*ACTA BIOLOGICA CRACOVIENSIA Series Botanica* 47/2: 27–32, 2005

# EFFICIENT IN VITRO PLANT REGENERATION OF *PINELLIA TERNATA* (THUNB) BREIT

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Received November 10, 2004; revision accepted March 30, 2005

An efficient plant regeneration system for *Pinellia ternata* (Thunb) Breit, an important wild Chinese herb, was established. The investigated factors influencing callus induction and plant regeneration included sterilizing agents, explant type, culture medium, and combinations of plant growth regulators in the medium. Ten min treatment of explants with 0.1% $HgCl_2$ was more suitable for sterilization of tuber explants than NaClO and $H_2O_2$. Tubers as explants were superior to leaves and petioles, and MS medium was better than N6 and White's media. It was found that 9.1 μM 2,4-dichlorophenoxyacetic acid (2,4-D) plus 4.4 μM 6-benzyladenine (BA) in the medium was the best hormone combination for promoting callus induction. The highest shoot regeneration frequency (99%) was achieved from callus cultured on MS medium supplemented with 9.3 μM kinetin (KN) and 2.3 μM 2,4-D. The rooted shoots were successfully transplanted in soil, with over 95% survival. Establishment of an efficient plant regeneration procedure provides a basis for rapid in vitro propagation of *P. ternata*, which is in great demand in China, and represents an excellent tool for further work on its genetic manipulation.

**Key words:** *Pinellia ternata*, in vitro culture, callus, plant growth regulators, regeneration, tuber explants.

## INTRODUCTION

*Pinellia ternata* (Thunb) Breit (Araceae) is a plant of medicinal importance. This herbaceous plant is native to China, where its tuber, *Rhizoma pinelliae*, is a valuable traditional Chinese medicine widely used for curing coughs and for treatment of tracheal dryness, phlegm, counterflow, vomiturition, glomus and lump (Li and Jiang, 1992). Its effect on coughs is similar to that of codeine but is nonaddictive (Chen and Yang, 1997; Mao and Peng, 2002). Prevention of vomiting is another application of *P. ternata*, employing its activation of the efferent nerves of the vagus (Xue et al., 1991; Akira, 1998; Kurata, 1998). Modern pharmacology studies have also indicated that *P. ternata* has an antitumor effect (Yang, 1991; Sun, 1992). A lectin extracted from *P. ternata* was found to have the effect of terminating early pregnancy (Tao, 1981, 1983; Xia, 1985; Yang, 1993). Because of its numerous medical applications, *P. ternata* is more and more in demand. Wild sources of *P. ternata* are in danger of being exhausted due to over-collection, inadequate propagation, and poor selection of desirable traits (Mao and Peng, 2002).

Wild *P. ternata* reproduces asexually with bulbils. Sexual reproduction of *P. ternata* is geitonogamous in nature, but its rate of flowering is very low (Gu et al., 1994). Sexual reproduction is therefore of minor importance. The tuber has no function of reproduction in nature because it only grows without developing young tubers or sprouts (Gu et al., 1994). The bulbil is a crucial organ for asexual reproduction of *P. ternata*. The bulbil, which arises from the top of the petiole, drops to the soil along with a blasted leaf in adverse conditions, and can develop a new plant in a favorable environment. The bulbil usually drops near the mater-

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PL ISSN 0001-5296

TABLE 1. Callus induction frequency (%) under different sterilization conditions

| Sterilizing agent | Concentration | 5 min | 10 min | 15 min | 20 min |
|---|---|---|---|---|---|
| $HgCl_2$ | 0.1% | 0 | 78.67 ± 5.03 ab | 59.67 ± 5.77 bc | 0 |
| | 0.2% | 46.67 ± 6.51 cd | 38.67 ± 0.13 d | 0 | 0 |
| | 0.5% | 37.67 ± 5.03 d | 0 | 0 | 0 |
| NaOCl | 1% | 0 | 0 | 14.33 ± 2.31 fg | 22.33 ± 6.81 ef |
| | 2% | 0 | 10.00 ± 2.68 g | 19.00 ± 1.73 f | 21.00 ± 1.73 f |
| | 5% | 3.33 ± 3.51 h | 22.33 ± 4.04 ef | 12.33 ± 5.03 g | 11.00 ± 3.46 g |
| $H_2O_2$ | 3% | 0 | 36.67 ± 3.51 de | 33.33 ± 3.51 de | 24.33 ± 2.31 ef |
| | 10% | 4.67 ± 4.04 h | 18.00 ± 1.73 f | 18.00 ± 1.73 f | 0 |

Data represent means ± SD based on three replicates. Different letters after means indicate significant differences at $p < 0.05$.

nal plant, the result being that the species grows in a concentrated area, which is disadvantageous to promulgation.

Due to poor reproduction in nature, artificial cultivation of *P. ternata* has been attempted to promote the production of this species, but with low propagation rates. It is therefore necessary to find a way to promote rapid production of *P. ternata*. Plant tissue culture and somatic regeneration has been demonstrated to be successful in achieving rapid propagation of various plant species (Gamborg, 2002). Callus induction and regeneration from *P. ternata* with leaf and petiole as explants and from protoplast culture have been reported (He, 1996; Zhang, 1999). To date, however, there are no reports on plant regeneration from tuber of *P. ternata*. In this work, a reliable regeneration system for *P. ternata* is described. Explant selection, medium type and growth regulators were evaluated in order to determine effective conditions for callus induction and plant regeneration.

## MATERIALS AND METHODS

### PLANT MATERIALS AND STERILIZATION TREATMENT

*Pinellia ternata* plants were collected from the Medical Plant Garden at the Second Military Medical University in Shanghai, and their leaves, petioles and tubers were used as explant materials. In order to solve the serious contamination problem encountered in tuber culture, three sterilizing agents for plant tissues, NaClO, $H_2O_2$ and $HgCl_2$, were tested at various concentrations for their sterilization efficiencies with tuber explants. The tubers were washed thoroughly with tap water and immerged in 75% ethanol for 60 sec followed by five rinses with sterile distilled water. The tubers were peeled and cut into blocks 8 $mm^3$ in volume. After sterilization for various durations, the blocks (explants) were rinsed five times with sterile distilled water and inoculated on 25 ml MS medium (Murashige and Skoog, 1962) supplemented with 4.5 µM 2,4-D, 2.2 µM BA and 30 g $dm^{-3}$ sucrose in a 9 cm Petri dish (10 explants per dish, total 96 dishes) at 25± 2˚C under a 12 h photoperiod (60 mol $m^{-2}$ $s^{-1}$, white fluorescent tubes) until callus developed. All culture media were solidified with 2.6 g $dm^{-3}$ Phytagel (Sigma) and adjusted to pH 5.8 prior to autoclaving for 20 min at 121˚C and 105 kPa. Thirty explants were used in each sterilization treatment, and each treatment was done in three replicates. The callus induction frequency was calculated as the total number of explants developing calluses divided by the total number of explants inoculated, and expressed as a percentage. The experimental data were analyzed by ANOVA. Fisher's least significant difference (LSD) was used to compare the means and standard deviations (SD).

### EFFECT OF CULTURE MEDIUM TYPE AND EXPLANT TYPE ON CALLUS INDUCTION

Four culture media [MS, half-strength MS, N6 (Chu et al., 1975), White's medium (White, 1943)] and three explant types (leaf, petiole, tuber) were tested for their ability to produce callus. For medium effect, the tubers were cultured in each of the four media containing 4.5 mM 2,4-D and 2.2 M BA. For explant effect, leaf, petiole and tuber were cultured on MS medium supplemented with 4.5 µM 2,4-D and 2.2 µM BA. Thirty explants were used in each treatment (three replicates), and the callus induction frequency was assessed.

### EFFECT OF PLANT GROWTH REGULATORS ON CALLUS INDUCTION, SHOOT REGENERATION AND ROOTING

Based on a previous study on callus induction of *P. ternata* (Zhang, 1999), several MS-based media containing different concentrations and combinations of plant growth regulators (NAA, KT, 2,4-D and BA) were investigated for their effects on callus induction from tuber explants. Subculture was carried out at 2-week intervals, and callus induction frequency was measured after 4 weeks.

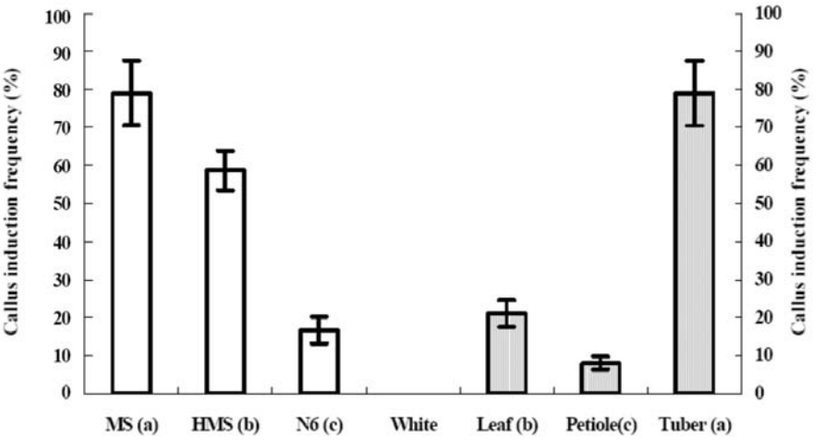


**Fig. 1.** Influence of medium type and explant type on callus induction frequency of *Pinellia ternata*. Vertical bars represent SD of means of three replicates. MS – Murashige and Skoog medium; HMS – half-strength MS medium; N6 – N6 medium; White – White's medium. All media contained 4.5 µM 2,4-D and 2.2 µM BA.

After 4 to 6 weeks of subculture, calluses of ~64 $mm^3$ were transferred onto MS-based regeneration media containing different concentrations of cytokinin (BA, KT) and auxin (NAA, 2,4-D) using a two-step regeneration procedure that included 50% reduction of gelling agent after 10 days as described previously (Tang et al., 2000). The callus was first transferred to regeneration medium solidified with 5.2 g $dm^{-3}$ Phytagel for 10 days, and then to the same medium but with a Phytagel concentration of 2.6 g $dm^{-3}$. The effects of different plant growth regulators on shoot regeneration were examined 1 month later. For callus induction and shoot regeneration, thirty explants were used in each treatment and each treatment was done in three replicates.

Shoots 1 cm in height were excised and transferred to 100 ml flasks containing 30 ml rooting medium: half-strength MS medium containing different concentrations of IAA (Indole-3-Acetic acid), NAA or IBA (Indole-3-Butyric acid). The shoots were first cultured for 3 days in the dark and then under a 16 h photoperiod for 20 days until roots developed. Then the rooting frequency was measured. Ten shoots were used in each treatment, and each treatment was done in three replicates.

The effect of plant growth regulators on callus induction, shoot regeneration and rooting was analyzed by ANOVA. Fisher's LSD was used to compare the means and standard deviations (SD).

### ACCLIMATION AND TRANSPLANTING OF PLANTLETS

The lids of the flasks were removed when the plants developed at least three roots. Two days later, the medium on the plantlet was washed off and the plantlets were transplanted to plastic vessels containing a 1:1:1 mixture of vermiculite, perlite and peat, and placed in the greenhouse. The plantlets were watered with a diluted solution of MS macronutrients (1:50) once per day. The light intensity in the greenhouse was 60 µmol $m^{-2}$ $s^{-1}$.

## RESULTS AND DISCUSSION

Tubers are difficult explants to disinfect. Our preliminary results on *P. ternata* showed that tubers peeled and cut into 8 $mm^3$ blocks were much easier to disinfect than whole tubers (data not shown). Cut blocks of tubers were used as explants in the present study. Of the three disinfectants, $HgCl_2$ was better than NaClO and $H_2O_2$ for sterilizing tuber explants to achieve high callus induction frequency. In the present study, 10 min treatment with 0.1% $HgCl_2$ was found best for sterilization and callus induction, and the callus induction frequency was 79% (Tab. 1).

Culture medium and explant type were found to significantly influence callus induction and regeneration. Four basal media (MS, half-strength MS, N6 and White's medium) containing the same hormones (4.5 µM 2,4-D and 2.2 µM BA) were used in this study. MS medium was the most favorable for callus proliferation, while callus could not be induced on White's medium (Fig. 1). Zhang (1999) studied callus induction from petiole and leaf of germinated *P. ternata* seedlings and found that leaf induced callus at higher frequency than petiole. In the present study, we also found that leaf explants were better for callus induction than petiole explants, but the tuber explants showed the highest

TABLE 2. Effect of plant growth regulators on callus induction, shoot regeneration and rooting of *Pinellia ternata* tuber explants

| Callus induction | | Shoot regeneration | | Rooting | |
|---|---|---|---|---|---|
| Plant growth regulator (μM) | Callus induction frequency (%) | Plant growth regulator (μM) | Shoot regeneration frequency (%) | Auxin (μM) | Rooting frequency (%) |
| 2,4-D 2.3 | 0 | KN 4.7 + 2,4-D 2.3 | 81.3 ± 5.1a | IAA 0.6 | 52.3 ± 5.0d |
| 2,4-D 4.5 | 0 | KN 9.3 + 2,4-D 2.3 | 99.0 ± 1.7a | IAA 1.1 | 75.0 ± 4.4b |
| 2,4-D 9.1 | 0 | KN 9.3 + 2,4-D 4.5 | 94.7 ± 4.0a | IAA 2.9 | 33.3 ± 3.5e |
| 2,4-D 4.5 + KN 2.3 | 34.3 ± 2.3c | BA 4.4 + 2,4-D 2.3 | 39.0 ± 5.3b | NAA 0.5 | 100.0 ± 0a |
| 2,4-D 4.5 + KN 4.7 | 8.0 ± 1.7de | BA 8.9 + 2,4-D 2.3 | 40.3 ± 2.5b | NAA 1.1 | 100.0 ± 0a |
| 2,4-D 9.1 + KN 4.7 | 42.3 ± 5.0c | BA 8.9 + 2,4-D 4.5 | 51.3 ± 5.1b | NAA 2.7 | 73.0 ± 3.0 |
| 2,4-D 4.5 + BA 2.2 | 79.0 ± 8.5b | KN 4.7 + NAA 2.7 | 0 | IBA 0.5 | 78.7 ± 5.1b |
| 2,4-D 4.5 + BA 4.4 | 67.7 ± 5.0b | KN 9.3 + NAA 2.7 | 14.3 ± 2.3cd | IBA 1.0 | 100.0 ± 0a |
| 2,4-D 9.1 + BA 4.4 | 100.0 ± 00a | KN 9.3 + NAA 5.4 | 0 | IBA 2.5 | 58.7 ± 5.1cd |
| NAA 5.4 + KN 2.3 | 9.0 ± 1.7d | BA 4.4 + NAA 2.7 | 11.0 ± 4.2d | No auxin | 16.7 ± 0.6f |
| NAA 5.4 + KN 4.7 | 0 | BA 8.9 + NAA 2.7 | 24.3 ± 5.1c | | |
| NAA 10.7 + KN 4.7 | 10.0 ± 3.0d | BA 8.9 + NAA 1.0 | 17.7 ± 5.0cd | | |
| NAA 5.4 + BA 2.2 | 11.0 ± 1.7d | No hormone, control | 0 | | |
| NAA 5.4 + BA 4.4 | 4.7 ± 4.0e | | | | |
| NAA 10.7 + BA 4.4 | 15.7 ± 2.3d | | | | |
| No hormone, control | 0 | | | | |

Data represent means ± SD based on three replicates. Different letters after means indicate significant differences at $p < 0.05$.

callus induction frequency among the tested three explant types (Fig. 1). The advantages of tuber as an explant, such as its easy acquisition and preservation, mean that the procedure can be carried out in all seasons.

Callus was induced using MS basal medium supplied with both auxin and cytokinin (Tab. 2). When inoculated on media containing no plant growth regulators, no callus was induced regardless of explant type. Application of 2,4-D alone showed no effect on callus induction, but the combined use of auxin and cytokinin significantly improved callus induction and growth. The use of 2,4-D and BA together were superior to the use of other auxin and cytokinin combinations tested for callus induction. The highest callus induction frequency (100%) was achieved from tubers cultured on medium containing 9.1 μM 2,4-D and 4.4 μM BA (Tab. 2). The calluses were white or light yellow. The calluses were maintained by subculture at 2-week intervals. The tuber block explants could not be induced to produce adventitious shoots directly.

A suitable combination of auxin and cytokinin in the regeneration medium was important for regeneration of *P. ternata* (Tab. 2). The use of KN and 2,4-D together was superior to the other combinations tested for regeneration. The highest shoot regeneration frequency (99%) was achieved with medium containing 2.3 μM 2,4-D and 9.3 μM KN. The maximum number of shoots per callus (7.3) and the longest shoots (2.3 cm) were achieved when calluses were cultured on medium containing 8.9 μM BA and 2.3 μM 2,4-D, and on medium containing 9.3 μM KN and 2.3 μM 2,4-D, respectively. The time of shoot emergence, varying from 13 days to 25 days, was also affected by plant growth regulator treatments. Calluses still had the potential to regenerate plants after 18 months of subculture (data not shown).

A one-step procedure using one gelling agent concentration yielded low frequency of shoot regeneration (data not shown), so a two-step procedure was adopted in which the callus was placed in regeneration medium with double the amount of Phytagel (5.2 g $dm^{-3}$) for 10 days, and then transferred to the same medium with the standard amount of Phytagel (2.6 g $dm^{-3}$). In the first step, in most cases the calluses cultured on regeneration medium became hard and turned green, and the whole callus developed into a protuberant structure in medium containing high Phytagel (5.2 g $dm^{-3}$). In the second step, subsequent transfer of such calluses to medium containing low Phytagel (2.6 g $dm^{-3}$) led to differentiation of shoots 10 to 15 days later, and leaves developed soon after shoot emergence. For

a few relatively soft calluses, many tiny green spots emerged on the surface of the calluses in the first step, and some of them subsequently differentiated into shoots 10 days later in the second step, resulting in multiple shoots (up to 7.3) derived from one callus. Earlier literature indicated that both increased osmolarity and decreased water content in the medium enhanced plant regeneration of rice callus and suspension cells (Tsukahara and Hirosawa, 1992; Jain and Wu, 1996). The stimulatory effect of two-step regeneration may share a similar mechanism.

On regeneration medium, shoots developed roots slowly. Rooting medium promoted more rapid root development. Vanegas et al. (2002) reported shoots of marigold developed roots well on hormone-free medium. In the present study, on hormone-free medium a few roots developed from shoots of *P. ternata* after 17 days, and the rooting frequency (number of shoots rooted after 20 days/total number of shoots) was 16.7%. Rooting efficiency was improved by the addition of auxin to the medium (Tab. 2). The highest rooting rate (100%) was achieved from shoots cultured on half-strength MS medium containing low concentrations (0.54 or 1.1 μM) of NAA or 1.0 μM IBA. Rooting time was also shortened to 3–4 days with the addition of low concentrations of NAA or IBA to the medium, and there were more roots per shoot on auxin-containing medium (average 6.2) than on auxin-free medium (average 1.3). After a month of acclimation, well-rooted plants were transplanted into pots with over 95% survival. The two-step procedure has been used successfully on plant regeneration from protoplast-derived calluses of rice (Tang et al., 2000).

In this study, we established a stable and efficient system for plant regeneration of *P. ternata*. Only 70 days were needed from callus induction to plantlet transplantation. No significant morphological variation was observed between the 54 plants regenerated by this procedure and the non-cultured donor plants.

Weeds are one of the most serious problems encountered in herb cultivation in China, and herbicides are widely used. However, *P. ternata* is sensitive to most of the commonly used herbicides such as Basta (active ingredient: phosphinothricin) and glyphosate; this is one of the main reasons for low reproduction of *P. ternata*. Genetic transformation, which has been demonstrated to be successful in developing herbicide-resistant crops such as soybeans (Carperter and Gianessi, 2000), may be the way to improve the resistance of *P. ternata* to herbicides. An efficient plant regeneration protocol for *P. ternata* is the first step toward developing herbicide resistance in it by transferring genes such as the *bar* gene, which gives resistance to the herbicide phosphinothricin (Toki et al., 1992), into *P. ternata*. This may ultimately lead to improved production of this valuable plant.

## ACKNOWLEDGEMENTS

This research was funded by the China National High-Tech "863" Program, China Ministry of Education, Shanghai Science and Technology Committee, and Shanghai Agriculture Committee.

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