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Research note

High frequency plant regeneration from callus culture of *Pleione formosana* Hayata

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Abstract

High frequency plant regeneration was induced from protocorm-derived callus cultured on half-strength of Murashige–Skoog medium with 2,4-dichlorophenoxyacetic acid (2,4-D, 0–5 mg l^{-1}) and 1-phenyl-3-(1,2,3-thiadiazol-5-yl, 0–1 mg l^{-1}) urea (TDZ) in the dark. Twelve totipotent callus lines were selected within 76 callus lines regenerated on half-strength of Murashige–Skoog (MS) medium with 0.5 mg l^{-1} TDZ. The proliferation rate was 4–5-fold in fresh weight after 30 days of culture on half-strength MS medium containing 5 mg l^{-1} 2,4-D and 0.5 mg l^{-1} TDZ in the dark. The maximum number of shoot buds generated by 0.01 g callus explant was 134 after 4 months of culture. These calli were regenerated to plantlets via protocorm-like bodies (PLBs) after 75–150 days of culture. The shoots, with two true leaves, were transferred to hormone-free medium, rooting and eventually formed plantlets. Totipotent callus lines of *Pleione formosana* Hayata have been successfully established in this study.

Abbreviations: 2,4-D – 2,4-dichlorophenoxyacetic acid; MS – Murashige–Skoog; TDZ – 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea; PLB – protocorm-like body

Pleione formosana Hayata, known as the one-foliate orchid, is a native terrestrial orchid in Taiwan (Chiang and Chen, 1986). However, habitat destructions and low reproductive abilities have made *P. formosana* an endangered species. Therefore, a reliable and rapid propagation method is desirable. Efforts to establish totipotent callus lines of orchids have succeeded for some orchid species in recent years. These calli give rise to plantlets via protocorm-like bodies (PLBs) (Ishii et al., 1998; Chen and Chang, 2000; Lin et al., 2000), somatic embryos (Chang and Chang, 1988; Chen and Chang, 2000), or rhizomes (Chang and Chang, 1988). The objective of the current study is to develop a reliable and effective protocol for obtaining healthy plantlets from protocorm-derived calli. To our knowledge, there has been no previous works on callus culture and regeneration of *Pleione* genus.

Seed-derived 45 days old protocorms were used as explants for callusing. They were incubated on the surface of basal medium containing half-strength MS salt (Murashige and Skoog, 1962), supplemented with (in mg l^{-1}): niacin (10), thiamine–HCl (1), pyridoxal–HCl (1), glycine (2.0), peptone (1000), NaH_2PO_4 (170), sucrose (20,000), gelrite (3000). Combination of 2,4-dichlorophenoxyacetic acid (2,4-D, 0–5 mg l^{-1}) and thiadiazuron (TDZ, 0–0.5 mg l^{-1}) were added to the basal medium. The pH of the media was adjusted to 5.3 with KOH or HCl prior to autoclaving for 15 min at 121 °C. Explants were incubated in 21mm × 50 mm culture tubes dispensed with 5 ml medium in the dark at 20 ± 2 °C. In each treatment, 30 replicates were cultured in six tubes (five explants/tube). Percentage of callusing from each experiment was scored after 3 months of culture. Proliferation rate was calculated by the final fresh weight of the explants divided by the original fresh weight on basal medium containing 5 mg l^{-1} 2,4-D and 0.5 mg l^{-1} TDZ. Ten replicates of 0.1 g callus per tube were used in each treatment.

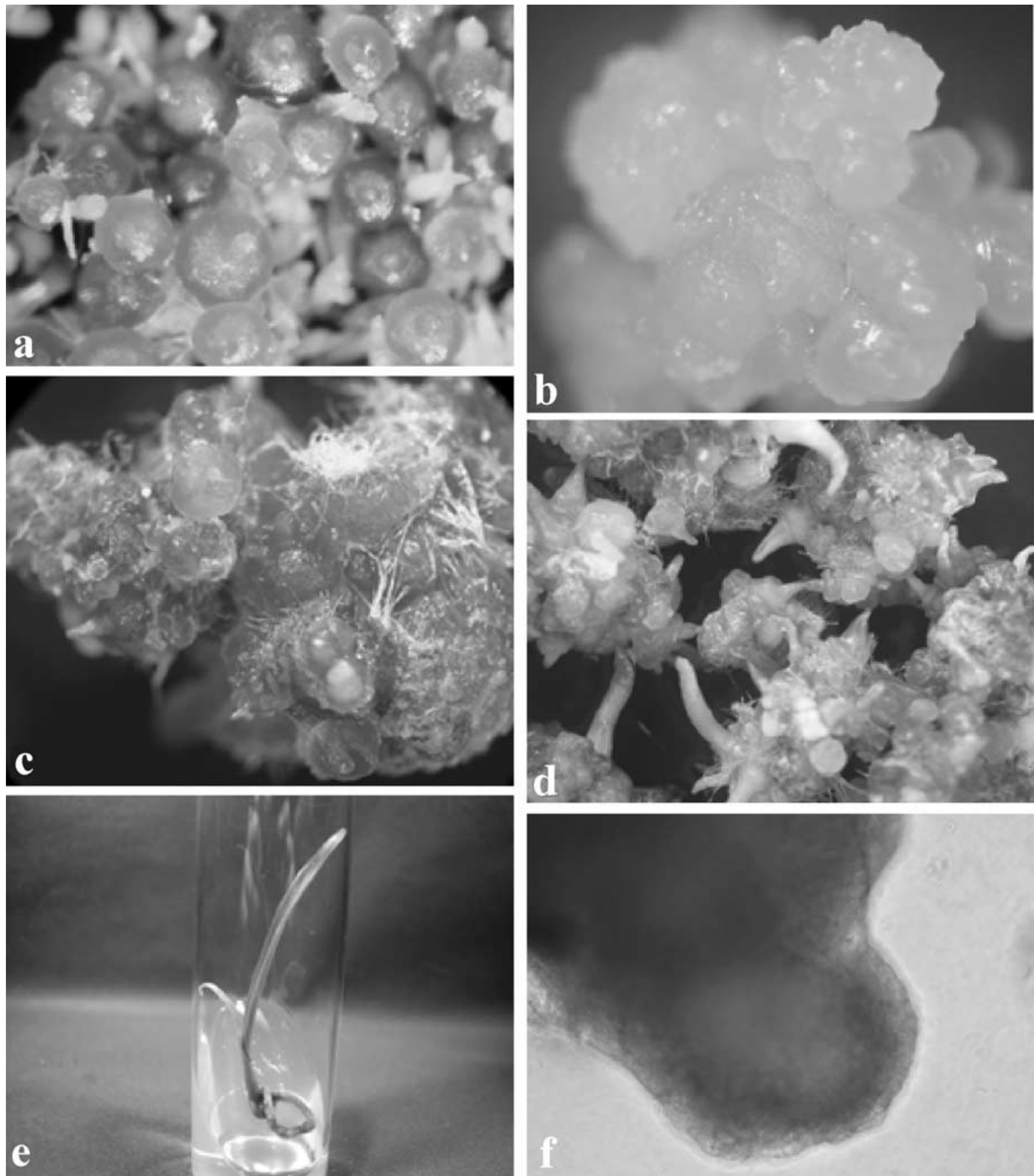

Figure 1. Plantlet regeneration from protocorm-derived calli of *P. formosana* Hayata. (a) Clusters of 45 days old protocorms used for callusing (35×); (b) subcultured protocorm-derived calli (30×); (c) a mass of globular structures covered by trichomes (15×); (d) clusters of shoot buds after 5 months of culture (7.5×); (e) a well-rooted plantlet and (f) globular embryo-like structure in callus line H12 (100×).

To evaluate plant regeneration ability, a piece of ca. 0.01 g (fresh weight) callus from each line was cultured on the basal medium with $0.5\,mg\,l^{-1}$ TDZ at $25 \pm 2\,°C$ under a 16-h photoperiod (ca. $80\,\mu mol\,m^{-2}\,s^{-1}$, Sylvania F40/Gro tube, 40 W, USA). In each treatment, 10 replicates in each cul-

Table 1. Regeneration abilities and proliferation rate of different callus lines induced from protocorms of *P. formosana* Hayata

Callus lines	Induced from basal medium containing combination of (mg l^{-1})		Proliferation rate[a]	Number of shoot bud[b]
	2,4-D	TDZ		
E12	1	0.1	–	16 ef
H12	3	0.1	4.1 bc	134 a
H22	3	0.1	–	9 f
H61	3	0.1	–	45 d
I20	3	0.5	4.5 ab	1 f
I42	3	0.5	4.0 bc	3 f
I51	3	0.5	3.3 c	72 c
J52	5	0	–	2 f
K13	5	0.1	–	104 b
L34	5	0.5	4.6 ab	22 e
L41	5	0.5	–	1 f
L43	5	0.5	5.1 a	8 f

[a] The proliferation rate was measured as the final fresh weight divided by the initial fresh weight. Means of 10 replicates with the same letters are not significantly different at $p < 0.05$ (Duncan, 1955). '–': not determined.
[b] Mean number of shoot buds per 0.01 g fresh weight of callus. Ten replicates were used in each experiment. Data were scored after 4 months.

ture tube were tested. After 4 months of culture, the number of shoot buds was counted. The shoots, with two true leaves, were transferred to hormone-free basal medium for rooting. The specimens were examined and photographed with stereozoom microscope (Nikon SMZ-U) or inverted microscope (Nikon TS100). Different between each treatment were scored with Duncan's multiple range test (Duncan, 1955).

About 7–53% of protocorms (Figure 1a) produced yellow, soft and granular callus mass (Figure 1b) after 3–5 months of culture on medium containing 2,4-D 1–5 mg l^{-1} and TDZ 0–0.5 mg l^{-1} (data not shown). Seventy-six callus lines were selected from 360 explants. The amount of calli per explant was estimated about 0.01–0.03 g. Calli showed a 4–5-fold increase in fresh weight after 30 days of culture on basal medium containing 5 mg l^{-1} 2,4-D and 0.5 mg l^{-1} TDZ in darkness (Table 1).

All callus lines were tested for regeneration abilities on basal medium containing 0.5 mg l^{-1} TDZ. Initially, the totipotent calli turned green and continued increasing weight. After 30–45 days of culture, a mass of globular structures covered by trichomes was formed. The globular structures turned into PLBs, germinated and produced shoot buds after 75–150 days of culture (Figure 1c, d). Calli regenerated via PLBs were also reported in *Phalaenopsis* (Ishii et al., 1998), *Oncidium* (Chen and Chang, 2000), and *Paphiopedilum* (Lin et al., 2000). Healthy plantlets were obtained following transferring these shoots to hormone-free basal medium (Figure 1e). By examining the number of regenerated shoots, 12 callus lines with totipotent abilities were selected within 76 callus lines. However, most callus lines (65%) showed rhizogenesis upon regeneration, and eventually became brown and necrotic.

The regeneration ability was affected by the combination of callusing medium, especially the concentration of 2,4-D and TDZ (Table 1). In general, callus lines originated from medium containing 2,4-D 1–5 mg l^{-1} and TDZ 0.1–0.5 mg l^{-1} had better totipotency (Table 1). Different degrees of regeneration abilities in callus lines of the same medium were found (Table 1). The maximum number of shoot buds was 134 (H12) (Table 1). A globular embryo-like structure was observed in callus line H12 (Figure 1f).

Calli originated from 2,4-D-alone medium showed little growth and, eventually, became brown and necrotic (Chang and Chang, 1998; Lin et al., 2000). A callus line (J52) originated from 2,4-D-alone medium produced two shoot buds (Table 1), which might suggest the high concentration of 2,4-D (5 mg l^{-1})

was the major determinant for dedifferentiation in *P. formosana* Hayata.

Orchid callus has been considered difficult to be induced and maintained in culture (Kerbauy, 1984; Arditti and Ernst, 1993). However, by combining of 2,4-D and TDZ, totipotent calli were induced in *Cymbidium* (Chang and Chang, 1998), *Oncidium* (Chen and Chang, 2000) and *Paphiopedilum* (Lin et al., 2000). The high frequency callus lines of *P. formosana* Hayata, induced from medium containing 2,4-D and TDZ, proved that callus culture had a high potential on the mass production of *Pleione* genus.

In conclusion, a reliable propagation method for *P. formosana* Hayata has been successfully established in this study. It is a promising beginning for developing suspension culture, mass production system and molecular breeding of *P. formosana* Hayata. Histological observations, genetic stability of plantlets, and callus induction from other tissues will be performed in the future.

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