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Propagation of Cymbidium eburneum in vitro by Immature Seed Culture

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Abstract. Orchids represent one of the largest families of angiosperms with innumerable hybrids and varieties producing flowers with high floricultural appeal. *Cymbidium eburneum* is one of most demanding orchids in international floricultural market because of their highly attractive large showy white flowers with sweet fragrance. However because of extensive exploitation of this orchid for personal gain through illegal collections and deforestation, the population is fast receding from its natural habitat. Proper conservation strategies should be adopted to protect this beautiful plant from extinction. The present study was conducted to establish an efficient regeneration protocol for this important orchid by culturing the immature seeds obtained from green capsules in Mitra and MS medium supplemented with varied combinations of different plant growth hormones. Seed germination was successfully observed in both the culture media incorporated with activated charcoal (AC) in the absence of any growth hormones. Highest seed germination was recorded in NAA supplemented Mitra medium while excellent root proliferation was also found in the same combination when AC was added. When BAP was present in association with IBA in both the culture media,

germination of seed was very poor with culture growth not observed beyond protocorm stage. However addition of AC alleviated the above problem and seedlings were successfully developed. Maximum leaf formation was found in BAP supplemented media with AC. The seedlings generated were successfully established in the green house after proper hardening. The *in vitro* protocol so developed from the present study can be utilized for regeneration of this Cymbidium orchid for commercial and conservational purposes.

Keywords: Orchidaceae, Cymbidium eburneum, in vitro, immature seeds, protocorm

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1 Introduction

Orchids the wondrous plants with extraordinarily beautiful plants are considered as one of the most advanced and largest family amongst the flowering plants with over 24000 species distributing across the world except in the Antarctic region. They exhibit remarkable diversity in form and growth habit and are regarded as truly luxurious plants producing highly attractive and fascinating flowers. The orchids are marketed both as cut flowers and potted plants with very expensive price tag making them out of reach from the common people [4]. Amongst the Cymbidium orchids, *Cymbidium eburneum* is one of the most popular and sought after orchids in international floriculture market because of their large beautiful showy white flowers with sweet fragrance. The natural population of this once thriving important orchid is fast declining mainly because of heavy collection by the locals and extensive habitat destruction due to deforestation and expansion of town and city. If the present trend of exploitation continues at the existing pace without adopting any conservational strategies, there is an ever increasing risk of this Cymbidium orchid being extinct from the wild. Proper conservation methods have

to be initiated both at the *in vitro* and *in vivo* level. One of the main obstacles to increasing the population of orchids in nature is the inability of the orchid seeds to germinate successfully without infection by mycorrhrizal fungi. However with the development of plant tissue culture techniques, immature seeds of orchids obtained from the green capsules are successfully grown *in vitro* without any fungal association [6]. This had opened a new door for effectively conservation and commercialization of a number of floriculturally important orchids. Using these techniques a number of orchids have been successfully regenerated *in vitro* by many workers [2, 3, 9, 12, 13]. Now understanding the gravity of the problem in hand, the present work has been attempted to successfully conserve the fast receding population of *Cymbidium eburneum* by culturing their immature seeds derived from green capsules *in vitro* using plant tissue culture methods.

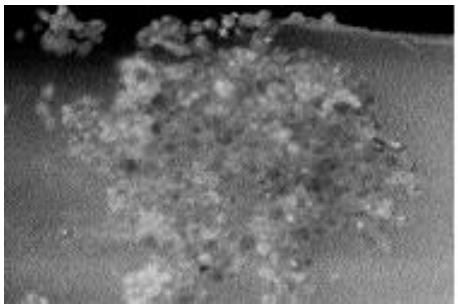

Figure 1: Immature seed germination resulting in spherules formation.

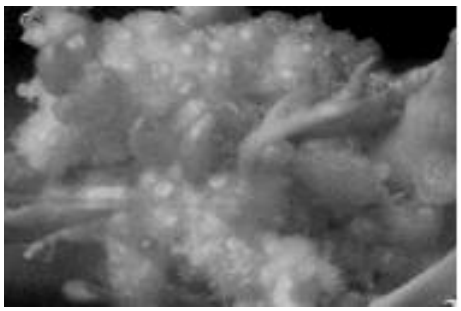

Figure 2: Rapid protocorm formation in AC incorporated Mitra medium.

2 Material and methods

Capsules obtained after 4 months of pollinations were used as explants. The capsules collected were first cleaned in running tap water for 10–15 minutes which was followed by surface sterilization with 0.4% of mercuric chloride solution for about 5–8 minutes

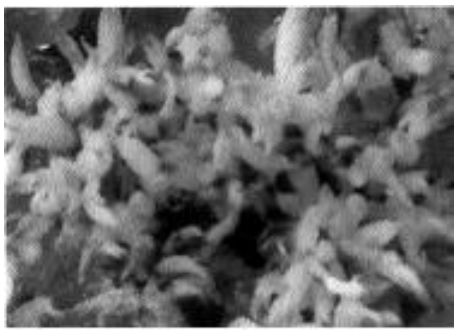

Figure 3: Induction of multiple soot proliferation in BAP and IBA supplemented medium with AC.

Figure 4: Complete plantlet formation in IBA enriched Mitra medium incorporated with AC.

with 1–2 drops of teepol as wetting agent. The capsules were washed finally with sterile distilled water to remove the mercuric chloride from the surface and were opened with knife under sterile condition in laminar flow cabinet to remove immature seeds which were then grown on the culture media to initiate culture. Mitra *et al.* [8] (M) and Murashige and Skoog [10] (MS) medium were used to grow the immature seeds. The culture media contained 0.9% agar, 2% sucrose with pH being adjusted at 5.8. The media were supplemented with plant growth regulators such as Indol-3-butyric acid (IBA), 6-benzylaminopurine (BAP) and Naphthalene acetic acid (NAA) which were used singly or in different combinations. The concentration of hormones in the media was kept at 1 g ml^{-1} for all the combinations. The growth response of the culture media was studied with or without activated charcoal (AC). When AC (0.2%) was incorporated to the culture media, swirling of the culture vessels was done to disperse completely the charcoal before the medium got solidified. Test tubes (25 $\times$ 200 mm) and conical flasks (150 ml) were used as culture vessels and appropriate quantity of the medium was dispensed to the culture vessels before they were tightly closed by cotton

plugs. The medium was then autoclaved at 1.1 kg/cm^2 pressure and 121 °C for 15 minutes and then placed in appropriate positions with test tubes in slanting and flask at vertical positions to allow the medium to gel properly. The cultures were maintained at $25 + 2$ °C with illumination of 3500 lux intensity for 12 hours a day using fluorescent tubes. Subculturing was performed at regular interval to ensure the growth of the culture. The parameters evaluated for the present study were spherule, protocorm, leaf primordium development as well as complete seedling formation in weeks. Regeneration of total number of leaves and roots in each combination was also recorded. 15 replicates were used for each set of experiment. The well developed seedlings complete with roots and leaves were washed sufficiently to remove agar and transferred to pots after proper acclimatization method in reduced concentration of minerals and salts of the culture medium. Sphagnum moss, brick pieces, sand and farmyard manure in 1:1:1 were used as potting mixtures during transplantation.

3 Results and discussion

The immature seeds when grown on the basal *M* medium showed sign of germination in just $2\frac{1}{2}$ weeks by swelling followed by the emergence of embryonal mass with intact tubular suspensor cells from the ruptured seed coat known as spherules in 4 weeks (Figure 1). The spherules after developing chlorophyll differentiated into protocorms in 8 weeks. Complete seedling with leaves and roots are formed in 17 weeks after germination (Table 1). The germination response was very low when the immature seeds were grown on MS medium and only 30% of the seeds germinated successfully. Protocorm development took place in 15 weeks but further development after this stage was not observed and seedlings were not developed (Table 2).

Incorporation of AC in both the culture media significantly improved the germina-

Table 1: *In vitro* regeneration response of *Cymbidium eburneum* in Mitra medium with different growth regulators (1 mg^{-1}).

Medium	Germination response (%)	Average time taken (in weeks) for development of				
		Spherules	Protocorm	1st leaf primordium	2nd leaf primordium	Seedlings
M (Basal)	80	4	8	12	13	17
M* (AC)	90	4	7	9	10	14
M + BAP	45	9	15	–	–	–
M* + BAP	86	4	7	9	10	15
M + NAA	95	4	7	8	9	11
M* + NAA	82	4	8	11	12	16
M + IBA	78	5	9	11	12	18
M* + IBA	85	4	8	10	12	15
M + IBA + BAP	44	10	17	–	–	–
M* + IBA + BAP	84	3	6	11	12	16

Results based on the average of 15 replicates.

*AC was incorporated into the medium.

tion rate with 90% of the germinated in M medium and 70% in MS medium respectively. It also induced rapid protocorm proliferation in the culture (Figure 2). Seedling development was observed in both the cases. When BAP was supplemented singly or in combination with IBA, the germination response was quite low with around 45% and 38% of seeds germinated in *M* and MS medium respectively. The germinated seeds developed into insignificant protocorms and the culture failed to develop beyond the protocorm stage. Healthy seedling formation after the protocorm development was eluded with the combinations. The poor seed germination in the presence of growth regulators suggested that either the young embryos might not have been receptive enough

Table 2: *In vitro* regeneration response of *Cymbidium eburneum* in MS medium with different growth regulators (1 mg^{-1}).

Medium	Germination response (%)	Average time taken (in weeks) for development of				
		Spherules	Protocorm	1st leaf primordium	2nd leaf primordium	Seedlings
MS (Basal)	30	11	15	–	–	–
MS* (AC)	70	7	10	13	14	21
MS + BAP	37	12	16	–	–	–
MS* + BAP	72	7	10	13	14	22
MS + NAA	28	14	17	–	–	–
MS* + NAA	80	8	9	13	14	17
MS + IBA	40	10	15	–	–	–
MS* + IBA	45	9	14	–	–	–
MS + IBA + BAP	38	12	15	–	–	–
MS* + IBA + BAP	75	7	10	13	16	20

Results based on the average of 15 replicates.

*AC was incorporated into the medium.

to evoke germination or the growth regulators might be inhibitory to the germination of the immature seeds. When AC was added in the above combination for both the culture media, the detrimental effect of these growth regulators on the germination and development of the culture was alleviated as there was significant increased in the seed germination. The presence of AC also promoted rapid protocorm formation and shoot multiplication (Figure 3). Leaf formation was significantly enhanced in the above combination for both the culture media (Table 3 & 4). Ernst [5] had indicated the beneficial properties of AC in asymbiotic orchid culture *in vitro* as it accelerated culture growth by absorbing the harmful phenolic exudates released during the culture and also helped

Table 3: Effect of different growth regulators on leaf and root formation of *C.eburneum* after 30 weeks of culture.

Medium	Growth regulators	Number of leaves	Number of roots	Seedling formation
M	–	3.8 ± 0.8	1.8 ± 0.4	Formed
M*	–	4.9 ± 0.5	2.7 ± 0.4	Formed
M	BAP	0	0	Not formed
M*	BAP	5.8 ± 0.6	2.6 ± 0.5	Formed
M	NAA	2.5 ± 0.4	4.2 ± 0.5	Formed
M*	NAA	3.1 ± 0.3	4.7 ± 0.4	Formed
M	IBA	4.0 ± 0.6	1.7 ± 0.4	Formed
M*	IBA	4.2 ± 0.6	2.4 ± 0.6	Formed
M	IBA + BAP	0	0	Not formed
M*	IBA + BAP	4.1 ± 0.9	2.5 ± 0.5	Formed

*Activated charcoal (AC) was incorporated into the medium.

□□ ± indicates the standard deviation values; $N = 15$.

in aerating the media. Similar beneficial effects of AC had also been observed by many workers during *in vitro* culture of several important orchids [1, 14].

Culture showed contrasting response to culture growth when NAA was supplemented in two culture media. In Mitra medium, there was phenomenal rise in the seed germination in presence of NAA with 95% of seed showing successful germination. Rapid protocorm proliferation was also observed which finally differentiated into healthy seedlings with accelerated root formation in 11 weeks. Similar observations for the beneficial effects of NAA on culture growth were earlier reported in other orchids [7, 11]. In MS medium, the positive response of NAA was not found if AC was

Table 4: Effect of different growth regulators on leaf and root formation of *C. eburneum* after 30 weeks of culture.

Medium	Growth regulators	Number of leaves	Number of roots	Seedling formation
MS	–	0	0	Not formed
MS*	–	5.6 ± 0.5	3.6 ± 0.5	Formed
MS	BAP	0	0	Not formed
MS*	BAP	3.6 ± 0.6	2.4 ± 0.5	Formed
MS	NAA	0	0	Not formed
MS*	NAA	4.8 ± 0.4	5.6 ± 0.5	Formed
MS	IBA	0	0	Not formed
MS*	IBA	0	0	Not formed
MS	IBA + BAP	0	0	Not formed
MS*	IBA + BAP	6.2 ± 0.5	4.8 ± 0.5	Formed

*Activated charcoal (AC) was incorporated into the medium.

□□ ± indicates the standard deviation values; $N = 15$.

not incorporated in the medium. Presence of AC in the culture medium was obligatory for successful seedling formation. Incorporation of IBA in the *M* medium produced satisfactory result by generating complete plantlets with leaves and roots either in the presence or absence of AC in the culture medium (Figure 4). However in case of MS medium, the presence of IBA did not help in the culture growth even in the presence of AC. Addition of AC failed to alleviate the inhibitory response of the growth hormone. Culture development did not proceed after protocorm stage and seedlings were not formed in this combination.

The above study indicated variable growth of *C. eburneum* in response to differ-

ent culture media with varied hormonal combinations in presence or absence of AC. The present work further revealed that germination of immature seeds could be successfully observed in basal *M* or MS media which were supplemented with AC. There was successful regeneration of seedlings in both the culture media. Maximum seed germination was observed in NAA supplemented M medium without AC. Proliferric growth of roots was found in NAA supplemented medium with AC and this combination could be seen as the appropriate rooting medium for this orchid species. BAP either singly or in combination with IBA showed detrimental affect to culture growth if AC was not added in the combination. AC improved the overall growth and development of the culture. Maximum leaf formation was recorded in BAP supplemented *M* medium enriched with AC. The presence of AC did not help much in promoting the culture growth in MS medium supplemented with IBA. The seedlings complete with 2–3 leaves and 1–2 roots regenerated successfully from the present study were gradually hardened and transplanted to community pots for necessary acclimatization to the nursery conditions. The *in vitro* protocol that have been established from the present work may be utilized for the rapid regeneration of this floriculturally important orchid on large scale for commercial and conservational purposes.

Acknowledgement

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References

[1] J. Arditti, M.A. Clements, G. Fast, G. Hadley, G. Nishimura and R. Ernst, *Orchid seeds germination and seedling culture—A manual*. In: J. Arditti [ed.], Orchid Biology: Reviews and Perspectives, II (1982) 243–370, Cornell University Press.

[2] J. Arditti and R. Ernst, *Physiology of germinating orchid seeds* In: J. Arditti [ed.], Orchid Biology: Reviews and Perspective, III (1984) 177–222. Cornell University Press.

[3] C. Chang, Y.C. Chen and H.F, Yen, *Protocorm or Rhizome? The morphology of seed germination in Cymbidium dayanum Reichb*., Bot. Bull. Acad. Sin., **46** (2005) 71–74.

[4] S. Chugh, S. Guha and I.U. Rao, *Micropropagation of orchids: A review on the potential of different explants*, Scientia Horticulturae, **122** (2009) 507–520.

[5] R. Ernst, *The use of activated charcoal in symbiotic seedling culture Paphiopedilum*, Amer. Orchid Soc. Bull., **43** (1975) 35–38.

[6] L. Knudson, *A nutrient for germination of orchid seeds*, Am. Orchid Soc. Bull., **15** (1946) 214–217.

[7] V.H. Mathews and P.S. Rao, *In vitro multiplication of Vanda hybrids through tissue culture techniques*, Pl. Sci. Letters. **17** (1980) 283–89.

[8] G.C. Mitra, R.N. Prasad and A.R. Choudhury, *Inorganic salts and differentiation of protocorm in seed callus of an orchid and correlation changes in its free amino acid content*, Indian J. Exp. Biol, **14** (1976) 350–351.

[9] K. Mitsukuri, G. Mori, M. Johkan, Y. Shimada, K.I. Mishiba, T. Morikawa and M. Oda, *Effects of explant position and dark treatment on bud formation in floret culture of Ponerorchis graminiflolia Rchb. f*, Scientia Horticulturae. **121** (2009) 243–247.

[10] T. Murashige and F. Skoog, *A revised medium for rapid growth and bioassays with tobacco tissue cultures*, Physiol. Pl., **15** (1962) 473–479.

[11] H. Shimura and V. Koda, *Micropropagation of Cypripedium marchanthus var. rebunense through protocorm-like bodies derived from mature seeds*, Pl. Cell Tiss. and Organ Cult., **78** (2004) 273–276.

[12] H.S. Tsay, S.F. Lo, S.M. Nalawade, C.L. Kuo and C.L. Chen, *Asymbiotic germination of immature seeds, plantlet development and ex vitro establishment of plants of Dendrobium tosaense Makino-A medicinally important orchid*, In Vitro Cell. Dev. Biol. Pl., **40** No. 5

(2004) 528–535.

[13] S. Vyas, S. Guha, M. Bhattacharya and I.U. Rao, *Rapid regeneration of plants of Dendrobium lituiflorum Lindl. (Orchidaceae) by using banana extract*, Scientia Horticulturae. **121** (2009) 32–37.

[14] M.A. Weatherhead, S.Y. Zee and G. Bairelto, *Some observations on early stages of development of Eulophia yushuiana. Mem. Hongkong, Nat. Soc*, **7** (1986) 85–90.