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Rapid clonal propagation of *Pinellia ternata* by tissue culture

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ABSTRACT

Adventitious buds or protocorm-like bodies were regenerated directly from excised explants without intervening callus. Differences in the ability of regeneration were observed among different plant organs with bulbils showing the highest regenerative ability followed by leaf blade and petiole. Ability of vegetative propagation of bulbil could be maintained by alternate solid and liquid-medium culture. Theoretically, 1.7 X 10^{27} plantlets could be produced from a single bulbil by this technique within one year based on the production and rapid growth of protocorm-like bodies and adventitious buds. Concentration of MS salts, NAA and sucrose influenced not only root formation from the differentiated adventitious buds, but also root number and length. For root formation, the best combination was one-half strength MS salts with 3-5% sucrose and 1 mg/l NAA. The high survival rate of 96% was recorded when plantlets were transplanted into a mixture of vermiculite:loam soil:peat moss (1:2:1). Plants from in vitro culture were morphological similar to field-grown plants. The acute toxicity of crude extracts from protocorm-like bodies was about one-fourth that of extracts from tubers of field-grown plants when tested with white mice. Tissue culture has potential for clonal propagation of Pinellia ternata plants for commercial use.

ABBREVIATIONS

MS: Murashige and Skoog (1962), 2,4-D: 2,4-dichlorophenoxyacetic acid, NAA: α-naphthaleneacetic acid, BA: 6-benzylaminopurine.

INTRODUCTION

Many medicinal plants have been propagated successfully by tissue or organ culture. These include a number of Chinese medicinal herbs such as Bupheurum falcatum (Hiroaka et al. 1983), Astragalus membranaccus (Fujioka et al. 1983), Dioscorea bulbifera (Forsyth and Van Staden 1982), Panax ginseng (Chang and Hsin 1979), Papaver somniferum (Hodges and Rapoport 1982) and Digitalis lantana (Erdei et al. 1981).

Pinellia ternata B. has been used widely for thousands of years in China to prevent vomiting and for analgesic and sedative effects. The tubers collected from plants growing naturally in the mountains are insufficient for medicinal use. The tissue culture method was therefore studied in an attempt to mass-propagate this medicinal plants.

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MATERIAL AND METHODS

Plants of Pinellia ternata grown in Wulin farm (2,000 m above sea level) were dug and bulbils about 2-4 mm in diameter forming at the base of leaf bledes or petioles were sterilized with 70% alcohol for 30 sec and 0.5% sodium hypochloride solution for 10 min and washed with sterilized water 3-4 times. The top half of the bulbils were excised and inoculated onto medium since a previous study showed that they were more regenerative than lower parts (Chen et al. 1989). Petioles and leaf blades excised from in vitro cultured plants were inoculated directly onto medium without surface sterilization.

The medium used in this study was MS (Murashige and Skoog 1962) basic salts supplemented with 3% sucrose and combinations of different concentrations of growth regulators BA(1-15 mg/l), NAA(0-0.2 mg/l) and 2,4-D(0.2 mg/l) as preliminary experiments showed better responses in these concentration ranges. The pH of the medium was adjusted to 5.7±0.1 before solidifying with 1% agar (solid medium) and autoclaving. The cultures were maintained at 26±1°C with a photoperiod regime of 16-hr light (135 μE/m^2s, fluorescent light) and 8-hr dark cycle. Explants were also cultured on a liquid medium without agar. Liquid cultures were maintained on a 100 rpm rotary shaker and received 50 μE/m^2s light for 16 hr a day.

Acute toxicity (a toxicity test of medicine to animals, usually white mouse) was also tested by calculating LD_{50} (lethal dose, 50%) of crude extracts from protocorm-like body (designated as PLBE) and field-grown P. ternata (W-PtE). The extracts were prepared by cleaning fresh samples followed by blending in a fruit mixer, and filtering with 20S defatted medical gauze and then with a Büchner funnel. The filtrate (can be stored in refrigerator) was diluted with normal saline to the needed concentrations for the test of acute toxicity. Prior to the test, 5 ml of undiluted fresh filtrate was dried at 50°C for 12 hrs and then weighed to determine dry matter content, or extract percentage of crude drug. The extract percentages of W-PtE and PLBE were, respectively, 1.25% and 0.9%. These values were used to calculate the dosage of LD_{50}. The ICR strain of white mice weighing in the range of 20-30 gm were used with 10 per treatment. The toxicity tests were done as described by Litchfield and Wilcoxon (1949). Standard error or 95% confidence limits of binomial variation were calculated for statistical analysis.

RESULTS

When the explants of P. ternata were cultured in solid medium containing MS salts with 2 mg/l BA and 0.2 mg/l NAA, the explants not only produced adventitious buds directly, but also gave raise to greenish large tissue masses, namely protocorm-like bodies (Figs. 1 and 2). Among the tissue explants tested, the bulbil was the best for adventitious bud formation followed by leaf blades and petioles (Table 1). Fig. 3 shows the time course of adventitious bud differentiation from a bulbil. It is clear that by 20-30 days the number of adventitious buds formed was near the maximum so subculturing could be carried out at this time.

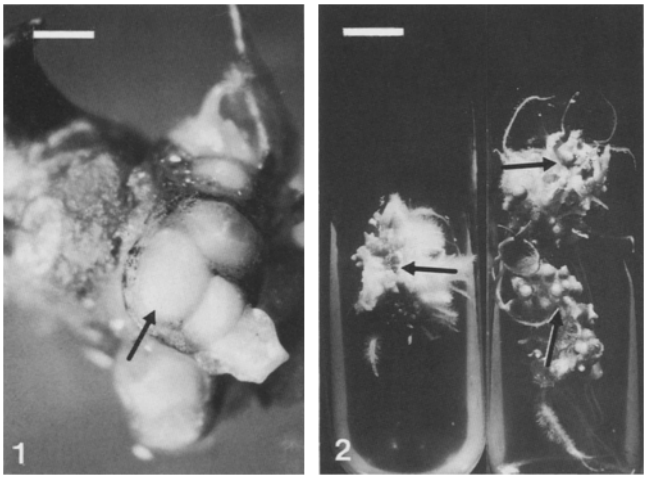

Fig. 1-2. The protocorm-like bodies (arrow) induced from bulbil of P. ternata cultured on solid MS medium with 2 mg/l BA and 0.2 mg/l NAA for 10 (Fig. 1) and 30 days (Fig. 2). (Bars equal 0.15 cm in Fig. 1 and 1 cm in Fig.2).

Table 1. The adventitious bud-forming ability of meristems from different plant tissues of P. ternata.*

Meristem source	Culture duration (days)	No. of adventitious buds formed per explant
Bulbil	30	22±3
	50	31±4
Petiole	30	8±4
	50	23±3
Leaf blade	30	10±3
	50	25±3

*Culture medium : MS salts with 2 mg/l BA and 0.2 mg/l NAA.

The protocorm-like bodies induced from BA and NAA-containing medium were then cut into pieces about 2x2x2 mm in size and transferred into a liquid medium containing different combinations of BA, NAA and 2,4-D. The effect of plant growth regulators on the differentiation ability of protocorm-like bodies of P. ternata in liquid medium was shown in Table 2. It was found that addition of 2,4-D into a medium containing 2 mg/l BA and 0.1 mg/l NAA promoted the growth of protocorm-like body and produced more adventitious buds from cultured protocorm-like bodies (Table 2). Fig. 4 showed the protocorm-like bodies which were cultured in liquid medium and under low light intensity of 20 days. Plant regeneration from protocorm-like bodies was observed after continuous culture in liquid medium (Fig. 5).

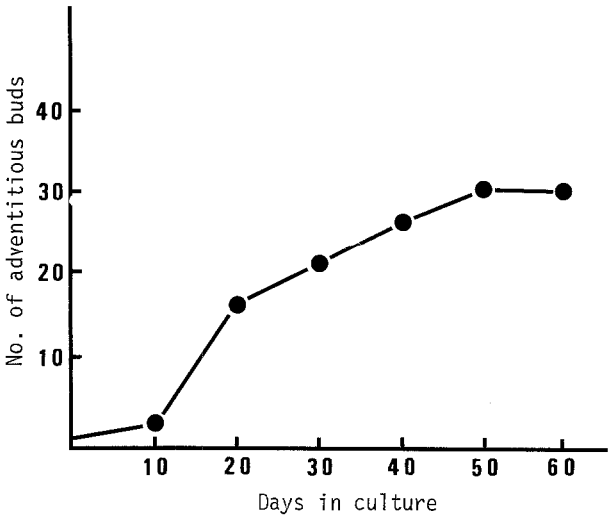

Fig. 3. Time course of adventitious bud formation from a bulbil of P. ternata. Culture medium: MS salts with 2 mg/l BA and 0.2 mg/l NAA.

In order to produce more secondary adventitious buds, the primary adventitious buds and protocorm-like bodies produced from liquid culture were transferred onto a solid medium containing different concentrations and combinations of BA and NAA (Table 3). It was found that medium containing 2 mg/l BA or 2 mg/l BA combined with 0.2 mg/l NAA produced the highest number of secondary adventitious buds from primary adventitious buds. Whereas medium containing 2-5 mg/l BA (without NAA) produced the highest number of new adventitious buds from protocorm-like bodies. Intact plants induced from protocorm-like bodies cultured on solid medium containing 2 mg/l BA and 0.2 mg/l NAA for 2 weeks. 4 weeks and 8 weeks were shown in Figs. 6,7 and 8, respectively.

Concentrations of MS salts, NAA and sucrose influenced not only root formation from the differentiated adventitious buds, but also root number and length. For root formation, the best combination was one-half strength of MS salts with 3-5% sucrose and 1 mg/l NAA (Tables 4 and 5 and Fig. 9).

The high survival rate of 96% was recorded when plantlets were transplanted into a mixture of vermiculite:loam soil:peat moss (1:2:1) (Table 6). Plants from in vitro culture were morphologically similar to field-grown plants. The basic histological structure of the protocorm-like body derived from cultured bulbils was similar to that of tubers of field-grown plants. The acute toxicity of crude extracts from protocorm-like bodies was about one-fourth the strength of that from tubers of field-grown plant (Table 7). However, the useful compounds have not yet been isolated and identified. We still do not know how much of the pharmaceutically active compounds are present in protocorm-like bodies. Nevertheless, the results suggested that protocorm-like bodies produced from bulbil culture are less toxic to the animal (mouse) than the tuber of field grown plants. Thus we concluded that tissue culture may have the potential for clonal propagation of P. ternata plants for commercial use.

DISCUSSION

Mass propagation of P. ternata by tissue culture was first reported by Shoyama et al. (1983). They reported that the addition of 0.5 mg/l 2,4-D and 1 mg/l kinetin stimulated production of a maximum number of regenerated plants. Theoretically, 4 X 10^{23}

Table 2. Influence of plant growth regulators on growth and differentiation ability of adventitious buds of P. ternata in liquid culture.*

MS salts with BA	NAA	2,4-D	% of protocorm-like bodies produced with diameter (cm) of			No. of adventitious buds produced per protocorm-like body
-mg/l-			0.3-0.5	0.6-1.0	1.1-1.5	
2			15(7-26)**	67(54-79)	18(9-30)	5±1
2	0.1		0(0-4)	78(64-88)	22(12-34)	5±1
2	0.1	0.2	0(0-4)	72(59-83)	28(17-41)	6±1
4			15(7-26)	85(74-93)	0(0-5)	5±1
4	0.1		21(12-33)	54(41-67)	25(15-38)	5±1

* Culture duration was 20 days. Size of protocorm-like bodies used for culture was 2x2x2 mm. 100 protocorm-like bodies were used for each treatment.
** Data in parentheses are the 95% confidence limits.

Table 3. Influence of plant growth regulator on adventitious bud formation from cultured adventitious bud and protocorm-like body of P. ternata on solid medium.

MS salts with BA	NAA	No. of new adventitious buds formed per adventitious bud cultured	No. of new adventitious buds formed per protocorm-like body cultured
-mg/l-			
1	0	15.0±3.1	55.5±6.3
1	0.2	12.1±2.0	46.0±5.7
2	0	21.8±3.6	73.0±6.8
2	0.2	23.0±3.4	60.2±6.1
5	0	11.7±2.6	70.0±7.2
5	0.2	21.5±2.9	56.1±5.9
10	0	3.7±0.9	48.1±6.6
10	0.2	16.9±2.6	28.5±4.2
15	0	2.0±0.4	26.2±3.8
15	0.2	9.0±1.8	25.4±5.4

* Culture duration was 30 days. 50 explants were used for each treatment.

Table 4. Influence of sucrose and NAA concentrations on root-formation from adventitious buds of P. ternata.*

Sucrose concentration (%)	NAA concentration (mg/l)	% of adventitious buds forming roots	Average root No.	Average root length (cm)
1	0	40(30-50)**	3.5±1.0	0.6±0.3
	0.5	56(46-66)	5.3±1.2	0.8±0.3
	1.0	100(96-100)	15.0±2.0	1.5±0.5
3	0	82(73-89)	6.2±1.0	1.8±0.5
	0.5	100(96-100)	9.5±1.3	2.2±0.5
	1.0	100(96-100)	21.8±2.1	2.4±0.4
5	0	85(76-91)	10.4±1.4	2.0±0.3
	0.5	100(96-100)	12.6±1.8	2.4±0.5
	1.0	100(96-100)	23.0±1.8	2.9±0.5
10	0.5	63(53-72)	8.0±1.4	1.1±0.4

* Culture medium: Full strength of MS salts. Culture duration was 30 days. 100 adventitious bud segments were used for each treatment.
** Data in parentheses are the 95% confidence limits.

Table 5. Influence of MS-salt strength on the rooting performance of adventitious buds of P. ternata.*

MS salts strength	% of adventitious buds forming roots	No. of root formed per adventitious bud	Average root length (cm)
1/4X	100	24±3	3.0±0.8
1/2X	100	29±2	5.0±1.0
1X	100	23±3	2.5±0.5

* Culture duration was 30 days. Culture medium contained 5% sucrose and 1 mg/l NAA. 100 adventitious buds were used for each treatment.

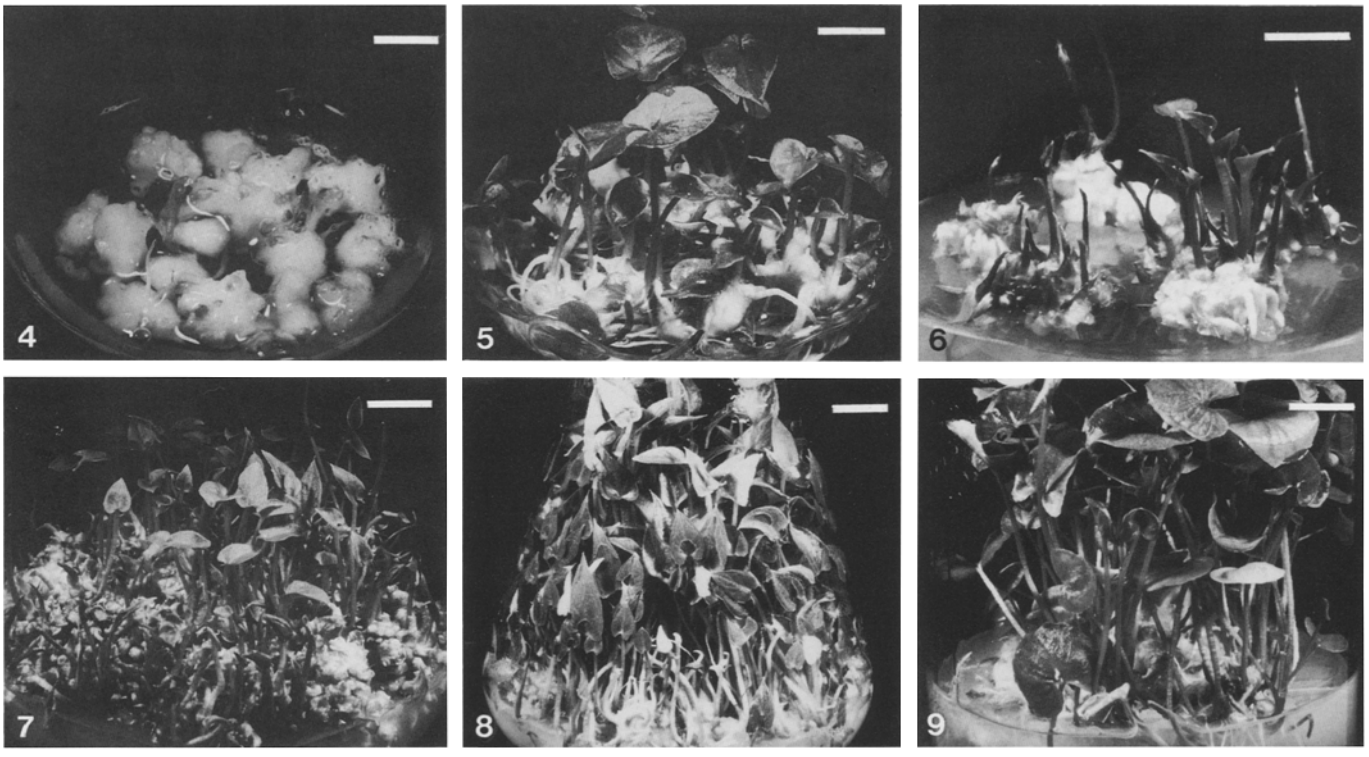

Fig. 4. Protocorm-like bodies about 2x2x2 mm in size were cultured in liqiud medium and low light intensity for 20 days.
Fig. 5. Continuous culture of the orotocorm-like bodies in 2,4-D-free liquid medium for 2 weeks results in plant regeneration.
Fig. 6-8. Plants induced from protocorm-like bidies cultured on solid medium containing 2 mg/l BA and 0.2 mg/l NAA for 2 (Fig. 6), 4 (Fig. 7) and 8 weeks (Fig. 8), respectively.
Fig. 9. Adventitious buds of P. ternata were cultured in half strength solid MS medium with 5% sucrose and 1 mg/l NAA for 4 weeks.
(Bars equal 1 cm in all Figures)

Table. 6. Influence of soil-mixture components on the survival of test-tube P. ternata plants after transplanting.*

Soil-mixture components**	No. of plants transplanted	Surviving plants No.	-%-
1 Vermiculite: 2 Soil: 1 Peat moss	200	192	96(93-99)***
1 Sand: 2 Soil: 1 Peat moss	190	137	72(66-78)
1 Sand: 2 Soil	190	116	61(58-64)

* Plants for transplanting had 20±5 roots with a length of 3.0±0.5 cm.
** Mixed by weight.
*** Data in parentheses are the 95% confidence limits.

Table 7. Acute toxicity of P. ternata extracts in mice.

Drug route	LD_{50}(mg/l)	95% Confidence limit
PLBE I.P.	27.1	15.5-47.0
W-PtE I.P.	7.1	5.9-8.6

PLBE : Crude drug extracts of protocorm-like bodies.
W-PtE : Crude drug extracts of wild-grown P. ternata.

plants could be produced from a single tuber in a year based on the multiplication rate. Our results show that protocorm-like bodies were first induced from bulbils cultured on solid medium followed by alternating culture on solid and liquid medium can not only produce large number of adventitious buds and protocorm-like bodies but also possess high capacity for plant regeneration. Theoretically 1.7×10^{27} plants could be produced from a single bulbil in a year. The average rate of increase is 40 times through protocorm-like bodies cultured on solid and liquid medium in a 21 day culture period. Since there can be 17 subcultures in a year the propagation rate will be $40^{17} = 1.7 \times 10^{27}$. A scheme of mass propagation of P. ternata is presented in Fig. 10.

Aitken-Christie et al. (1987) described a "meristematic multiplication" system based on continuous production of meristematic tissue on solid medium. The system, which generates naturally separated "meristematic nodules", has been maintained as a Pinus radiata plant regeneration system. McCown et al. (1987) have also developed a liquid culture system for Populus in which "nodules" are the predominant structure. These nodules all go through the same developmental sequence. They enlarge and multuple, and have a high capacity for plant regeneration. Our results show that the protocorm-like bodies produced from bulbils of P. ternata were quite similar to

those of "meristematic tissue" or "nodules structure". In addition, the multiplication rate of these protocorm-like bodies could be maintained in solid or liquid medium for at least 2 years.

McCown et al. (1987) also suggested that the nodule culture system may be useful for secondary metabolite production. In cell cultures, differentiation usually coincides with cessation of active growth. The nodules in liquid culture system have the capacity to multiply similar to suspension cultures, and yet are highly differentiated. Our solid-liquid culture system of protocorm-like bodies also has the biological capacity to multiply. Although the useful medicinal compounds of P. ternata have not yet been isolated, we have found that the basic histological structure of protocorm-like bodies derived from cultured bulbils and tubers of field-grown plants are similar. Plants from in vitro culture are also morphologically similar to field-grown plants. Thus we conclude that alternating solid-liquid culture method of these protocorm-like body is a very useful system for mass propagation of P. ternata.

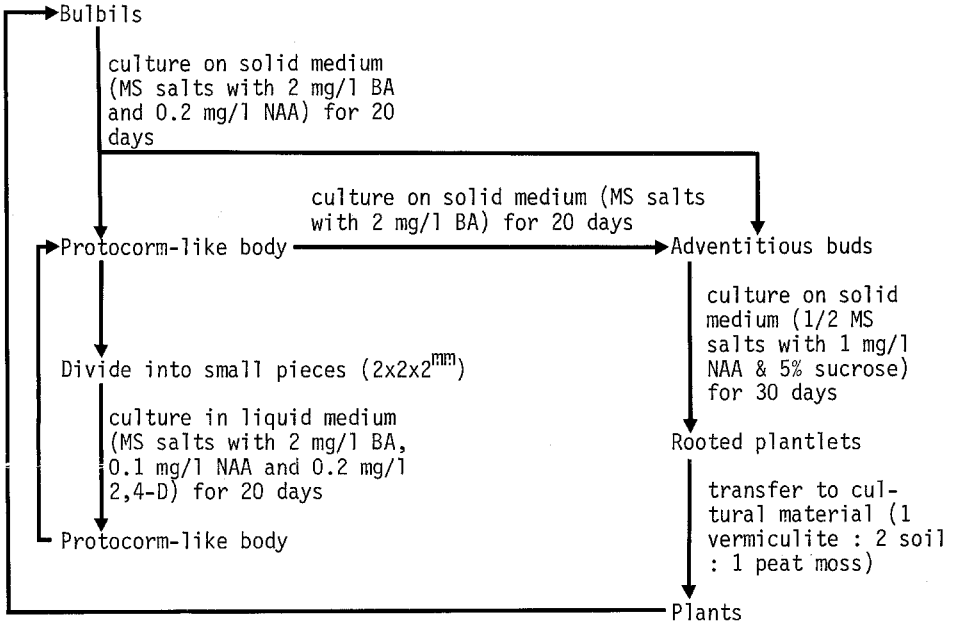

Fig. 10. Scheme of mass propagation of P. ternata.

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