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PLANT REGENERATION FROM PROTOCORM-DERIVED CALLUS OF *CYPRIPEDIUM FORMOSANUM*

YUNG-I LEE* AND NEAN LEE

Department of Horticulture, National Taiwan University, No. 1, Sec. 4, Roosevelt Rd., Taipei, Taiwan

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Summary

Totipotent callus of *Cypripedium formosanum*, an endangered slipper orchid species, was induced from seed-derived protocorm segments on a quarter-strength Murashige and Skoog medium containing 4.52 μ*M* 2,4-dichlorophenoxyacetic acid and 4.54 μ*M* 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea (thidiazuron). This callus proliferated well and was maintained by subculturing on the same medium. On average, 13 protocorm-like bodies could be obtained from a piece of 4 mm callus after being transferred to the medium with 4.44 μ*M* N^6-benzyladenine after 8 wk of culture. The regenerated protocorm-like bodies formed shoots and roots on medium containing 1 g l^{-1} activated charcoal and 20 g l^{-1} potato homogenate. After 24 wk of culture on this medium, well-developed plantlets ready for potting were established.

Key words: Cypripedium formosanum; callus induction; plant regeneration; protocorm-like body.

Introduction

Cypripedium formosanum, commonly known as slipper orchid, is a popular terrestrial orchid with attractive pinkish flowers which has the potential for increased use in perennial gardening and as a pot flower crop. The wild populations are now considered to be extinct as a result of over-collection and habitat destruction. For commercial requirements and the conservation of endangered species, it is desirable to establish protocols for rapid and large-scale clonal propagation.

Methods of micropropagation have been documented in many orchids, but the slipper orchids are still difficult to propagate *in vitro* (Arditti and Ernst, 1993). In *Paphiopedilum* (a relative of *Cypripedium*), Stewart and Button (1975) reported that a few plantlets could be regenerated from shoot apex-derived callus, but that the callus eventually failed to survive during subcultures. Recently, a procedure with reproducible results to obtain plantlets from callus culture of a *Paphiopedilum* hybrid was reported (Lin et al., 2000). However, there has been no such report for *Cypripedium*. We report here a reliable *in vitro* regeneration procedure of *C. formosanum* from subcultured protocorm-derived callus. In the investigation reported here, seed-derived protocorm segments were used for callus induction and subsequent plant regeneration.

Materials and Methods

Plant material. Plants of *C. formosanum* were cultivated in a greenhouse at Mei-Fung farm (2000 m above sea level) in Taiwan. A number of flowers were self-pollinated manually by transferring pollinia onto the stigma of the same flower in April (mean temperature 10°C). Green capsules were collected 3 mo. after pollination. Capsules were surface-sterilized with a 1% sodium hypochlorite solution for 20 min and rinsed three times with sterile distilled water. Capsules were cut open and the seeds were scooped out with forceps onto the quarter-strength macro-elements and full-strength micro-elements of MS medium (Murashige and Skoog, 1962) supplemented with (mg l^{-1}): *myo*-inositol (100), niacin (0.5), pyridoxine HCl (0.5), thiamine HCl (0.1), glycine (2.0), tryptone (1000), sucrose (20 000), and Gelrite™ (2200).

The seed cultures were incubated in darkness at 25 ± 2°C for 12 wk to develop into small round protocorms, then transferred to illuminated conditions at 25–30 μmol $m^{-2} s^{-1}$ (daylight fluorescent tube FL-20D/18, 20 W, China Electric Co., Taipei). Twenty weeks after sowing, cone-shaped protocorms (approximately 5–6 mm in diameter) were bisected transversally. The segments were then used as explants for callus induction.

Callus induction from protocorm segments. For callus induction, 1/2-strength and 1/4-strength macro-elements of MS basal salts (1/2-MS and 1/4-MS) with full-strength micro-elements supplemented with (mg l^{-1}): *myo*-inositol (100), niacin (0.5), pyridoxine HCl (0.5), thiamine HCl (0.1), glycine (2.0), sucrose (20 000), and Gelrite™ (2200) were selected for this trial. Plant growth regulators were added prior to autoclaving as optional additives according to the experimental objectives. 2,4-Dichlorophenoxy-acetic acid (2,4-D; 0, 0.45, and 4.52 μ*M*), N^6-benzyladenine (BA; 0, 4.44, and 22.19 μ*M*), and thidiazuron (TDZ; 0, 0.45, and 4.54 μ*M*) were added to the basal media in this experiment. The pH of the media was adjusted to 5.5 with 1 *N* KOH or HCl prior to autoclaving at 121°C for 15 min. Six protocorm segments were inoculated onto 10 ml of Gelrite-solidified medium in 25 × 100 mm culture tubes and incubated in darkness.

Protocorm-like body (PLB) formation. One piece of callus mass (4 mm in diameter) was placed on 1/4-MS basal medium supplemented with (mg l^{-1}): *myo*-inositol (100), niacin (0.5), pyridoxine HCl (0.5), thiamine HCl (0.1), glycine (2.0), sucrose (20 000), and Gelrite™ (2200). The combinations of plant growth regulators were the same as callus induction except the removal of 2,4-D. The explants were cultured in 10 ml of medium in 25 × 100 mm culture tubes and incubated under illumination.

Plantlet regeneration. About 6 mm-long PLBs were placed on 1/4-MS basal medium supplemented with (mg l^{-1}): *myo*-inositol (100), niacin (0.5), pyridoxine HCl (0.5), thiamine HCl (0.1), glycine (2.0), sucrose (20 000), activated charcoal (1000), and Gelrite™ (2200). Peptone (0.5 and 1 g l^{-1}), potato homogenate (20 and 50 g l^{-1}), and banana homogenate (20 and 50 g l^{-1}) were added to the basal media in this experiment. Potato and ripe banana were purchased from a local supplier, then peeled and cut into about 1 cm^3 sections. These fresh materials were boiled for 10 min with 100 ml of distilled water and homogenized with a kitchen blender. The homogenate

*Author to whom correspondence should be addressed: Email leeyungi@hotmail.com

was added to the basal medium as required before the pH was adjusted. Explants were incubated in 25 × 100 mm culture tubes under illumination.

Cultural conditions. All cultures were incubated at 25 ± 2°C, and under a 12/12-h photoperiod at 25–30 μmol $m^{-2} s^{-1}$ (daylight fluorescent tubes FL-20D/18, 20 W, China Electric Co.) or in the dark.

Histological observation. Some callus masses after 2 and 4 wk of culture were fixed in a solution of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 *M* sodium phosphate buffer (pH 7.2) at 4°C, overnight. After three 15-min buffer rinses, the materials were postfixed in 1% OsO_4 in the same buffer for 4 h at room temperature and then rinsed in three 15-min changes of buffer. Following fixation, the materials were dehydrated in an acetone series and embedded in Spurr's resin (Spurr, 1969). Serial sections, 1 μm thick, were cut using a glass knife on a Richert-Jung Ultracut E ultramicrotome. These sections were stained with periodic acid Schiff (PAS) reagent and counter-stained with 1% toluidine blue O (Yeung, 1984).

Statistical analysis. Experiments were performed in a randomized design and repeated twice. Eighteen replicates were taken for each treatment in the experiment of callus induction, and six explants were planted in each culture tube. Observations were made after 24 wk of culture. For PLB formation, 27 replicates were used for each treatment, and one explant was planted in each culture tube. Observations were made after 8 wk of culture. For plantlet regeneration, 20 replicates were used for each treatment, and two explants were planted in each culture tube. Observations were made after 8 wk of culture. The data were statistically analyzed and means were compared using Duncan's multiple range test (Duncan, 1955).

Results

Induction and subculture of callus. Initially, callus formation was extremely slow, and browning of protocorm segments was common in most cases. The cultures on 1/4-MS medium showed better induction of callus compared to those on 1/2-MS medium (Table 1). More phenolic compounds were released in the 1/2-MS medium that not only retarded the formation of callus but also caused lethal effects on the explants. About 11.6–55.8% of explants produced yellowish friable callus from the incision areas of protocorm segments (Fig. 1*A*) in the dark on 1/4-MS basal media containing both 2,4-D (0.45–4.52 μ*M*) and TDZ (0.45–4.54 μ*M*) after 16–24 wk of culture. In contrast, 10.6–19.7% of callus formation was obtained on 1/2-MS basal media. Small amounts of yellowish and non-friable callus formed on 1/4-MS basal medium containing 4.52 μ*M* 2,4-D alone, but they failed to proliferate. The results showed that BA was neither able to induce callus formation alone, nor in combination with 2,4-D. TDZ alone also failed to induce callus formation. In most cases, explants formed multiple shoots on media containing BA. The percentage of callus induction was greater (55.8%) on medium containing 4.52 μ*M* 2,4-D and 4.54 μ*M* TDZ as compared to the other combinations of 2,4-D and TDZ.

TABLE 1

EFFECT OF COMBINATIONS OF 2,4-D WITH TDZ OR BA ON CALLUSING OF SEED-DERIVED PROTOCORM SEGMENTS OF *CYPRIPEDIUM FORMOSANUM* WITH 1/2-STRENGTH AND 1/4-STRENGTH MACRO-ELEMENTS OF MS BASAL SALTS (AFTER 8 WK OF CULTURE IN THE DARK)

Plant growth regulators (μ*M*)			% Callusing	
2,4-D	BA	TDZ	1/2-MS	1/4-MS
0	0	0	0 c	0 d
0	4.44	0	0 c	0 d
0	22.19	0	0 c	0 d
0	0	0.45	0 c	0 d
0	0	4.54	0 c	0 d
0.45	0	0	0 c	0 d
0.45	4.44	0	0 c	0 d
0.45	22.19	0	0 c	0 d
0.45	0	0.45	0 c	11.6 c
0.45	0	4.54	10.6 ab	16.1 bc
4.52	0	0	5.6 ab	11.6 c
4.52	4.44	0	0 c	0 d
4.52	22.19	0	0 c	0 d
4.52	0	0.45	19.7 a	28.3 b
4.52	0	4.54	19.7 a	55.8 a

Means of 18 replicates with the same *letters* are not significantly different at $P < 0.05$ (Duncan, 1955).

Protocorm-derived callus was subcultured at 8 wk intervals on 1/4-MS medium in combination with 2,4-D (0.45–4.52 μ*M*) and TDZ (0.45–4.54 μ*M*) (data not shown). After two subcultures, some yellowish and friable callus differentiated into globular structures on medium containing 0.45 μ*M* 2,4-D and 0.45 μ*M* TDZ. On the other hand, most of the proliferated callus still remained yellowish with a friable surface on the medium containing 4.52 μ*M* 2,4-D and 4.54 μ*M* TDZ. Hence, the medium supplemented with 4.52 μ*M* 2,4-D and 4.54 μ*M* TDZ was selected as the standard maintenance medium for proliferation of totipotent callus.

PLB formation. Within 24 wk of subculture (subcultured bimonthly), each callus mass (4 mm diameter) was transferred onto the media under illumination for PLB formation studies (Table 2). About 11.1–96.2% of the callus produced globular granules within 4 wk of culture. These globular granules transformed into PLBs after 8 wk of culture (Fig. 1*B*, *C*). The optimum growth regulator concentration for regeneration from totipotent callus was found to be 4.44 μ*M* BA, where the highest percentage of PLB formation (96.2%) and the maximum number of PLBs per explant (13 PLBs) were observed. The medium with no growth regulators or containing 2,4-D alone resulted in PLB formation, but with a lower number of PLBs. On the media with combinations of 2,4-D and TDZ, most callus turned yellowish green and became compact in appearance under illumination that gave rise to a lower percentage of PLB formation (11.1–12.9%).

Histological examination revealed that globular-shaped granule formation occurred at the periphery of the callus mass. Small clusters of cytoplasmic cells subtended by a few vacuolated cells were discernible after 2 wk of culture on the medium containing 4.44 μ*M* BA (Fig. 2*A*). These clusters of cells continued to grow, and the globular-shaped granules started to emerge (Fig. 2*B*). They were characterized by globular-shaped embryo proper, formed by small cytoplasmic cells. Small starch grains began to appear within the cells at the center of the embryo proper, and a differentiated protoderm proper was observed at this time.

Regeneration of plantlets from PLBs. Different concentrations of peptone, potato homogenate, and banana homogenate were tested in the experiments (Table 3). Survival percentage of PLBs was high on media with 1% peptone and 20 g l^{-1} potato homogenate. In contrast, survival of PLBs was poor on media with 20 and 50 g l^{-1} banana homogenate. Shoot buds and root formation occurred after 3 wk of culture (Fig. 1*D*). The optimum rooting response was obtained on the medium with 20 g l^{-1} potato homogenate. In this medium, plantlets produced an average of 7.2 thick, healthy roots, each 4.7 cm in length within 10 wk of culture (Fig. 1*E*). In a period of 24 wk of culture, PLBs grew into plantlets about 6–7 cm tall which were ready for transplanting to pots (Fig. 1*F*).

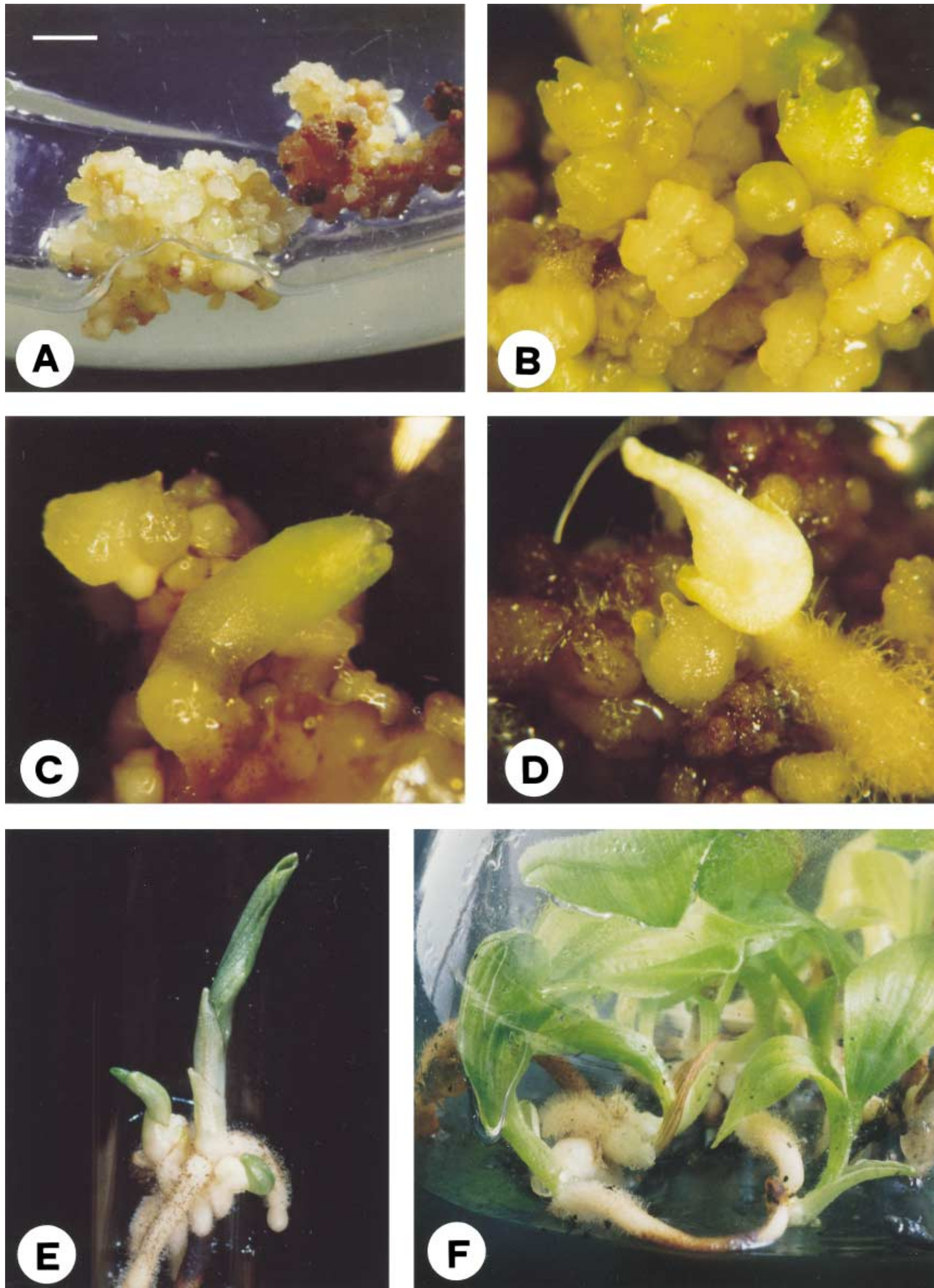

FIG. 1. *In vitro* plantlet regeneration from protocorm-derived callus of *Cypripedium formosanum*. *A*, Totipotent callus obtained from a seed-derived protocorm segment after 24 wk on basal medium with 4.52 μ*M* 2,4-D and 4.54 μ*M* TDZ following induction (*bar* = 12 mm). *B*, PLBs formed after 8 wk of culture from protocorm-derived callus on basal medium with 4.44 μ*M* BA (*bar* = 1.2 mm). *C*, An elongated PLB (*bar* = 1.2 mm). *D*, A PLB developed into a shoot bud and a root (*bar* = 1.5 mm). *E*, Lateral buds proliferating from a sprouting shoot (*bar* = 4 mm). *F*, Twelve-month-old PLB-derived plantlets ready for potting (*bar* = 12 mm).

TABLE 2

EFFECT OF COMBINATIONS OF 2,4-D WITH TDZ OR BA ON PROTOCORM-LIKE BODY FORMATION FROM PROTOCORM-DERIVED CALLUS OF *CYPRIPEDIUM FORMOSANUM* (AFTER 8 WK OF CULTURE)

Plant growth regulators (μ*M*)				
2,4-D	BA	TDZ	Percent of explants producing PLBs	Number of PLBs per explant
0	0	0	88.8 a	1.6 d
0	4.44	0	96.2 a	13.0 a
0	22.19	0	92.5 a	9.0 ab
0	0	0.45	33.3 c	4.7 cd
0	0	4.54	25.5 cd	5.8 bc
4.52	0	0	18.5 d	1.1 d
4.52	4.44	0	51.8 b	6.7 b
4.52	22.19	0	33.3 c	11.3 a
4.52	0	0.45	12.9 d	6.4 b
4.52	0	4.54	11.1 d	3.4 cd

Means of 27 replicates with the same *letters* are not significantly different at $P < 0.05$ (Duncan, 1955).

Discussion

The four genera (*Cypripedium*, *Paphiopedilum*, *Phragmipedium*, and *Selenipedium*) of the subfamily Cypripedioideae are considered to be difficult to culture *in vitro* (Arditti and Ernst, 1993). Efforts to develop tissue culture methods have not had much success (Stewart and Button, 1975). Recently, plant regeneration from callus culture of a *Paphiopedilum* hybrid was demonstrated by Lin et al. (2000). In the present report, we have described a procedure for callus induction and plant regeneration from seed-derived protocorm segments of *C. formosanum*.

In the present study, of the two cytokinins, BA and TDZ, in combination with 2,4-D tested for callusing, BA did not show any response. The superiority of TDZ in promoting callus induction and *in vitro* morphogenesis was observed in several orchid species (Ernst, 1994; Chang and Chang, 1998; Chen et al., 1999). TDZ alone was efficient at inducing direct somatic embryogenesis from the leaf explants of *Oncidium* (Chen et al., 1999), but, as shown here, was not effective for protocorm segments of *C. formosanum*. Thus, the exogenous hormonal requirements for inducing *in vitro* morphogenesis of orchid species are quite different. Previously, Lin et al. (2000) used the medium containing 4.52–45.2 μ*M* 2,4-D and 0.45–4.54 μ*M* TDZ for induction of totipotent calluses of *Paphiopedilum* hybrids. In the present study, both 2,4-D and TDZ were essential for inducing totipotent callus of *C. formosanum*, even at a low concentration (0.45 μ*M*). This suggests that the combination of TDZ with auxins played a crucial role in callus induction of slipper orchids. Such an effect of the combination of TDZ with auxins in maintaining long-term totipotent callus culture of *Cymbidium* has also been observed (Chang and Chang, 1998). A small amount of callus was observed on the medium containing 4.52 μ*M* 2,4-D alone; but these calluses failed to survive during subcultures. This result appeared to be similar to that of shoot apex-derived callus of *Paphiopedilum* (Stewart and Button, 1975). Although the effect of organic supplements is complex, many organic forms of nitrogen and natural organic compounds have been used in media in the micropropagation of orchids, such as peptone, tryptone, coconut water, and banana homogenate (Arditti and Ernst, 1993). Of the three organic supplements used, peptone and potato homogenate significantly promoted rooting of the plantlets. The highest survival rate (80%) of PLBs and the optimum rooting were obtained at $20\,g\,l^{-1}$ potato homogenate. However, banana homogenate showed inhibitory effects for PLB survival, as Ichihashi and Islam (1999) found on callus growth in *Phalaenopsis*, *Doritaenopsis*, and *Neofinetia*. Effects of organic supplements on various orchid species may indicate different requirements for organic compounds. Which factors are particularly responsible for PLB growth were not made clear by our study and could be the subject for further investigation.

In the presented regeneration protocol, one small protocorm-derived callus mass (4 mm diameter) yielded 13 PLBs. A large

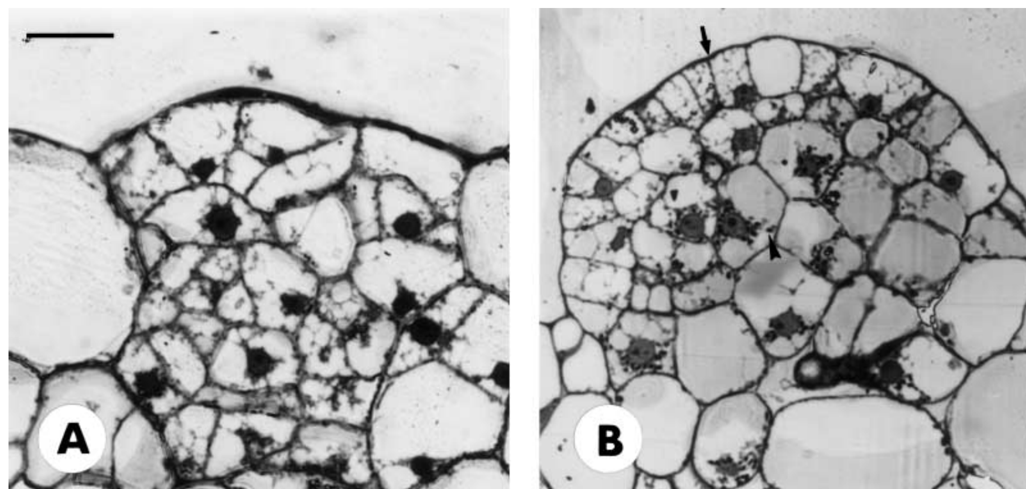

Fig. 2. Formation of globular-shaped granules from protocorm-derived callus of *Cypripedium formosanum*. *A*, Small clusters of cytoplasmic cells at the periphery of calluses were discernible after 2 wk of culture on the regeneration medium with 4.44 μ*M* BA (*bar* = 20 μm). *B*, Globular-shaped granules were present within 4 wk of culture. They were characterized by a differentiated protoderm (*arrow*), and starch grains (*arrowhead*) begin to appear within the cells at the center of the embryo proper (*bar* = 50 μm).

TABLE 3

EFFECT OF PEPTONE, POTATO, AND BANANA HOMOGENATE ON PROTOCORM-LIKE BODY SURVIVAL AND ROOTING OF PLANTLETS OF *CYPRIPEDIUM FORMOSANUM* (AFTER 8 WK OF CULTURE)

Organic supplement	($g\,l^{-1}$)	Survival rate of PLBs (%)	Mean root number per plantlet	Mean root length (cm)
Control[z]		70 ab	5.2 c	2.3 c
Peptone	0.5	75 ab	5.5 c	3.8 ab
	1	80 a	6.4 b	4.0 ab
Potato homogenate	20	80 a	7.2 a	4.7 a
	50	75 ab	6.5 b	4.5 a
Banana homogenate	20	15 c	6.4 b	3.0 b
	50	10 c	6.0 bc	3.6 ab

[z] Quarter-strength macro-elements and full-strength micro-elements of MS medium lacking organic supplements served as control.
Means of 20 replicates with the same *letters* are not significantly different at $P < 0.05$ (Duncan, 1955).

number of plantlets can be obtained within 1 yr if the totipotent callus was induced from protocorm segments. This appeared to be a more reliable method for mass propagation as compared to seed propagation with low germination frequency. Application of this protocol to other *Cypripedium* species or hybrids also needs to be assessed. In addition, this regeneration system may provide a suitable procedure for attempting transformation of *Cypripedium* species.

In summary, totipotent calluses of *C. formosanum* were initiated from excised protocorm segments on basal medium containing 4.52 μM 2,4-D and 4.54 μM TDZ. For plant regeneration, the subcultured callus was transferred to medium containing 4.44 μM BA. This callus demonstrated its totipotency by plant regeneration via PLBs as an intermediate step. The PLBs developed into plantlets after further incubation on basal medium plus 1 $g\,l^{-1}$ activated charcoal and 20 $g\,l^{-1}$ potato homogenate. Following the protocol described herein, we estimate that approximately 10 plantlets could be formed from one piece of protocorm-derived callus (4 mm diameter) after 16 wk of culture.

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References

Arditti, J.; Ernst, R. *Paphiopedilum*. Micropropagation of orchids. New York: John Wiley & Sons, Inc.; 1993:434–466.

Chang, C.; Chang, W. C. Plant regeneration from callus of *Cymbidium ensifolium* var 'misericors'. Plant Cell Rep. 17:251–255; 1998.

Chen, J. T.; Chang, C.; Chang, W. C. Direct somatic embryogenesis on leaf explants of *Oncidium* 'Gower Ramsey' and subsequent plant regeneration. Plant Cell Rep. 19:143–149; 1999.

Duncan, D. B. Multiple range and multiple F test. Biometrics 11:1–42; 1955.

Ernst, R. Effects of thidiazuron on *in vitro* propagation of *Phalaenopsis* and *Doritaenopsis* (Orchidaceae). Plant Cell Tiss. Organ Cult. 39:273–275; 1994.

Ichihashi, S.; Islam, M. O. Effects of complex organic additives on callus growth in three orchid genera, *Phalaenopsis*, *Doritaenopsis* and *Neofinetia*. J. Jap. Soc. Hort. Sci. 68:269–274; 1999.

Lin, Y. H.; Chang, C.; Chang, W. C. Plant regeneration from callus culture of a *Paphiopedilum* hybrid. Plant Cell Tiss. Organ Cult. 62:21–25; 2000.

Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473–497; 1962.

Spurr, A. R. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26:31–43; 1969.

Stewart, J.; Button, J. Tissue culture studies in *Paphiopedilum*. Am. Orchid Soc. Bull. 44:591–599; 1975.

Yeung, E. C. Histological and histochemical staining procedures. In: Vasil, I. K., ed. Cell culture and somatic cell genetics of plants, vol. 1. Laboratory procedures and their applications. Orlando, FL: Academic Press; 1984:689–697.