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Callus formation and plant regeneration from callus through somatic embryo structures in *Cymbidium* orchid

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Abstract

Embryogenic calli were induced from longitudinally bisected segments of protocorm-like bodies (PLBs) of *Cymbidium* Twilight Moon 'Day Light', a hybrid orchid, on modified Vacin and Went medium [Bot. Gaz. 110 (1949) 605] supplemented with 1-naphthaleneacetic acid (NAA) or 2,4-dichlorophenoxyacetic acid (2,4-D) alone or in combination with *N*-phenyl-*N*′-1,2,3-thiadiazol-5-yl urea (TDZ) within 1 month. The medium containing the combination of 0.1 mg l^{-1} NAA and 0.01 mg l^{-1} TDZ was optimal for callus formation. Calli proliferated very well when being subcultured every 4 weeks on the same medium. The PLB formation from callus was achieved when callus was transferred to the medium without plant growth regulators. Histological observation proved the somatic embryo structure formation. Callus-derived PLBs converted into normal plants with well-developed shoots and roots on the medium without plant growth regulators after about 4 months, which were acclimatized in the greenhouse with 100% survival rate. Among 103 twenty-month-old regenerated plants, no morphological variations were observed.

Keywords: Callus formation; *Cymbidium*; Plant regeneration; Protocorm-like bodies; Somatic embryo structures

1. Introduction

Cymbidium is a genus of about 50 species from Asia. Most of the cultivated Cymbidiums are hybrids [1]. Many attractive hybrids of *Cymbidium* orchid have become commercially important in cut flower and potted plant industries. *Cymbidium* was the first orchid genus to be propagated using shoot-tip culture by Morel [2]. Since then, there are many reports on micropropagation of *Cymbidium* using shoot-tip culture or protocorm-like bodies as explants [3–8]; however, there are very few reports on callus cultures in *Cymbidium* as well as other orchids, this might be due to the slow growth and a necrotic tendency of orchid callus [9–13]. Begum et al. [13] reported that globular compact calli were induced from inner tissue of *Cymbidium* PLB, but these structures could not be subcultured, turned brown and died after 2 months of incubation. Chang and Chang [14] successfully obtained calli from pseudobulbs, rhizomes, and root explants and maintained these in subculture as well as regenerated plants from callus in *Cymbidium ensifolium*, a terrestrial orchid species; however, this study did not focus on callus induction from explants. Plant regeneration from callus of orchid is usually achieved through PLB formation, a process that is suggested to involve somatic embryogenesis [13,15–17]. However, clear evidence of somatic embryogenesis in callus cultures of orchid is still limited.

This study presented optimal conditions for callus formation from PLB segments, callus proliferation, and plant regeneration from callus in *Cymbidium* Twilight Moon 'Day Light'.

2. Materials and methods

2.1. Plant material and culture condition

Protocorm-like bodies (PLBs) of *Cymbidium* Twilight Moon 'Day Light' originated from shoot-tip culture were subcultured every 2 months on modified Vacin and Went medium [18] supplemented with 0.1 mg l^{-1} 1-naphthaleneacetic acid (NAA) and 0.1 mg l^{-1} kinetin,

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and solidified with 8 g l^{-1} Bacto agar (Difco Laboratories, USA). PLBs were cut tops and then longitudinally bisected. The PLB segments were used as explants for callus induction.

Modified Vacin and Went culture medium [18] supplemented with 1 ml l^{-1} Nitsch microelements [19], 2 g l^{-1} tryptone, and 20 g l^{-1} sucrose and solidified with 8 g l^{-1} Bacto agar was used as a basal medium, unless otherwise noted. In all media, the pH was adjusted to 5.3 with 1 N NaOH or HCl before autoclaving for 17 min at 100 K Pa. Cultures were kept at 25 °C, under a 16-h photoperiod with a light intensity of 45 μmol m^{-2} s^{-1} provided by plant growth fluorescent lamps (Homo Lux, Matsushita, Japan).

2.2. Effects of plant growth regulators on callus formation from PLB segments

The basal medium was supplemented with NAA (0, 0.05, 0.1, 0.25, 0.5, 0.75, 1.0, and 2.0 mg l^{-1}) or 2,4-dichlorophenoxyacetic acid (2,4-D; 0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.25, and 0.5 mg l^{-1}) alone or in combination with *N*-phenyl-*N*′-1,2,3-thiadiazol-5-yl urea (TDZ; 0, 0.001, 0.01, and 0.1 mg l^{-1}) for examining the effects of plant growth regulators on callus formation from PLB segments. Ten longitudinally bisected PLB segments were cultured in 100-ml Erlenmeyer flasks containing 40 ml of medium. Forty explants were used for each treatment and the experiment was repeated four times. Data were recorded after 1 month of culture.

2.3. Effects of tryptone on callus proliferation

Callus pieces of about 40 mg induced from PLB segments were cultured on to the same medium containing different concentrations of tryptone (0, 2, and 4 g l^{-1}) to examine the effects of tryptone on callus proliferation. Five callus pieces were cultured in 100-ml Erlenmeyer flasks containing 40 ml of medium. Thirty explants were used for each treatment and the experiment was repeated three times. Callus growth was evaluated using the ratio between the final fresh weight and the initial fresh weight. Data were recorded after 4 weeks of culture.

2.4. Effects of coconut water and sucrose on PLB formation from callus

Five subcultured callus pieces (about 40 mg each) were cultured in 100-ml Erlenmeyer flasks containing 40 ml of the basal medium or the basal medium supplemented with 200 ml l^{-1} coconut water, which was from green coconuts, in the presence or absence of sucrose to examine the effects of coconut water and sucrose on PLB formation from callus. Thirty explants were used for each treatment and the experiment was repeated three times. The number of PLBs per explant, and fresh and dry weights of PLB clusters regenerated from each callus were recorded after about 2 months of culture.

2.5. Histological observation

Tissues for histological observation were fixed in FAA (formalin:acetic acid:70% ethanol = 1:1:18 v/v/v), dehydrated in a butanol series, and embedded in paraffin wax. The materials embedded in paraffin were sectioned at 10 μm and stained with hematoxylin. The observation and photography were done using light microscope (Olympus, Japan).

2.6. Plant regeneration

Single PLBs derived from callus were transferred to 100-ml Erlenmeyer flasks containing 40 ml of basal medium for plant regeneration. Regenerated plants were transplanted and grown in the greenhouse.

The data were subjected to analysis of variance and means were separated using Duncan's multiple range test.

3. Results and discussion

3.1. Effects of plant growth regulators on callus formation from PLB segments

PLB segments were cultured on media containing different combinations of NAA, 2,4-D, and TDZ for callus induction. The basal medium without plant growth regulators was used as a control. Yellow and granular calli were formed from PLB segments within 1 month (Fig. 1). While there was no callus formation in the control, calli were formed from PLB segments in most treatments with different frequencies (Tables 1 and 2). The highest frequency of callus formation from PLB segments was observed on the medium containing 0.1 mg l^{-1} NAA and 0.01 mg l^{-1} TDZ. Besides forming only callus, few explants formed callus together with PLBs on some media. Treatments with 0.001, 0.01, 0.1 mg l^{-1} TDZ, 0.05, 2.0 mg l^{-1} NAA, 0.001 mg l^{-1} 2,4-D with or without 0.001 mg l^{-1} TDZ, 0.005, 0.1, and 0.25 mg l^{-1} 2,4-D resulted in the formation of PLBs from explants, but not of callus. For a given concentration of NAA or 2,4-D, the addition of TDZ increased percentage of explants forming calli. Furthermore, NAA (0.05, and 2.0 mg l^{-1}) or 2,4-D (0.005, 0.1, and 0.25 mg l^{-1}) used alone induced no callus from PLB segments, but induced callus when combined with TDZ. The concentration of 0.5 mg l^{-1} of 2,4-D resulted in death of all explants when utilized alone or with TDZ. Among tested treatments, NAA proved to be more effective than 2,4-D in callus induction from PLB segments. However, there was no significant difference between calli formed on NAA-containing media and those formed on 2,4-D-containing media.

In orchid, successful reports on callus formation is limited. Callus formation in *Cymbidium* was reported by Begum

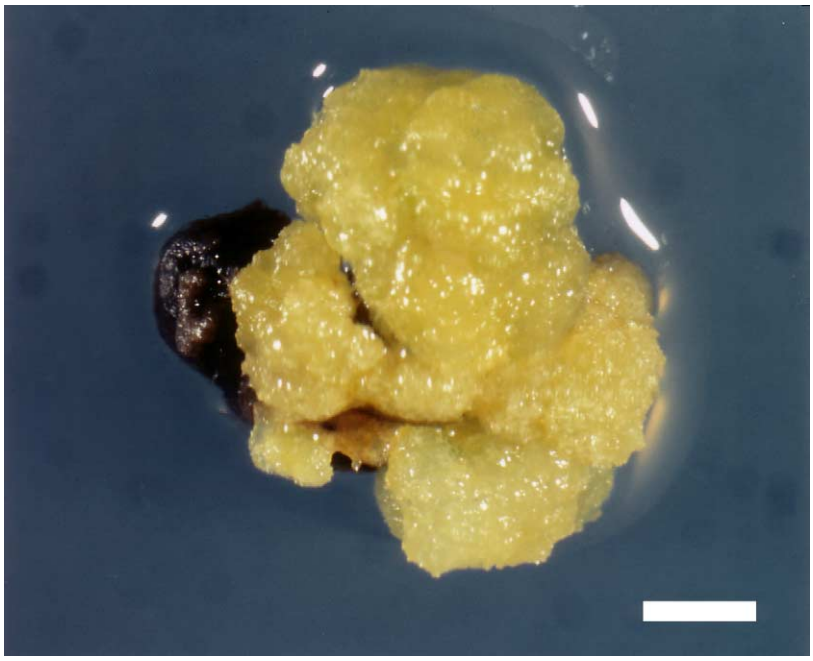

Fig. 1. Callus formation from PLB segment. Bar: 0.2 cm.

et al. [13] but these callus structures could not be maintained and died after 2 months in culture. More recently, callus was obtained from several kinds of explants in *C. ensifolium*, a terrestrial orchid [14]; however, this study did not focus on callus induction, in which, callus formation from explants was carried out on one medium containing plant growth regulators at high level after a long time. In the research presented here, callus formation from PLB segments in *Cymbidium* Twilight Moon 'Day Light' was examined with various combinations of NAA, 2,4-D, and TDZ. The results indicate the important role of exogenous plant growth regulators on callus formation in *Cymbidium*. This

Table 1
Effects of NAA and TDZ on callus formation from PLB segments of *Cymbidium* Twilight Moon 'Day Light'

Plant growth regulators ($mg\,l^{-1}$)		Explants formed callus and/or PLBs (%)			
NAA	TDZ	Callus	Callus + PLBs	PLBs	Died
0	0	0 h	0 a	92.5 a	7.5 k
0	0.01	0 h	0 a	92.5 a	7.5 k
0	0.1	0 h	0 a	90.0 a	10.0 j,k
0.05	0	0 h	0 a	92.5 a	7.5 k
0.05	0.01	10.0 d,e	5.0 a	75.0 b,c	10.0 j,k
0.05	0.1	7.5 e,f	2.5 a	80.0 b	10.0 j,k
0.1	0	17.5 c	0 a	67.5 d	15.0 i,j,k
0.1	0.01	50.0 a	2.5 a	30.0 j	17.5 h,j,k
0.1	0.1	25.0 b	2.5 a	47.5 f	25.0 g,h
0.25	0	10.0 d,e	0 a	70.0 c,d	20.0 h,i
0.25	0.01	20.0 c	2.5 a	42.5 f,g,h	35.0 e,f
0.25	0.1	17.5 c	5.0 a	45.0 f,g	32.5 f,g
0.5	0	5.0 f,g	0 a	57.5 e	37.5 d,e,f
0.5	0.01	27.5 b	2.5 a	32.5 i,j	37.5 d,e,f
0.5	0.1	20.0 c	2.5 a	42.5 f,g,h	35.0 e,f
0.75	0	2.5 g,h	0 a	55.0 e	42.5 c,d,e
0.75	0.01	12.5 d	0 a	47.5 f	40.0 d,e,f
0.75	0.1	10.0 d,e	0 a	45.0 f,g	45.0 b,c,d
1.0	0	2.5 g,h	0 a	45.0 f,g	52.5 b
1.0	0.01	12.5 d	2.5 a	40.0 g,h	45.0 b,c,d
1.0	0.1	12.5 d	0 a	37.5 h,i	50.0 b,c
2.0	0	0 h	0 a	30.0 j	70.0 a
2.0	0.01	10.0 d,e	0 a	27.5 j,k	62.5 a
2.0	0.1	7.5 e,f	0 a	22.5 k	70.0 a

Means within a column followed by the same letters are not significantly different at $P < 0.05$ according to Duncan's multiple range test.

Table 2
Effects of 2,4-D and TDZ on callus formation from PLB segments of *Cymbidium* Twilight Moon 'Day Light'

Plant growth regulators (mg l^{-1})		Explants formed callus and/or PLBs (%)			
2,4-D	TDZ	Callus	Callus + PLBs	PLBs	Died
0	0	0 g	0 b	92.5 a	7.5 k
0	0.001	0 g	0 b	92.5 a	7.5 k
0	0.01	0 g	0 b	92.5 a	7.5 k
0	0.1	0 g	0 b	90.0 a,b	10.0 j,k
0.001	0	0 g	0 b	90.0 a,b	10.0 j,k
0.001	0.001	0 g	0 b	90.0 a,b	10.0 j,k
0.005	0	0 g	0 b	85.0 b,c	15.0 i,j
0.005	0.001	2.5 f,g	0 b	82.5 b,c	15.0 i,j
0.01	0	5.0 e,f	0 b	70.0 d	25.0 h
0.01	0.01	27.5 a	7.5 a	45.0 e,f	20.0 h,i
0.05	0	7.5 d,e	0 b	47.5 e	45.0 f
0.05	0.01	20.0 b	2.5 b	40.0 f,g	37.5 g
0.1	0	0 g	0 b	40.0 f,g	60.0 d
0.1	0.01	15.0 c	0 b	32.5 h	52.5 e
0.1	0.1	10.0 d	0 b	35.0 g,h	55.0 d,e
0.25	0	0 g	0 b	20.0 i	80.0 b
0.25	0.01	7.5 d,e	0 b	20.0 i	72.5 c
0.25	0.1	5.0 e,f	0 b	20.0 i	75.0 b,c
0.5	0	0 g	0 b	0 j	100.0 a
0.5	0.01	0 g	0 b	0 j	100.0 a
0.5	0.1	0 g	0 b	0 j	100.0 a

Means within a column followed by the same letters are not significantly different at $P < 0.05$ according to Duncan's multiple range test.

is in agreement with other researches on callus formation in orchid [13,17,20,21]. Though TDZ alone induced no callus, the addition of TDZ to auxin-containing media improved callus formation in both cases of NAA and 2,4-D. Similar stimulatory effects of TDZ in combination with 2,4-D to callus formation were also observed in *Paphiopedilum* [20]. On the contrary to long time required for callus formation in some other reports such as 12–18 months for callus formation from pseudobulb sections, rhizomes, and roots of seed-derived plantlets in *C. ensifolium* [14] or 7 months for callus formation from shoot-tips excised from flower stalk buds in *Phalaenopsis* [22], only about 1 month was required for inducing callus from PLB segments in this research. This might depend on species, culture condition, as well as explant tissue.

3.2. Effects of tryptone on callus proliferation

Calli induced from PLB segments were subcultured on the medium containing 0.1 mg l^{-1} NAA, 0.01 mg l^{-1} TDZ, and different concentrations of tryptone to investigate the effects of tryptone on callus growth. Callus pieces continued to proliferate after subculture. The presence of tryptone in medium significantly improved the growth of callus (Table 3). Callus proliferated very well on the medium supplemented with 2 g l^{-1} tryptone (Fig. 2). The higher concentration of tryptone (4 g l^{-1}) was not suitable for callus growth; some explants had small brown parts.

In orchid, the maintenance of induced callus is often difficult and the growth of callus is often slow. The low growth rate of callus derived from root apices was reported in *Cattleya* [10]. Begum et al. [13] obtained calli from inner tissue of protocorm-like bodies in *Cymbidium* but these structures turned brown and died after 2 months in incubation. In contrast, callus induced in this work exhibited good growth on the medium supplemented with 0.1 mg l^{-1} NAA, 0.01 mg l^{-1} TDZ, and 2 g l^{-1} tryptone with approximately 10-fold fresh weight increase after 4 weeks in culture. The callus has been maintained by subculturing every month without loss of regeneration ability for about 2 years.

3.3. Effects of coconut water and sucrose on PLB formation from callus

Pieces of subcultured calli were cultured on the basal medium without plant growth regulators, with or without coconut water supplementation in the presence or absence

Table 3
Effects of tryptone on callus proliferation of *Cymbidium* Twilight Moon 'Day Light'

Medium containing[a] (g l^{-1})	Callus growth[b]	Morphogenesis
Tryptone 0	3.1 c	Callus
Tryptone 2	9.7 a	Callus
Tryptone 4	6.1 b	Callus

Means followed by the different letters are significantly different at $P < 0.05$ according to Duncan's multiple range test.

[a] Modified Vacin and Went medium [18] containing 0.1 mg l^{-1} NAA and 0.01 mg l^{-1} TDZ.

[b] Ratio between the final fresh weight and the initial fresh weight.

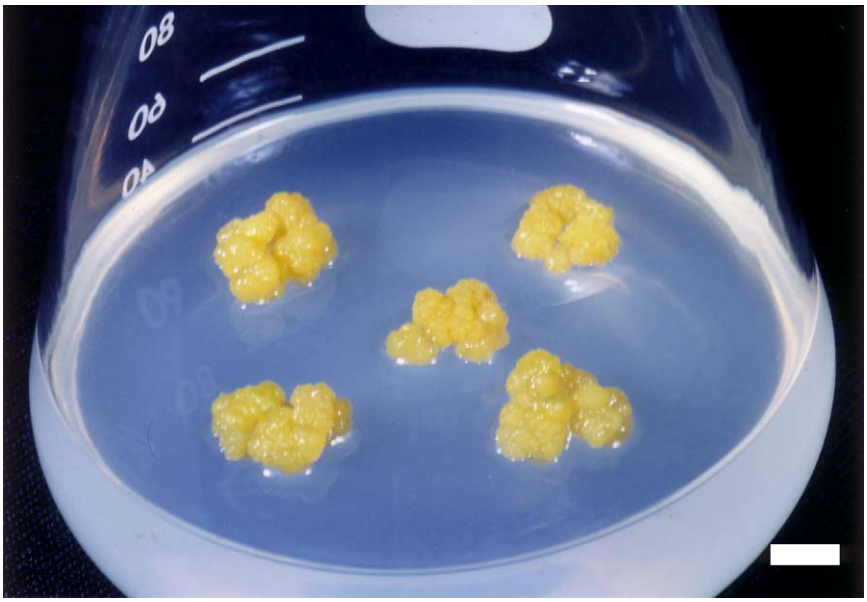

Fig. 2. Callus proliferation on the medium supplemented with 0.1 mg l^{-1} NAA, 0.01 mg l^{-1} TDZ, and 2 g l^{-1} tryptone. Bar: 0.5 cm.

of sucrose to examine the effects of coconut water and sucrose on PLB formation from callus. After being transferred on to the media treatments, calli continued to proliferate, gradually turned green, and began to form PLBs. The formation of PLB was best on the plant growth regulator-free medium with an average of 90.7 PLBs per cultured callus (Table 4; Fig. 3). The supplementation of coconut water also produced many PLBs, but these PLBs were not uniform with some misshapen and rather smaller than those produced on the plant growth regulator-free medium. The medium with no sucrose but supplemented with coconut water was not effective for PLB formation from callus in comparison with the plant growth regulator-free medium and the plant growth regulator-free medium supplemented with 200 ml l^{-1} coconut water in terms of both number and size of formed PLBs. On this medium, PLBs were often small and some PLBs died within the last month of culture.

Table 4
Effects of coconut water and sucrose on PLB formation from callus of *Cymbidium* Twilight Moon 'Day Light'

Medium[a]	No. of PLBs per explant	Fresh weight of PLB cluster (mg)	Dry weight of PLB cluster (mg)
MV-W S^+CW^-	90.7 a	1588.5 a	141.3 a
MV-W S^+CW^+	91.3 a	1319.2 b	118.5 b
MV-W S^-CW^+	38.5 b	431.2 c	36.6 c

Means within a column followed by the same letters are not significantly different at $P < 0.05$ according to Duncan's multiple range test.

[a] MV-W S^+CW^-: modified Vacin and Went medium [18] without plant growth regulators containing 20 g l^{-1} sucrose (basal medium); MV-W S^+CW^+: modified Vacin and Went medium without plant growth regulators containing 20 g l^{-1} sucrose was supplemented with 200 ml l^{-1} coconut water. MV-WC S^-CW^+: modified Vacin and Went medium without plant growth regulators was supplemented with 200 ml l^{-1} coconut water in the absence of sucrose.

In this research, the PLB formation from callus in *Cymbidium* was very good on the plant growth regulator-free medium. The medium without plant growth regulators was also used for PLB formation from callus in some other orchid researches [9,16,21,23]. Ishii et al. [16] used the medium containing coconut water and no sucrose for PLB formation from callus in *Phalaenopsis* but the results obtained in this research showed that the supplementation of coconut water in the absence or presence of sucrose was not effective for PLB formation from callus in *Cymbidium*. Similarly to this finding, Kerbauy [10] reported that coconut water did not improve PLB formation from callus in *Cattleya*. The effect of coconut water on PLB formation from callus might be dependent on species.

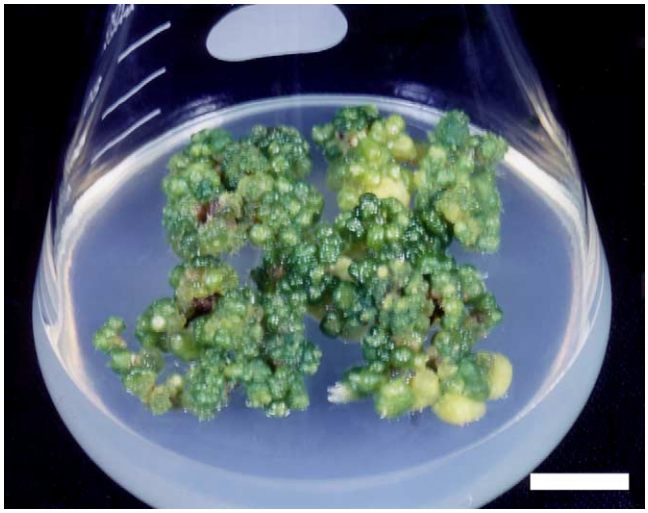

Fig. 3. PLB formation from callus on plant growth regulator-free medium. Bar: 1 cm.

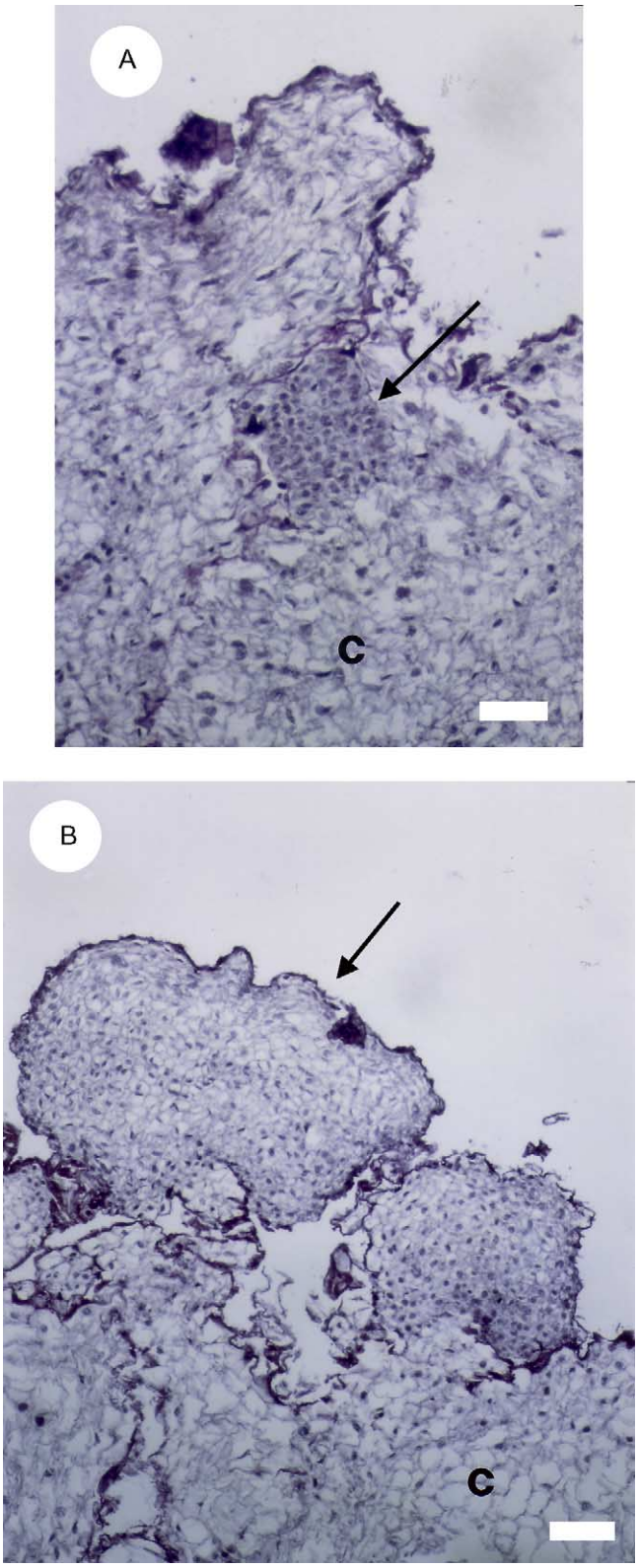

Fig. 4. Longitudinal sections of callus cultured on plant growth regulator-free medium. (A) Somatic embryo structure composed of cells with dense cytoplasm and large nuclei in callus mass. (B) Somatic embryo structures emerged from callus and turned into PLBs. C: callus tissue. Bar: 50 and 100 μm, respectively.

3.4. Histological observation

The longitudinal section of callus cultured on plant growth regulator-free medium showed somatic embryo structures composed of cells with dense cytoplasm and large nuclei in callus mass (Fig. 4A). These structures were almost similar to zygotic embryos developing in seeds of *Cymbidium sinense* [24,25]. These somatic embryo structures emerged from callus and turned into PLBs without any vascular connection with each other and callus tissue (Fig. 4B). These PLBs were similar in shape to protocorms formed from seeds of *Cymbidium*. The histological observation suggested that the plant regeneration from callus was through somatic embryo structures and the callus induced in this work was embryogenic.

3.5. Plant regeneration

Callus-derived PLBs developed both shoots and roots into individual plantlets on plant growth regulator-free medium (Fig. 5). After about 4 months, plantlets (6–7 cm in height) with well-developed shoots and roots were transplanted into the greenhouse with 100% survival rate. Among 103 twenty-month-old regenerated plants, no morphological variation was detected (Fig. 6).

This rapid and efficient system for embryogenic callus induction from PLB segments and plant regeneration from callus through somatic embryo structures in *Cymbidium* orchid could be useful for micropropagation as well as genetic transformation in this plant.

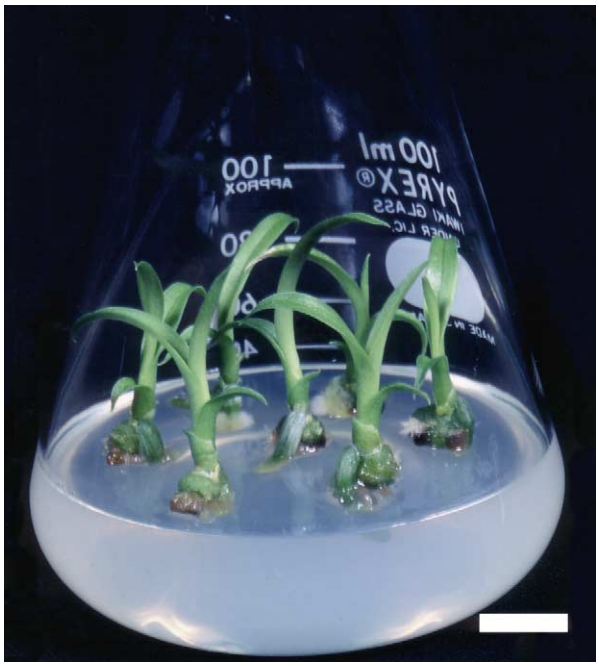

Fig. 5. Callus-derived PLBs developed both shoots and roots into individual plantlets on plant growth regulator-free medium. Bar: 1 cm.

Fig. 6. Twenty-month old callus-derived plant in the greenhouse. Bar: 5 cm.

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