra, G.C., Prasad, R.N. and Chwdhary , A.R. 1976. Inorganic salts and differentiation of protocorms in seed callus of an orchid and correlated changes in its free amino acid content. *Indian J. Exp. Biol*. 14 : 350-51.

Morel, G. 1964. Tissue culture-a new means of clonal propagation of orchids. *American Orchid Society Bulletin*. 33 : 473-478.

Nasiruddin, K.M., Begum, R. and Yesmin, S. 2003. Protocorm like bodies and plantlet regeneration from *Dendrobium formosum* leaf callus. *J. Plant Sci.* 2(13) : 955-957.

Paudel, M.R. and Pant, B. 2013. A Reliable Protocol for Micropropagation of *Esmeralda* clarkei Rchb.f. (Orchidaceae). *As. Pac. J. Mol. Biol. Biotech.* 21(3) : 114-120.

Philip, V.J. and Nainar, S.A.Z. 1988. *In vitro* transformation of root meristem to shoot and plantlets in Vanilla planifolia. *Ann Bot.* 61 : 143-99.

Pongener, A. and Deb, C.R. 2011. *In vitro* regeneration of plantlets of *Cymbidium iridiodes* D.Don using nodal segments as explants. *Int.J.of Appl.Biotech. and Biochem*. 1(4) : 389-400.

Pramanick, D.D. 2016. Pharmacognostic studies on the pseudobulb of *Coelogyne cristata* Lindl. (Orchidaceae) –an epiphy- tic orchid of ethno-Medicinal importance. *Journal of Pharmacognosy and Phytochem.* 5(1) : 120-123.

Ramesh, T., Renganathan, P., Prabhakaran, M. 2019. In vitro studies on Dendrobium fimbriatum Hk.F – An endangered orchid. *J. of Applied and Advanced Research*. 4(4) : 100-103.

Regmi, T., Pradhan, S. and Pant, B. 2017. In vitro Mass Propagation of an Epiphytic Orchid, *Cymbidium aloifolium* (L.) Sw., through Protocorm Culture, Biotech. *J. Internation.* 19 (1) : 1-6.

Sarmah, D., S., Kolukunde M., Sutradhar, B. K., Singh, T. and Mandal, M.N. 2017. A Review on: *In vitro* Cloning of Orchids. *Int. J. Curr. Microbiol. App. Sci*. 6(9) : 1909-1927.

Sharma, V. 1996. *Orchid Propagation for Conservation and Commercialization-A study In vitro*. Ph.D. Thesis, Panjab University, Chandigarh, India.

Sharma, V. and Vij, S.P. 1997. Effect of $CuSO_4.5H_2O$ on in

vitro regenerative capacity of foliar explants excised from mature *Vanda cristata* Lindl. plant. *Phytomorphology*. 47 (2) : 203-208.

Sharma, V. 2017. Regeneration competence of pseudobulb explants of endangered orchid genera:a study *in vitro*. *Int. J. of Recent Sci. Res*. 8(11) : 21722-21724**.**

Shibu, B.S., Wesley, P.S., Moin, S. and Devi, B.C. 2014. *In vitro* regeneration of *Coelogyne nervosa* A. Rich. and *Eria pseudoclavicaulis* Blatt. Threatened orchids of Westarn Ghats, India. *Indian J. Biol*. 52 : 658-663**.**

Singh, D.R., Kishore, R., Kumar, R., Singh, A. 2016. Orchid preparing. In*: Techinical Bull*. (ed. Singh *et al*., 2016), ICAR National Research Centre for Orchids, Pakyong, Sikkim: 1-51.

Sunitibala, H. and Kishor, R. 2009. Micropropagation of *Dendrobium transparens* L. from axenic pseudobulb segments. *Indian J. Biotechnol*. 8 : 448452.

Sungkumlong and Deb CR. 2009. Regeneration competence of *Tainia latifolia* (Lindl.) Benth ex Hook. pseudobulb segments: An *in vitro* study. *Indian J. Biotechnol*. 8 : 121126.

Taji, A., Kumar, P. and Laskhmanan, 2002. *In vitro* plant breeding. In: *Micropropagation* (ed. Taji *et al.*, 2002). New York, The Haworth Press Inc. pp. 15-32

Tsering, J., Ngilyang, T., Tag, H., Gogoi, B.J. and Apang, O. 2017. Medicinal Orchids of Arunachal Predesh: A Review. *Bull. of Arunachal Forest Research*. 32(1&2): 1-16.

Vij, S.P., Kher, A. and Pathak, P. 2000. Micropropagation of *Bulbophyllum careyanum* (Hook.) Spring. using pseudobulb segments. *The Journal of The Orchid Society of India*. 14 : 47–56.

Zhanga, S., Yanga, Y., Li, J. 2018. Physiological diversity of orchids. *Plant Diversity.* 40 :196-208.