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IN VITRO PROPAGATION OF *CYMBIDIUM ALOIFOLIUM* SW. THROUGH SHOOT-TIP CULTURE

Short communication

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ABSTRACT

The research aimed to investigate the regenerative capacity of *Cymbidium aloifolium* shoot tips obtained *in vitro*. Shoot-tip explants (< 1–2 mm) of *C. aloifolium* were cultured on Mitra medium supplemented with cytokinins and auxins individually and in combinations. The regenerative response, proliferation of regenerants, and subsequent plantlet development were evaluated. Explants (< 1 mm) remained resistant to regeneration, whereas those 1–2 mm responded to the chemical stimulus. Auxins were highly beneficial in initiating the regenerative response and early plantlet development. Protocorms multiplied best on medium containing sucrose at a concentration of 15 $g \cdot L^{-1}$.

Key words: regeneration, protocorm-like body, auxins, cytokinins

INTRODUCTION

Cymbidium Sw. comprises nearly 70 species of tropical Asian origin with magnificent flowers in erect, semierect, or pendulous racemes (Zotz 2013). In India, this genus is represented by 24 species (Jalal & Jayanthi 2015; Mandal et al. 2018). Cymbidiums are important cut flowers. Most interspecific and intergeneric *Cymbidium* hybrids are known for their long-lasting and exquisitely beautiful flowers. Cymbidiums are among the top ten cut flowers in the international floricultural trade (Chadha 1994). In addition to their importance in floriculture, most *Cymbidium* species are used for their medicinal properties, as a diuretic – *C. ensifolium*, antifertility agent – *C. mandidum*, remedy for dysentery – *C. canaliculatum*, chronic diseases and paralysis – *C. macron* and *C. aloifolium*, kidney diseases – *C. tenuifolium*, and as a flavoring agent – *C. virescence* and adhesive agent – *C. canaliculatum* and *C. lancifolium* (Lawler 1984; Arditti 1992). Cymbidiums were one of the first floricultural crops cultured *in vitro* (Morel 1960). Moreover, some of the *Cymbidium* species are used for culinary purposes (https://www.permacultureproject.com/orchids-are-edible-wildcrafting-indoors-through-the-cold-months/). Due to the corrosive extermination of their natural populations due to indiscriminate collecting for illegal trade and ever-increasing demand, several species such as *C. hookerianum, C. macinnoni, C. macrorhizon, C. tigrinum*, and also *C. aloifolium* have become endangered (Pradhan et al. 2013). *Cymbidium aloifolium* (L.) Sw., is an epiphytic orchid of ornamental value. The species may become extinct if its collection from the wild is not protected for commercial purposes. The entire Orchidaceae family is classified in CITES Appendix I and II (CITES 2024).

In vitro shoot-tip culture is advantageous as it provides (as a rule) clones that exhibit uniformity of phenotypic appearance and genotypic stability.

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In vitro techniques such as shoot-tip culture, meristem culture, micrografting, cryotherapy, etc. have been used to eliminate pathogens from infected stocks to produce disease-free plants (Akin-Idowu et al. 2009). Presently, an attempt has been made to adopt *in vitro* techniques using shoot tips as a material for conserving elite plant *C. aloifolium* as an alternative for *in situ* collections.

MATERIALS AND METHODS

C. aloifolium (Sw.) shoot tips (<1.0–2.0 mm), sourced from 35-week-old axenic cultures, were used as explants. Mitra medium (M) was used as a source of nutrition (Mitra et al. 1976). The medium was gelled with 0.9% agar powder (Hi-Media, Mumbai, India) and sucrose (20 $g \cdot L^{-1}$). The effect of plant growth regulators such as cytokinins (4.44 µM 6-benzylaminopurine – BAP and 4.65 µM furfuryl aminopurine – KN) and auxins (5.37 µM α-naphthaleneacetic acid – NAA and 5.71 µM indole-3-acetic acid – IAA) (Hi-Media, India) was also assessed in multiplication of the regenerants in Mitra medium with activated charcoal (2 $g \cdot L^{-1}$ AC) (Table 1). The pH of the medium was adjusted to 5.8 after adding growth regulators. In another experiment, the effect of different concentrations of sucrose (5, 10, 15, 20, 25, and 30 $g \cdot L^{-1}$) on the multiplication of protocorms was assessed.

The medium was dispensed in the test tubes (25 × 150 mm, Borosil, India) and autoclaved at 121°C at a pressure of 1.06 kg cm^{-2} for 15 min. The cultures were incubated at 25 ± 2 °C under a 12 h photoperiod of 40 µ mol m^{-2} s^{-1} light intensity provided by white fluorescent tubes (40W, Philips India). Eight replicates were used for each treatment. The experiments were repeated, to check the reproducibility of the protocol.

The experiment was set following a complete randomized design. The effect of medium on regeneration percentage, invocation of regeneration response in the explants, the average number of regenerants per explant, and time necessary to form plantlets was checked by applying Tukey's multiple comparison tests ($P \leq 0.05$) in one-way ANOVA to separate of significantly different groups. Statistical analysis was done using SPSS (USA, version 17) software. The results are shown as means ± SD of eight replicates per treatment.

Table 1. Effect of growth regulators on regeneration of *Cymbidium aloifolium* from shoot tip explants in Mitra medium

Treatment	Regeneration frequency (%)	Initiation of response (weeks)	Regeneration pathway	Complete plantlet development (weeks)
M	50.0 ± 0.5[b]	6.3 ± 0.61[a]	C → PLB	28.6 ± 0.02[c]
M+AC	47.0 ± 0.1[c]	6.1 ± 0.52[a]	C → PLB	28.0 ± 0.00[c]
+ BAP + KN	25.0 ± 0.6[d]	5. 0 ± 0.29[b]	PLB	20.0 ± 0.00[d]
+ BAP + KN+AC	25.0 ± 0.3[d]	5.0 ± 0.60[b]	PLB	20.1 ± 0.00[d]
+ IAA	95.0 ±1.2[a]	2.0 ± 0.00[e]	PLB	18.0 ± 0.00[de]
+ IAA+AC	91.3 ±1.0[a]	2.0.00[e]	PLB	17.0 ± 0.00[f]
+ NAA	95.0 ±0.9[a]	2.0 ± 0.00[e]	PLB	18.3 ± 0.90 [de]
+ NAA+AC	91.1 ±1.0[a]	2.0 ± 0.00[e]	PLB	17.0 ± 0.00[f]
+ BAP+IAA	50.0 ± 0.40[b]	4.0 ± 0.50[c]	C → PLB	35.0 ± 0.40[ab]
+ BAP+IAA+AC	50.1 ± 0.3[b]	3.0 ± 0.71[d]	C → PLB	35.6±0.29[ab]
+ KN+IAA	25.6 ± 0.1[d]	4.0 ± 0.20[c]	C → PLB	38.5±0.29[a]
+ KN+IAA+AC	25.1 ±0.8[d]	4.0 ±0.10[c]	C → PLB	36.6±0.29[ab]

M – Mitra medium, PLB – protocorm-like body, C – callus; AC – activated charcoal 2 $g \cdot L^{-1}$, BAP – 6-benzylaminopurine 4.44 µM, KN – furfuryl aminopurine 4.65 µM, IAA – 5.71 µM indole-3-acetic acid, NAA – α-naphthaleneacetic acid 5.37 µM, sucrose 20 $g \cdot L^{-1}$; Data are shown as mean ± standard deviation; Values in a column with the same superscripts are not significantly different at $p \leq 0.05$

RESULTS AND DISCUSSION

In vitro shoot-tip culture is widely used for germplasm multiplication and mass propagation of genotypes, but also for maintaining cultures as a virus-free collection. Both mass propagation and gene bank collections must be clonal without any genetic changes, which is provided in direct regeneration without the callus phase.

In our experiment, explants regenerated in simple medium (Mitra et al. 1976), indicating the low nutritional requirements of explants, but MS medium was also successfully used to initiate a regenerative response (Pradhan et al. 2016; Paul et al. 2019). On the contrary, in experiments by Mulgund et al. (2011), it was found that the initiation of the regenerative response is obligatory and the use of growth adjuvants initiated the response at the shoot tips of *Xenikophyton smeeanum*.

The explants < 1.0 mm in size did not regenerate; they turned necrotic and perished soon, whereas those bigger up to 2 mm responded positively to regenerate protocorm-like body (PLB) via callus. Morphologically, the callus was creamy yellow, compact, and organogenetic (Fig. 1A). On the basal Mitra medium regenerated 50% shoot tips in a time of 6.3 weeks, and plantlets were obtained within 28 weeks. Adding charcoal to the basal medium evoked a similar response, but by 3% smaller. In this experiment, such a response could have happened due to the large size of the explants (larger surface area), which efficiently absorbed more nutrients for survival and initiated the regeneration response (Langens-Gerrits et al. 2003).

Cytokinins (BAP and KN) with and without activated charcoal elicited a response in 25% of initial explants in five weeks. The meristemoids developed directly without any intervening callus stage in the explants and underwent multiplication (Fig. 1B–C). Plantlets were formed within 20 weeks. Induction of the callus stage is not a preferred regeneration pathway as it can lead to genetic variations in the regenerants.

The presence of auxin IAA or NAA with or without AC proved to be highly beneficial. Up to 92% of explants regenerated within 2 weeks (Table 1) by direct development of PLB without intervening callus. Plantlets developed within 17–18 weeks of culture (Fig. 1D). The PLBs multiplied profusely at the base of plantlets in M+IAA+AC supplemented medium (Fig. 1E).

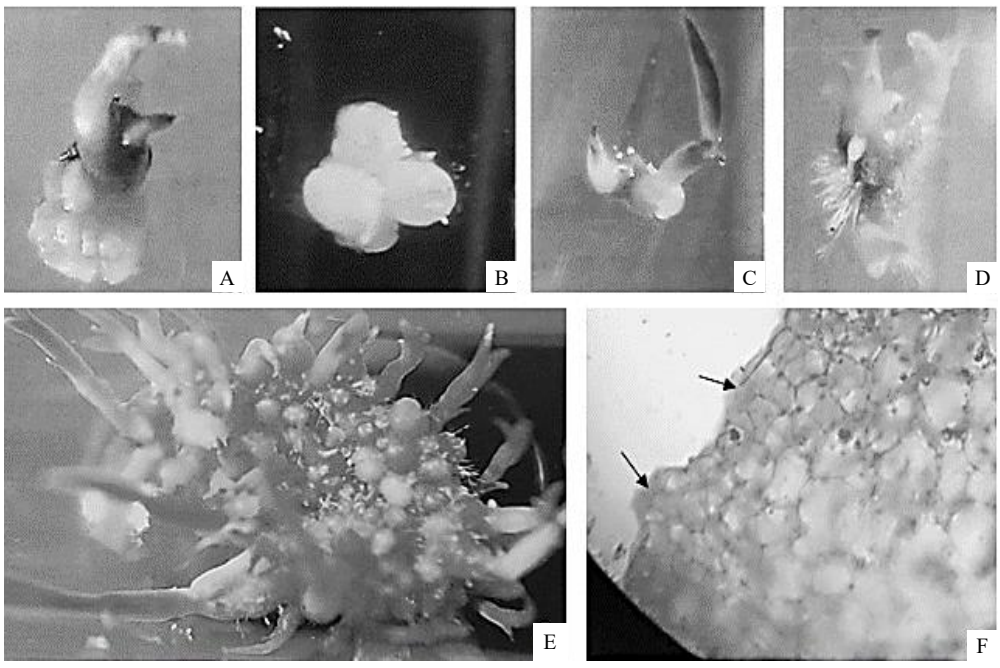


Figure 1. *Cymbidium aloifolium* regeneration from shoot tips on Mitra medium: callus-mediated regeneration of protocorm-like body (PLB) (A), direct regeneration of PLB on BAP and BAP + AC medium (B, C), plantlet formation on IAA (D), profuse multiplication of PLBs and plantlet development on M + IAA +AC (E), histological section showing the origin of the regenerants as epidermal and originating from a group of cells (arrows) (F)

In the present experiment, explants combined with IAA and BAP caused the reaction of 50% shoot tips within 3–4 weeks. The combination of IAA with KN was even worse because only 25% of shoots regenerated. The regeneration began after 3–4 weeks, and shoots were formed after 35–38 weeks. The explants followed the mediated regeneration pathway of regeneration via direct PLB, which is less risky for genetic identity. Earlier studies reported the induction of callus-mediated regeneration of PLBs in a combination of 0.5 mg l^{-1} NAA and 1 mg l^{-1} BAP in *Dendrobium densiflorum* shoot tip explants (Roy & Banerjee 2003). A synergistic action of auxin NAA and cytokinin BAP was reported in the *Vanda tessellate* cultures, where multiple shoots on the shoot meristem explants were formed (Rahman et al. 2009), in *Rhynchostylis retusa* where the combination of IAA and KN favored shoot multiplication in the shoot-tip cultures (Malabadi et al. 2005) and in *Geodorum densiflorum* (Seeni & Latha 2000; Sheelavantmath et al. 2000).

Malabadi et al. (2005) used triacontanol (plant growth regulator) to initiate the regeneration response in the shoot tips of *Dendrobium nobile.* Protocorm-like bodies or proliferating shoot buds were also induced in thin shoot-tip sections cultured on Vacin and Went (1949) medium supplemented with 11.35 μM TDZ (Mulgund et al. 2011).

In this experiment, sucrose concentration influenced regeneration differently. Without sucrose, no regeneration was observed. The pattern of regeneration without callus mediation was obtained at 5, 15, and 20 $g \cdot L^{-1}$. The most effective was 15 $g \cdot L^{-1}$, which regenerated 100% of shoots beginning from the fourth week (Table 2). Higher levels of sucrose (2.5 and 3.0%) reduced regeneration in the cultures and stimulated callus-mediated PLB regeneration. In the present study, profusely multiplying PLBs at low concentrations of sucrose (Fig. 1E) indicates its simple nutritional requirements.

In plant tissue culture, sucrose is an obligatory carbohydrate source to provide energy to cells at all stages of morphogenesis because it can move across the cell membrane (Kumara Swamy et al. 2010). Mainly, it was used at a concentration of 20–30 $g \cdot L^{-1}$ (de Faria et al. 2004; Yoon et al. 2007; Baque et al. 2011). According to the literature study, a higher amount of sucrose hampers the growth and development of cells *in vitro* by terminating the cell cycle (Wu et al. 2006). A similar kind of response was also observed in our experiment, where higher sucrose concentration led to the necrosis of the protocorm-like bodies. In *Dendrobium nobile*, a higher sucrose concentration leads to decreased protocorms number (Udomdee et al. 2014).

Histological observations showed the neoformations (PLBs) to be hypodermal in origin (Fig. 1F). According to Huan et al. (2004), somatic embryogenesis or PLBs formation is the starting point of regeneration in orchids. Somatic embryos can be induced directly from individual somatic cells or indirectly from embryonic callus (Yang & Zhang 2010; Horstman et al. 2017). A similar kind of response was observed in *Malaxis acuminata* (Kaur & Bhutani 2010) *Bulbophyllum careyanum* (Vij et al. 2000), *Dendrobium chrysotoxum* (Kaur 2017). Furthermore, it also indicates the ability of PLBs to have an inherent proliferative potential, and their multiplication seems to be regulated by endogenous hormone levels.

Table 2. The effect of sucrose concentration on the regeneration of *Cymbidium aloifolium* in Mitra medium

Sucrose concentration ($g \cdot L^{-1}$)	Regeneration frequency (%)	Response initiation (weeks)	Regeneration pathway
0	0.0 ± 0.00^{a}	0.0 ± 0.00^{a}	-
5	50.0 ± 0.2^{c}	6.0 ± 0.20^{c}	PLB
10	50.0 ± 0.17^{c}	4.4 ± 0.08^{b}	C → PLB
15	100.0 ± 0.00^{e}	4.0 ± 0.01^{b}	PLB
20	50.0 ± 0.20^{c}	4.3 ± 0.00^{b}	PLB
25	75.0 ± 0.00^{d}	5.4 ± 0.08^{bc}	C → PLB
30	75.0 ± 0.32^{d}	5.2 ± 0.00^{bc}	C → PLB

Note: see Table 1

CONCLUSION

NAA and IAA proved to be the best for maximal regeneration and multiplication of protocorm-like bodies on Mitra medium with 15 $g \cdot L^{-1}$ sucrose. The addition of cytokinin reduced the regeneration frequency and delayed plantlet development. The present study results could be the basis for the *in vitro* conservation of *Cymbidium aloifolium* to prevent its extinction.

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