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Morphogenesis of *Cymbidium atropurpureum in-vitro*

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ABSTRACT Shoot tips excised from the orchid, *Cymbidium atropurpureum,* produced protocorm-like bodies (PLB) on hormone-free Vacin and Went medium and hormone supplemented Vacin and Went (VW) medium. Explants showed the highest PLB formation (63.33%) on the medium supplemented with 5.0 mg/l of α-naphthaleneacetic acid (NAA). The PLBs obtained were regenerated on hormone-free Murashige and Skoog (MS) medium. Besides PLB formation, callus formation from shoot tips was also observed in this study. Pseudobulb, rhizome and root explants produced callus on 1/2 strength MS medium supplemented with 10 mg/l 2,4-dichlorophenoxyacetic acid and 0.1 mg/l of thiadiazuron. The callus was maintained by sub-culturing in the same medium. Further experiments to determine if the callus obtained is organogenic or embryogenic is in progress.

ABSTRAK Eksplan hujung pucuk daripada *Cymbidium atropurpureum* berjaya megaruhkan struktur menyerupai protokorm (PLB) apabila di kultur di atas media Vacin dan Went (VW) yang berhormon dan tidak berhormon. Medium yang mengadungi 5.0 mg/l α-naphtaleneacetic asid (NAA) berjaya mengaruhkan PLB yang terbanyak iaitu sebanyak 63.33% daripada eksplan hujung pucuk. Plantlet yang lengkap dapat diperolehi dari PLB dengan menggunakan media Murashige dan Skoog (MS) tanpa hormon. Pertumbuhan kalus juga diperhatikan pada eksplan hujung pucuk. Eksplan pseudobulb, rhizom dan akar juga berjaya meghasilkan kalus apabila dikultur di atas medium MS yang mengandungi 10 mg/l 2,4-dichlorophenoxyacetic asid dan 0.1 mg/l thiadiazuron. Kalus yang terbentuk di sub-kultur di atas media yang sama. Kalus yang terbentuk digunakan untuk eksperimen selanjutnya yang masih dijalankan bagi menentukan samada kalus yang terbentuk adalah organogenik atau embriogenik.

(Protocorm-like bodies, shoot tip, morphogenesis, regeneration, callus, pseudobulb).

INTRODUCTION

The *Cymbidium* orchid is either terrestrial or epiphytic. This genus consists of about 50 species, found in Madagascar, in Asia from Ceylon and India to Japan, through Malaysia to Australia [1]. Most of the species are decorative, and some are very handsome, especially those from the mountains of Burma. According to Holtum [1], in Malaysia nine species have been reported, and a tenth probably occurs; one of the nine species is however somewhat doubtful. Initially orchids were propagated vegetatively as well as generatively. With vegetative propagation, the progeny is identical to the parent plants. However, with generative propagation (by seed), identical progeny are rarely obtained. Therefore, if seeds from a cultivated orchid are used, the progeny will be extremely heterogeneous, seldom identical to the starting material. In principle, cultivated orchids can only be propagated vegetatively. Orchid cloning *in-vivo* is a very slow process, requiring sometimes 10 years before a clone is obtained [2]. In 1960, Morel [3] developed a conventional method for vegetative propagation of orchids to obtain virus-free *Cymbidiums* by shoot tip culture. Protocorm-like bodies (PLBs) were obtained from these shoot tips, which were extremely similar to those already well known from seed germination. The PLBs can be proliferated by sectioning the PLBs into pieces, and each piece will produce new PLBs. When the sectioning stops, each PLB develops into normal plant as does the embryo. Sagawa *et al.* [4] developed one of the early methods for the culture of *Cymbidium* shoot tip explants. He obtained PLB from the shoot tip explants after six weeks of culture on hormone-free Vacin and Went (VW) medium [5]. Kim and Kako [6] also obtained PLBs from shoot apices

of *Cymbidium* cultured on solid medium of Murashige and Skoog [7] supplemented with 0.1 mg/l of 6-benzylaminopurine (BAP). However, according to Hasegawa et al [8], propagation of the genus *Cymbidium*, either by seed or by *in-vitro* culture, is still considered difficult.

Orchid callus is considered to be difficult to induce and subculture [9, 12,13,14,]. For example, Begum [9] obtained globular compact callus from PLB inner tissue of a *Cymbidium* sp. These calli turned brown and died after 2 months and subculture was not achieved. Steward and Mapes [10] in his paper reported that, *Cymbidium* plants could be indefinitely multiplied as cell cultures (as distinct from proliferated calluses), and made to give rise, at will, to globular forms, which then develop into PLBs and thence form plants. He obtained callus from shoot apex of *Cymbidium* cultured on solid White's medium [15] supplemented with 10% (v/v) coconut water (CW) and 5.0 mg/l α-napthaleneacetic acid (NAA). The resulting callus was inoculated into a liquid medium supplemented with 5.0 mg/l of 2, 4-dichlorophenoxyacetic acid (2,4-D) and CW for the production and multiplication of free cells. In *Cymbidium ensifolium*, totipotent calli were induced from sections of pseudobulb, rhizomes and roots, which was used for morphogenetic studies [11]. On the other hand, Colli and Kerbauy [12] induced callus from root tip of *Catasetum fimbriatum* in the presence of exogenous auxins in the media. Kerbauy [13] reported that high concentration of NAA favoured callus induction from the root tip culture of *Oncidium varicosum* where by root apices of *Cattleya* form callus when cultured on media containing 2, 4-D [14]. In this paper, we describe the growth and multiplication of PLBs of *Cymbidium atropurpureum* through the conventional shoot tip culture and also callus induction from different types of explants of this species.

EXPERIMENTAL METHODS

Explant Materials

Explants were excised from *C. atropurpureum* plantlets raised *in-vitro*. The plantlets from which the explants were derived were maintained in the culture room with 16 hour photoperiod, at 25 ±1°C.

PLB Induction

The donor plantlets were surface sterilized with 2% freshly prepared calcium hypochlorite (v/v) before excising the shoot-tips. The shoot tips were excised under sterile condition using sterile tools. The excised shoot tips were immediately placed on VW medium [5] with 15% coconut water (v/v), 2% sucrose (w/v) and 0.8% difco-bacto agar (w/v). The VW medium was supplemented with various concentrations of BAP and NAA either individually or in various combinations (Table 1). The pH of the media was adjusted to 5.2-5.5 prior to autoclaving at 121°C for 25 minutes (min). The cultures were kept under 16 hour photoperiod at 25±1°C. The PLBs derived from the shoot tip cultures were propagated in the MS [7] liquid culture medium with 3% sucrose (w/v). The cultures were incubated at the same condition as the shoot tip cultures on a rotary shaker at 120 rotations per minute (rpm). After 2 months of culture, the percentage of explants forming PLB was recorded and the morphology of the PLBs was observed.

Callus Induction

In this investigation, callus induction method described by Chang [11] with minor modifications, was followed. Leaves, roots, pseudobulbs and rhizomes excised from *in-vitro* grown plantlets were used as explants for callus induction. Explants were placed on the basal medium (BM) with 1/2 strength macro- and microelements of MS medium [7] supplemented with (mg/l) myo-inositol (100), nicotinic acid (0.5), pyridoxine HCL (0.5), thiamine HCL (0.1), glycine (2.0), peptone (1000), sucrose (20,000) and geltrite (2200). The BM was supplemented with 10mg/l of 2,4-dicholorophenoxyacetic acid (2,4-D) and 0.1mg/l of *N*-phenyl-*N'*-1,2,3-thidiazol-5-ylurea (TDZ) as plant growth regulators. The pH of the medium was adjusted to 5.2-5.5 prior to autoclaving at 121°C for 25 min. Cultures were incubated at 25±1°C in total darkness. Callus formation was observed after 1-2 months of culture. After 1-2 months of culture, the percentage of explants forming callus was recorded and the morphology of callus was observed.

RESULTS AND DISCUSSION

PLB induction

Shoot tips of *C. atropurpureum* produced PLB on hormone supplemented media, as well as,

hormone-free VW medium (Table 1). The initiation of PLB formation was observed in hormone supplemented media, 60 days after culture, whereas in hormone-free VW medium, initiation of PLB was delayed and was observed after 75 days of culture. About 43.33 % of explants cultured on hormone-free medium produced light green callus.

The pattern of PLB formation was similar in both hormone supplemented and hormone-free medium. The whole explant starts swelling, small meristematic areas appear in the axis of the leaves or at their bases. Most of these outgrowths remain undifferentiated for a month and gradually increases in size and turn into a green globular PLB, often some leaf primordium extends slowly.

Media supplemented singly with 5.0 mg/l NAA increased the number of explants producing PLBs (Table 1). About 63.33% explants produced large green globular PLBs on media supplemented with 5.0 mg/l NAA (plate 1) The media supplemented with 1.0/1.0 mg/l NAA/BAP and 1.0/1.5 mg/l NAA/BAP produced small green PLBs with extended leaf primordium. The PLBs obtained were propagated on MS liquid culture medium supplemented with 3% sucrose (w/v) and placed on a rotary shaker at 120 rpm in 25 ±1 °C. Plants were regenerated from these PLBs by placing the PLBs on hormone-free MS solid medium (plate 2).

There are many reports on micropropagation of *Cymbidium* using shoot-tips or PLB as explants [6, 16, 17, 18, 19]. Many orchid species requires auxins and /or cytokinins for PLB formation and plantlet development [20]. The combinations, concentration and the ratio of auxin to cytokinin for PLB formation vary from species to species. This experiment showed that the number of PLB was high on media supplemented with NAA alone compared to on media containing NAA and BAP in combination. Although the number of PLB on media supplemented with 1.0/1.0 mg/l NAA/BAP and 1.5/1.0 mg/l NAA/BAP was higher than the hormone-free medium, it was lower than on media supplemented with NAA singly at high concentration. These results suggest that PLB formation was enhanced by addition of NAA to the medium (Table 1). Ueda and Torikata [16] reported that Knudson C medium (with Nitsch's microelements) supplemented with NAA produced shoots in *C. goeringii*. Kim and Kako [6] obtained similar results as to when shoot-tips were used as explant. But Wang *et al* [17] reported that modified White's medium supplemented with NAA produced PLBs in *C. ensifolium* shoot-tip culture. These results indicate that *Cymbidium* may respond to different NAA levels in different way.

Besides PLB formation, callus formation was also observed in this experiment. Small nodular callus was observed on media supplemented with 1.0 mg/l NAA. Fourty percent of the explants cultured on this media was able to produce callus, where, 36.67% of explants produced large yellow callus on media supplemented with 10.0 mg/l of NAA. Small, soft yellow callus was observed on media supplemented with 5.0/0.5 mg/l NAA/BAP.

There are very few reports on the callus formation of *Cymbidium* and it is considered to be rather difficult. Steward and Mapes [10] obtained callus from the shoot apex of *Cymbidium*. It was reported that they also used NAA for callus induction. There have been a number of reports on callus formation in other orchids, in which explants inducing callus in *Vanda* shoot-tips by supplementing the VW media with coconut milk; Morel [22] obtained similar results with contained meristematic or epidermal cells [21,22]. Kunisaki *et al* [21] succeeded in *Paphiopedilum* using 2,4-D

Callus Induction

Callusing from explants was rather fast compared to the results obtained by Chang [11]. About 80% of the pseudobulb explant, 48% of the rhizome explant and 10% of the root explant successfully produced calli after 1-2 months. Swollen and granular callus formed from the lateral buds of rhizomes. Soft, creamy callus mass proliferated from the psuedobulb explants. Brownish and soft calli proliferated from the necrotic tissues of the root explants. No callus was observed from the leaf explants. The callus was maintained by sub-culturing on the same medium.

Orchid plants produce callus from shoot-tips, leaves, bud, and PLB [9, 10, 13, 22]. But basically these works were focused on macroscopic findings. It is not certain whether the callus obtained is organogenic or embryogenic. Begum *et al* [9] and Steward and Mapes [10] reported the formation of

embryogenic callus in *Cymbidium*. Ishii *et al* [23] suggest that PLBs derived from calli could be considered as somatic embryos and the callus induced as embryogenic. Further experiments to determine if the callus obtained from this study is organogenic or embryogenic is in progress.

Table 1. Effects of BAP and NAA on PLB induction from shoot-tip of *Cymbidium atropurpureum.*

VW media supplemented with hormone (mg/l)		Percentage of explants showing results		Morphology
NAA	BAP	PLB	CALLUS	
0.0	0.0	43.33	-	Yellowish green PLB
1.0	0.0	-	40.00	Small nodular callus
1.0	1.0	53.33	-	Small green PLB with leaf primordium
1.5	1.0	46.67	-	Small green PLB with leaf primordium
5.0	0.0	63.33	-	Large, green, globular PLB
5.0	0.5	-	33.33	Small, soft, yellow callus
10.0	0.0	-	36.67	Large, yellow callus

Note: Results of other combinations of BAP and NAA is not presented in Table 1 because it does not show the significance of this experiment.

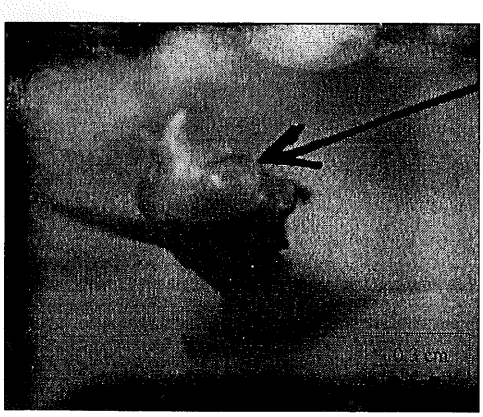

Plate 1. Large green globular PLB form on VM media supplemented with 5.0 mg/l NAA (arrow indicates PLB).

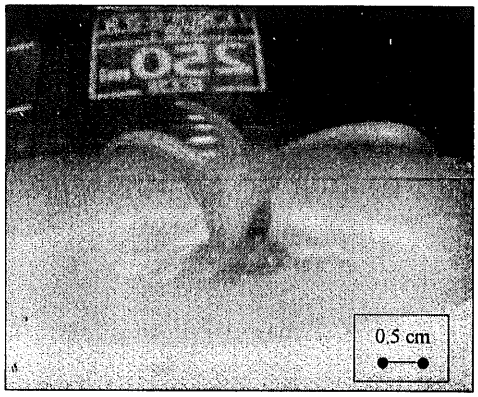

Plate 2. Plantlet formation on hormone-free MS medium

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