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Protoplast culture and plant regeneration of *Pinellia ternata*

Yikun He*, Changfu Zhu, Mengyuan He, and Shui Hao

Institute of Genetics and Cytology, Northeast Normal University, Changchu 130024, P. R. China
* *Present address:* 804 Group, Plant Biotechnology Laboratory, Institute of Genetics, Academia Sinica, Beijing 100101, P. R. China

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Abstract. A study was undertaken to develop a protoplast regeneration system for pinellia. A yield of 19-29 $\times 10^5$ protoplasts/g F. W. could be obtained from cell suspension cultures incubated in a digestion enzyme solution with 2% cellulase Onzuka R-10, 1% pectinase (Sigma), 0.01% pectolyase Y23. K8P and modified MS media were used to culture protoplasts in: a) liquid, b) liquid-solid double layer, or c) agarose embedded protoplast culture. The former two were conducive to colony formation from protoplast-derived cells. The frequency of cell division was about 8% after 3 days in culture. Gradually adding fresh medium of lower osmotic pressure into the medium for protoplast culture favored cell division. Calli(1-2 mm in diameter) formed after 30-40 days in culture. The calli transferred onto medium supplemented with KT (0.5 mg l^{-1}) and NAA (0.2 mg l^{-1}) could regenerate plants after 40-50 days. Of 47 plantlets transplanted into plots, 29 flowered and were fertile.

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; NAA, α-naphthaleneacetic acid; KT, kinetin; CH, casein hydrolysate

Introduction

Pinellia (*Pinellia ternata* Breit), a perennial Araceae herb, is an important Chinese medicinal plant that has been used in clinical practice for over 2000 years. In 558 Chinese prescriptions, pinellia ranked 22nd among the 30 most widely used drugs (Zheng 1980). The wild pinellia resources are exhausted by over-gathering, so the cultivation of pinellia will be an important strategy to meet the medicinal needs (Gu 1990). Cultivated pinellia is propagated by tubers and bulbils, but the yield and quality of tubers are gradually reduced over generations because of viral diseases (Shen 1992). To ensure the highest possible yield and quality, improved plants should be provided to growers. Major efforts have focused on utilization of in vitro culture technology for pinellia rapid propagation, and plantlets have been regenerated from tubers (Shoyama et al. 1983, Su 1989), leaves and roots (Ren et al. 1983, Choi et al. 1986) and bulbils (Tsay et al. 1989, Kim et al. 1990). A protoplast regeneration system can be an important genetic manipulation technology for crop improvement. So far, there is only one report of protoplast culture in pinellia, in which mesophyll protoplasts were used to regenerate plantlets via organogenesis and somatic embryogenesis (Wu et al. 1986), but the regeneration frequency was not reported. We isolated numerous viable protoplasts from suspension cultures and obtained plantlets which could flower and were fertile in plots.

Materials and Methods

Plant materials. Tubers of pinellia, which were collected from the suburban field of Nanchong city in Sichuan province and had been cultivated for three generations, were germinated in wet sand in a greenhouse at 25-28℃ with natural light. When leaves grew to 3-5 cm long in 10 days, petioles were excised and washed with tap water, then sterilized with 0.1% $HgCl_2$ for 8 min prior to being washed 5 times with sterile distilled water. Petiole segments (3-5 cm long) were cultured on MS medium supplemented with 2.0 mg l^{-1} 2,4-D and 0.5 mg l^{-1} KT. Calli

Correspondence to: Y. He

could be induced from the end and the surface of explants, and were subcultured once every month. After 3-4 months, friable calli could be selected for use afterward (He et al. 1994).

Establishment of suspension cell cultures. Approximately 3 g of callus were transferred into 200-ml flasks containing 30 ml liquid MS medium (Murashige and Skoog 1962) with 2.5 mg l^{-1} 2,4-D and 0.5 mg l^{-1} KT. The flasks were incubated on a shaker at 120 rpm in the dark at 27 ± 2℃, and were subcultured every 5 days. In the original subcultures, the bigger clumps of tissue were gradually removed. After 3 months in culture, a suspension cell line was established.

Table 1. Enzymes of isolation solutions

Isolation solutions \ Enzymes(%)	Cellulase (Onzuka, R−10)	Pectinase (Sigma)	Pectolyase Y23 (Seishin)
solution I	4	2	0.1
solution II	3	1	0.1
solution III	2	1	0.05

Protoplast isolation and culture. About 1 g of suspension cell cultures was digested from 5 to 20 h with 10 ml of enzyme solution in a 6-cm Petri dish agitated at 30 rpm, in the dark at 25 ± 1℃. The enzyme solutions contained cellulase, pectinase (Table 1), 1470 mg l^{-1} $CaCl_2.2H_2O$, 95 mg l^{-1} KH_2PO_4 and 0.6 mg l^{-1} glycine, pH 5.8. Following incubation, the crude digests were successively passed through 85-μm and 46-μm copper sieves and collected into centrifuge tubes. The mixture was centrifuged at 500 rpm for 5 min. Protoplasts were washed twice with the enzyme-free isolation solution and once with medium M_1 or M_3 respectively (Table 2). Protoplasts were cultured in liquid K8P medium (Kao al. 1975) or improved MS medium (Table 3) in 6-cm Petri dishes containing 2-3 ml of M_1 or M_3 medium respectively. The initial plating density was $5\text{-}7 \times 10^5$ protoplasts per ml. Three culture methods were used: a) liquid, b) solid-liquid double layer culture [the upper layer was 2 ml of protoplast mixture, the lower layer was 1.5 ml of the same medium solidified with 0.8% agar (Shanhai Chemical Reagent Co. China)], and c) agarose (Sigma) embedment culture (protoplasts were plated in solid medium by mixing 1.5 ml of protoplast mixture with an equal volume of molten agarose medium with 1.6% agarose). After a week, the cultures were gradually diluted with the corresponding lower osmotic M_2 or M_4 medium respectively (namely, M_2 to M_1, M_4 to M_3. Table 2).

Table 2. Protoplast culture and plant regeneration media

Compound \ Media[b]	M_1	M_2	M_3	M_4	M_5	M_6
Basal medium[a]	K8P	K8P	Improved MS	Improved MS	MS	MS
Sucrose(%)	2.0	1.0	2.0	1.0	3.0	3.0
Glucose(%)	9.0	3.0	9.0	3.0		
2,4-D(mg l^{-1})	1.0	2.0	1.0	2.0	2.0	
KT(mg l^{-1})	0.2	0.5	0.2	0.5	1.0	0.5
NAA(mg l^{-1})						0.2
Agar(%)					0.6	0.6

[a] The other components, except glucose and sucrose, were the same as those of K8P, improved MS, or MS medium.
[b] M_1-M_4 media were filter-sterilized, M_5 and M_6 media were sterilized by autoclaving.

These cultures were maintained at 25 ± 2℃ in the dark. Three to five plates of plated protoplasts were observed for each treatment, and differences between treatments at different incubation time were evaluated using the Newman and Keuls test ($p = 0.05$) (Newman 1939).

Plant regeneration. After 40-50 days in culture, protoplast-derived cell clumps grew into microcalli visible to the naked eye, which were transferred to M_5

Table 3. Organic elements added in improved MS medium[a] (mg l^{-1})

compound	Content	compound	Content
Sucrose	20,000	Vitamin B_1	10
Glucose	90,000	Vitamin B_6	1.0
Fructose	200	Vitamin C	1.0
Ribose	200	Pantothenic acid calcium	0.5
Inositol	100	Nicotinic acid	0.5
CH	300	Folic acid	0.2

[a] Inorganic elements were half−strength MS salts (without NH_4NO_3)

medium for proliferation in the light (50 μmol m^{-2} s^{-1}, 16 h). Plantlets could be produced from calli transferred onto M_6 medium (Table 2), and regeneration frequency was calculated 30 days later as the percentage of calli plated onto M_6 medium which regenerated into plants. The plants were transplanted into pots. Initially, the pots were covered with glass beakers to ensure high humidity, avoiding direct exposure to sunlight. After 10 days, plantlets were transplanted into plots.

Results and Discussion

Protoplast isolation. Enzyme concentration and incubation time had an influence on the yield and viability of protoplasts (Table 4). Protoplasts isolated from solution I almost disintegrated after 2-3 days in culture. In order to avoid an adverse influence of the higher concentration of enzyme on protoplast viability, protoplasts used in subsequent experiments were isolated in enzyme solution Ⅲ for 8 h.

Table 4. Yields of protoplasts in different enzyme solutions and incubation times.*

Enzyme solutions	Yields of protoplasts($\times 10^5$) at incubation times(h)			
	5	8	11	20
solution Ⅰ	18±3	25±6	12±3	15±7
solution Ⅱ	17±4	26±3	24±7	31±5
solution Ⅲ	5±2	19±3	17±8	18±4

* means±standard error of 3-5 replicates per treatment (protoplast number per g F. W.)

The protoplast yield was about 2.0×10^6/g F. W. The freshly isolated protoplasts were bright, small in size and densely cytoplasmic (Fig. 1A). After 20 h culture in liquid and solid-liquid double layer media, or 70 h culture in agarose embedment medium, some protoplasts become elongated, indicating formation of the cell wall. First divisions (Fig. 1B) were seen at a frequency of 3-9 % three days after plating, depending on the culture method (Table 5). By the 7th day in liquid culture, 4-cell clumps were found (Fig. 1C). Protoplasts embedded in agarose become triangular because of partial cell wall regeneration (Fig. 1D), and would not continue to divide after the first division. It was necessary that the cultures be diluted initially with 0.2-0.3 ml of the corresponding lower osmotic M_2 or M_4 medium, respectively. When an appropriate amount of fresh medium was added, cell volume increased slightly, and cytoplasmic streaming increased. By two weeks in culture, small colonies were formed (Fig. 1E), and many cell colonies were visible to the naked eye 40 days later (Fig. 1F). The results of culture between K8P and modified MS media were not markedly different ($p>0.05$) at the 3rd or 7th days, but were significantly different from embedment culture at 7 days ($p<0.05$).

Table 5. Effect of culture methods on division of protoplast-derived cells and calli formation*

Culture methods	K8P medium			Improved MS medium		
	3d	7d	40d	3d	7d	40d
liquid culture	6±2	17±4	++	5±1	12±3	++
liquid-solid culture	8±1	14±3	+++	9±1	13±2	+++
agarose embedment culture	3±1	5±1	−	5±2	7±3	−

* Frequency of protoplast-derived cell division (FPC) was calculated after 3 or 7 days in culture:

$$FPC = \frac{\text{No. of cells dividing at least once}\times 100}{\text{No. of surviving cells}} \text{ (means} \pm \text{SE)}$$

Calli were observed after 40 days: ++: moderate, +++: good, −: none

Plant regeneration. Small calli derived from protoplasts and transferred onto M_5 medium proliferated rapidly, but looked watery and soft. After several subcultures, a pale yellow friable callus was selected, and transferred onto M_6 medium. After 40-50 days in culture, tubercles differentiated from the callus (Fig. 1G) and gradually developed into plantlets (Fig. 1H), at a frequency of 21% in the protoplast-derived calli. Forty seven plantlets were transplanted to soil, with a survival rate of 93%. Twenty nine plants flowered and were fertile (Fig. 1 I, J).

The plant regeneration frequency of calli from petioles was over 90% (He et al. 1994), but only 21% from protoplast-derived calli. The reduction of plant regeneration frequency may result from genetic mutations or physiological and biochemical changes induced during the process of culture and regeneration (Evans and Sharp 1983). When the agar concentration or osmotic component content was adjusted during subcultures of protoplast-derived calli, a higher frequency of plant regeneration was obtained (Liu et al. 1985, Li et al. 1985). These methods may be

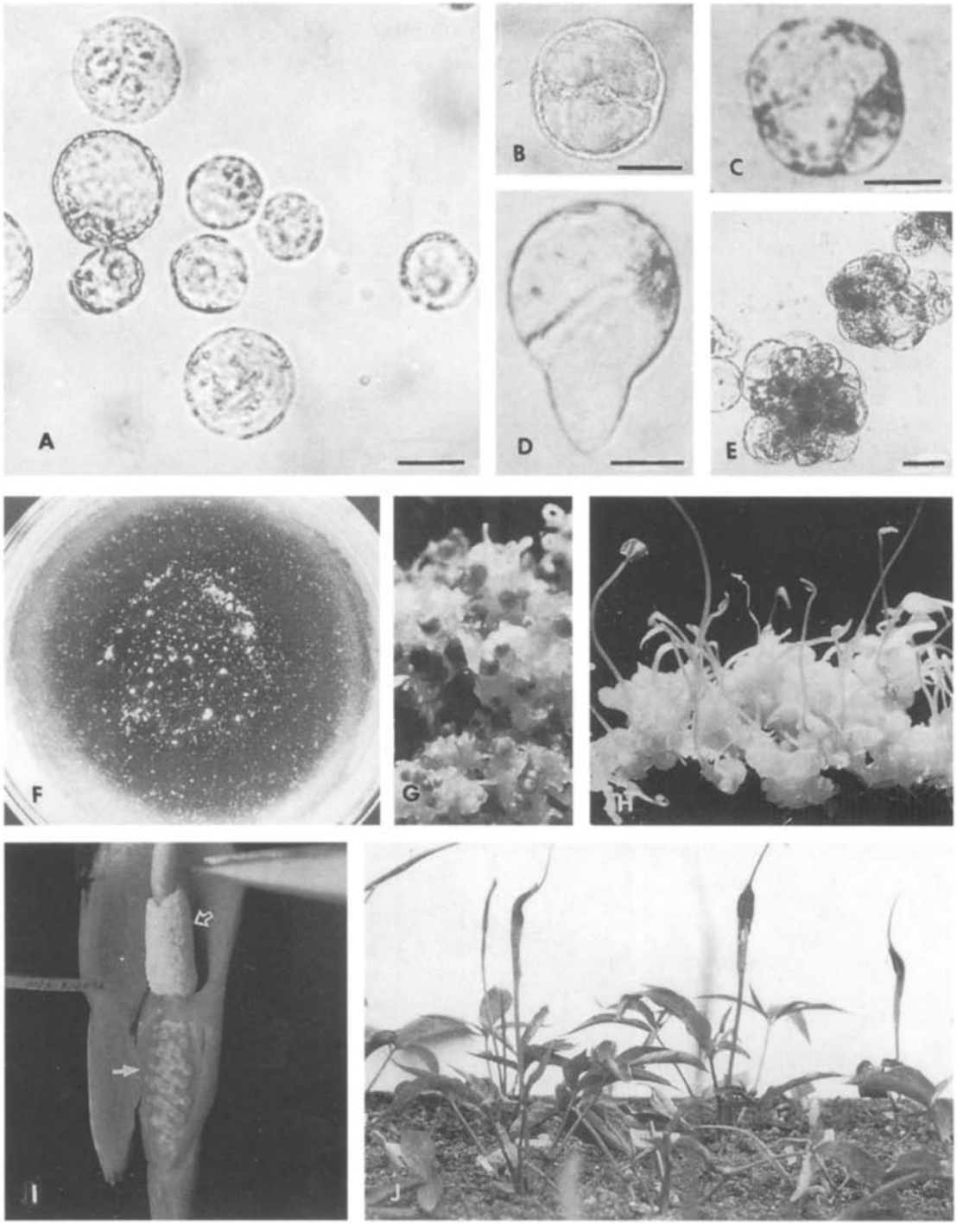

Fig. 1. Plant regeneration from protoplasts of *Pinellia ternata* (Bar = 30 μm).
(A) Freshly isolated protoplasts. (B) First division of a protoplast-derived cell. (C) A four-cell clump. (D) A protoplast-derived triangular cell in agarose. (E) protoplast-derived cell clumps. (F) Microcalli growing in a dish. (G) Tubercles induced from protoplast-derived calli. (H) Plantlets regenerated from protoplast-derived calli. (I) A spathe with anthers (black arrow) and ovaries (arrow). (J) Fertile protoplast-derived plants.

used to increase the frequency of plant regeneration from protoplast-derived calli of pinellia.

Dilution of protoplast cultures with lower osmotic pressure medium was effective for sustaining protoplast-derived cell division (Sun et al. 1991, Murata 1992, Yang et al. 1994). When Wu et al. (1986) used the liquid-solid double layer culture, the osmoticum concentration was 0.44 mol l^{-1}in the upper-layer liquid medium and 0.09 mol l^{-1}in the lower-layer solid medium. It was suggested that the osmotic pressure of the upper-layer medium would be gradually reduced by diffusion of osmotica in both layers. Regeneration was achieved from mesophyll cells of pinellia. We observed that the amount of lower-osmotic-pressure medium added to the protoplast cultures was critical. Initially, only 0.2-0.3 ml could be added. Otherwise, protoplasts or protoplast-derived cells could bulge and be further disrupted.

The regeneration system developed from our experiments was stable, and might be useful for genetic transformation studies of pinellia.

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