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REGENERATION COMPETENCE OF LEAF EXPLANTS IN *CYMBIDIUM EBURNEUM* LINDL. (ORCHIDACEAE)

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Abstract

Present paper reports the regeneration competence of leaf segments in a commercially important orchid, *Cymbidium eburneum* Lindl., for its mass multiplication. Nearly 83.3% regeneration response could be evoked on M (Mitra *et al.,* 1976) medium supplemented with 6-Benzyladenine (BA, 0.5 mgl^{-1}) and α-Naphthaleneacetic acid (NAA, 2 mgl^{-1}) within 5.25 wks, and 15.7 plantlets per explants could be regenerated after 30 wks of culture initiation. Cytological studies confirmed the uniform somatic chromosome number of 2n=40 in the regenerants. The protocol can be utilized for rapid mass multiplication of this orchid and aid in alleviating the collection pressures on its natural populations.

Introduction

CYMBIDIUMS, THE robust pseudobulbous orchids possessing long narrow leaves and beautiful fragrant flowers, constitute a group of well prized ornamental plants. Their highly floriferous habit and large sized flowers of vibrant colours make them indispensable in the breeding programmes. *Cymbidium* was the first floricultural crop to have been propagated commercially through tissue culture techniques (Morel, 1960). In addition to their exceptionally beautiful flowers, cymbidiums are also acclaimed for their therapeutic properties and have been widely used in the local systems of medicine (Pal *et al.,* 2020). Many countries like Thailand and Singapore earn a major part of their economy through orchid based trade (Reddy, 2008). India, with its varied climate, affordable labour, and increasing market demand is favorable for orchid based floriculture (Janakiram and Baskaran, 2018; Prakash and Pathak, 2019).

Cymbidium eburneum Lindl. is an epiphyte having short, stout pseudobulbs, linear leaves, and large ivory white, fragrant flowers (Fig. 1A), growing well in warm to cold climates. It is widely distributed in India (Arunachal Pradesh, Darjeeling, Manipur, Meghalaya, Sikkim), Nepal, Burma, and South China within an altitude of 300-1600 m amsl and flowers during the months of March-April (Misra, 2019). Habitat loss and extensive collections for commercial purposes have pushed the species towards rarity. Owing to its immense importance, an effort was made to develop reproducible protocol for *in vitro* propagation of this commercially significant and endangered epiphytic orchid by using its leaf explants. The efficacy of foliar explants for mass propagation has been positively tested by many researchers in a number of orchids including *Aerides multiflora* (Vij *et al*., 2004)*, Cymbidium aloifolium* and *C. iridioides* (Deb and Pongener, 2013), *Dendrobium* sp. (Goswami *et al*., 2015), *Oncidium flexuosum* (Mayer *et al*., 2010), *Phalaenopsis* cv. 'Surabaya' (Balilashaki *et al*., 2015), *Rhynchostylis gigantea* (Pathak *et al*., 2017), *Rhynchostylis retusa* and *Satyrium nepalense* (Vij and Pathak, 1990), *Tolumnia* hybrids (Chookoh *et al*., 2019; Shen *et al.,* 2018), *Vanda coerulea* (Seeni and Latha, 2000), *Vanda tessellata* (Bhattacharjee and Islam, 2017), and *Vanilla planifolia* (Janarthanam and Seshadri, 2008). Present study reports the development of reproducible regeneration protocol by using leaf explants in *Cymbidium eburneum*.

Material and Methods

Leaves (0.5-0.7 cm long) from 30 wks old *in vitro* cultures were segmented into apical and basal halves and cultured on M (Mitra *et al*., 1976) medium and its combinations with two plant growth regulators (PGRs), 6-Benzyladenine (BA) and α-Naphthaleneacetic acid (NAA) at various concentrations (Table 1). The cultures were incubated in the ambience of 25±2°C and 12 hr photoperiod of 3500 Lux light intensity (Florescent tubes-40W; Philips India Ltd., Mumbai). These were subcultured regularly at 4-6 wks intervals depending upon the growth of the cultures. Visual observations were recorded by evaluating the regeneration response, time taken for the initiation of response, regeneration pathway, number of meristematic loci evoked per explant, number of plantlets obtained per explant after 30 wks, and any other significant morphological variation. Mitotic studies were conducted to analyze the cytological fidelity of the regenerants using actively growing root tips. For this, the root tips were pre-treated

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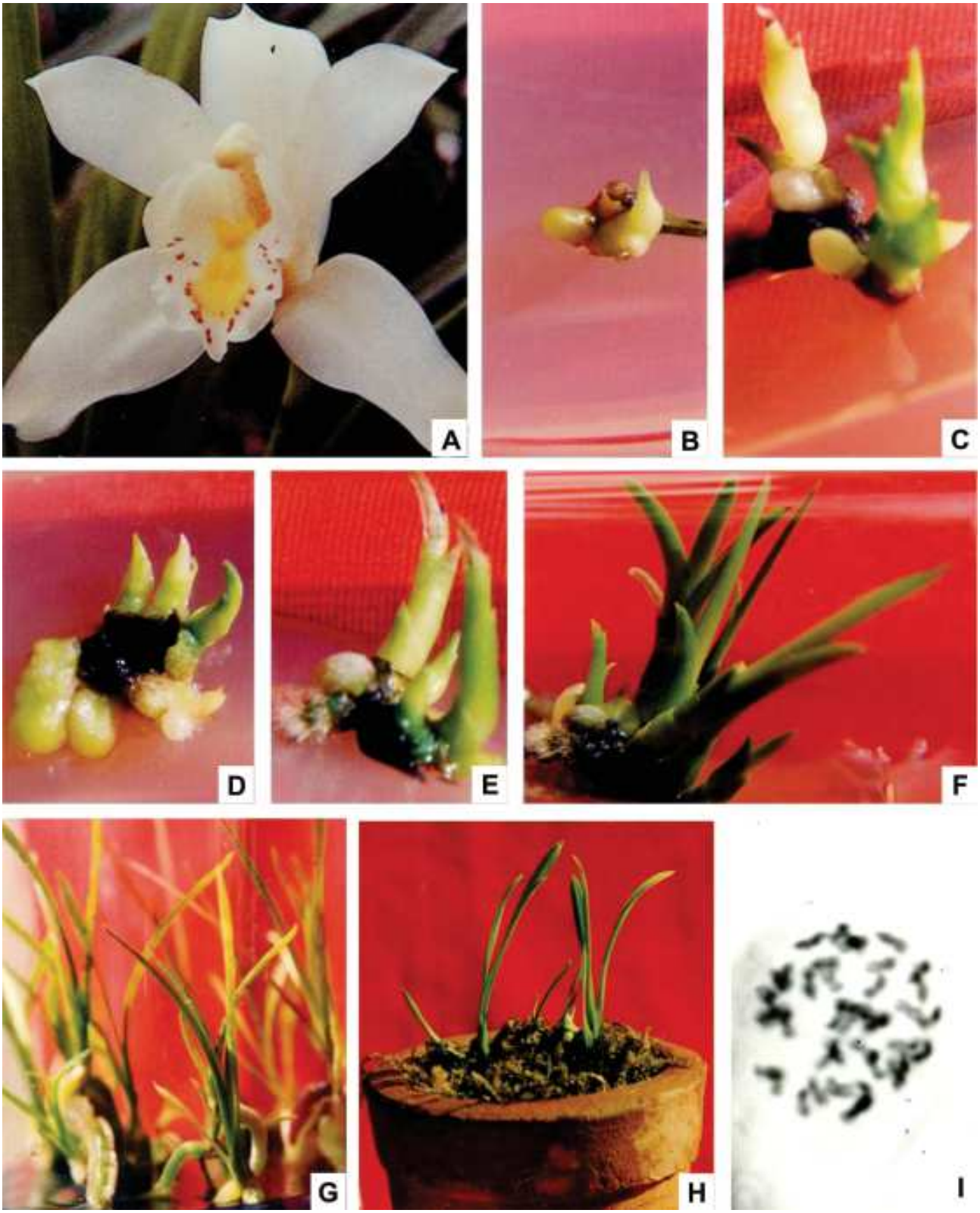

Fig. 1. A-I. Regeneration competence of leaf explants in *Cymbidium eburneum* Lindl.: A, Close up of a large ivory white flower; B, Leaf segment showing regeneration response at its basal region; C, Direct development of PLBs [BA (0.5 mgl^{-1}), NAA (0.5 mgl^{-1})]; D, Enhanced regeneration frequency in medium containing BA (0.5 mgl^{-1}) and NAA (2 mgl^{-1}); E-F, PLB formation and their subsequent development into plantlets [BA (1.0 mgl^{-1}), NAA (0.5 mgl^{-1})]; G, Plantlets with well developed leaves and roots; H, Plantlets transferred to greenhouse; I, Regenerants showing somatic chromosome number of 2n=40.

Table 1. *In vitro* regeneration response of *Cymbidium eburneum* leaf explants on M medium.

Additives (mgl^{-1})	Regeneration response (%)	Regeneration pathway	Number of meristematic loci evoked	Time taken for initiation of response (wks)	Number of plantlets obtained after 30 wks
-	-	-	-	-	-
$BA_{0.5}+NAA_{0.5}$	66.6^{ab}	PLBs-PL	1.00^{a}	4.22^{a}	8.7^{d}
$BA_{0.5}+NAA_{2}$	83.3^{b}	PLBs -PL	1.00^{a}	5.25^{b}	15.7^{e}
$BA_{1}+NAA_{0.5}$	58.3^{ab}	PLBs-PL	1.00^{a}	8.15^{d}	6.7^{c}
$BA_{1}+NAA_{2}$	58.3^{ab}	PLBs -PL	1.00^{a}	10.40^{e}	6.2^{bc}
$BA_{2}+NAA_{2}$	66.6^{ab}	PLBs -PL	1.00^{a}	6.32^{c}	5.2^{ab}

Values in column no. 2, 4, 5, and 6 with same superscripts are not significantly different at $p \leq 0.05$. PLBs, Protocorm like body(ies); PL, Plantlet.

with 0.002M Hydroxyquinoline solution, fixed in 45% Acetic acid solution, and stained with Feulgen Nuclear Reaction. Squashes were prepared in Acetocarmine and mounted on Euparol (Darlington and LaCour, 1942). Results were analyzed using Completely Randomized Design (CRD) by subjecting to One Way Analysis of Variance (ANOVA) to detect the significant differences ($p \leq 0.05$) amongst the treatments using SPSS (Version 17.0) software package.

Results

Vegetative propagation using conventional techniques of rhizome division and back bulb culture has lost its relevance as it is time consuming. Efforts were, therefore, directed towards developing a mass propagation system for the present species using leaf explants (0.5-0.7 cm long and segmented into two halves) sourced from 30 wks old *in vitro* grown plantlets. The results have been summarized in Table 1 and presented here in detail. In the basal medium, the explants remained recalcitrant to regeneration, exuded profusely, turned brown, and perished within 10 wks. Even repeated subculturing proved futile for culture survival. A combined treatment with BA and NAA, proved effective by stimulating a PLBs mediated regeneration response from the basal regions of the explants.

The response, whenever elicited, was invariably restricted to the basal regions of the leaf segments (Fig. 1B). In combinations containing 0.5 mgl^{-1} each of BA and NAA, 66.6% explants responded to direct development of PLBs (Fig. 1C) within 4.22 wks, each of which soon differentiated into a plantlet. On an average, 8.7 plantlets, each with 2-3 leaves and 1-2 roots, could be obtained within 30 wks. The regeneration frequency, however, could be enhanced to 83.3% by increasing the concentration of NAA to 2 mgl^{-1} in the above combination (Fig. 1D). In combinations containing BA (1 mgl^{-1}) and NAA (0.5 mgl^{-1}), 58.3% explants responded via PLBs formation, and further developed into plantlets (Fig. 1E-F). A combination containing 2 mgl^{-1} each of BA and NAA also favored a PLBs mediated regeneration in 66.6% explants within 6.32 wks. More than 5 plantlets complete with 2-3 leaves and 1-2 roots were obtained from each of the PLBs within 30 wks.

The plantlets with 5-6 leaves and 3-4 roots (Fig. 1G) were hardened *in vitro* by gradual elimination of nutrients. They were deflasked into loosely capped jars in the controlled laboratory conditions and kept there until the appearance of new growth signs. These were finally transferred to the greenhouse conditions (Fig. 1H) under low light and high relative humidity conditions. Nearly 60% plantlets survived. Cytological fidelity of the plantlets was confirmed with a uniform somatic chromosome number of 2n=40 (Fig. 1I).

Discussion

Orchids produce large number of minute seeds. Their germination in nature, however, is very poor due to dependency on a suitable symbiotic fungal association. Furthermore, these plants are strictly outbreeders. Therefore, in order to produce true-to-type plantlets, which is highly desirable in pot-plant and cut-flower trade, efforts have been focused on explant based micropropagation methods in orchids (Arditti, 2008; Yam and Arditti, 2009). Evoking of the adventitious meristems for micropropagation has also proved to be beneficial over the use of shoot apical meristem, as it can be easily obtained without harming the mother plant. Wimber (1965) reported for the first time, the regeneration response through PLBs from *Cymbidium* leaves. In the present study, regenerative potential of leaf segments of *Cymbidium eburneum* was positively assessed on M (Mitra *et al.,* 1976) medium. The response proved to be a function of the chemical stimulus in the nutrient pool. Only young leaves (<1 cm in length) were selected as explants as these showed better

morphogenetic responses due to their physiologically active state. Such a differential responses due to the age of the explant under identical nutritional conditions has also been reported earlier (Murthy and Pyati, 2001). In present cultures, the leaves regenerated only along the basal regions which can be attributed to accumulation of nutrients due to the proximity with the axis (Bhattacharjee and Islam, 2017; Chung *et al*., 2007). The response was initiated by formation of PLBs which is a common morphogenetic pathway in orchids (Balilashaki *et al*., 2015; Bhattacharjee and Islam, 2017; Chookoh *et al*., 2019; Janarthanam and Seshadri, 2008; Lukatkin *et al*., 2019; Martin and Madassery, 2006; Vij *et al*., 2002, 2004; Vasundhra *et al.,* 2019). The initiation of meristematic activity in the explants, however, was obligatory to the use of specific PGRs at appropriate concentrations which is in accordance with other reports (Pathak *et al*., 2017; Vij *et al*., 2002) Presently, a combination containing cytokinins and auxins [BA (0.5 mgl^{-1}) + NAA (2 mgl^{-1})] proved to be the best to initiate regeneration in leaf explants; it favoured high regeneration frequency (83.3%), early response, and high proliferations. The requirement of synergistic combinations of cytokinins and auxins for stimulating proliferation and further differentiation is well reported in orchid literature (Balilashaki *et al*., 2015; Bhattacharjee and Islam, 2017; Decruse *et al*., 2003). Cytological fidelity of the plantlets was confirmed with a uniform somatic chromosome number of 2n=40 which is in conformity with the earlier reports by Vij *et al.* (1986).

Conclusion

Cymbidium eburneum Lindl. could be efficiently regenerated using a nutrient mixture containing BA (0.5 mgl^{-1}) and NAA (2 mgl^{-1}). Nearly, 15.7 plantlets could be obtained from each of the explant within 30 wks. This protocol can be effectively used to alleviate the commercial collection pressures faced by this species thereby aiding in its conservation.

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