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【Short Report】

Efficient Plant *in vitro* Regeneration of *Pinelliae Cordatae* Breit

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Pinelliae cordatae, one species of *Pinellia* genus, is a kind of rare wild plant growing in the dank around rocks of hill or cliff. It also has been a traditional Chinese medical herb applied on treating snake biting bite, stomachache and lumbago for years. Moreover, it may be used as a good ornamental plant for its diminutive stature, nice leaves and inflorescence. With the development of this traditional Chinese medicine exploiting nowadays, *P. cordatae* is more and more in demand. The wild sources of *P. cordatae* are in danger of being exhausted due to overcollection, inadequate propagation, and rigorous growing environment limitation. It is, therefore, important to find an effective way of propagation of *P. cordatae* to protect wild resource and solve the shortage problems. Plant tissue culture and somatic regeneration has been demonstrated to be successful in achieving rapid propagation of various plant species (Gamborg, 2002). To date, however, there have been no reports on the plant culture *in vitro* of *P. cordatae*.

In this paper, we report an efficient plant regeneration program of *P. cordatae*. Factors such as explant type, medium type and growth regulator combination were evaluated for determination of effective.

Materials and Methods

1. Plant materials and sterilization treatment

P. cordatae plants were collected from the Medical Plant Garden of the Second Military Medical University in Shanghai, and their tubers were used as explant materials after sterilized. The tubers peeled and cut into blocks of 2 mm^3 in volume were washed thoroughly with tap water, then immerged in 75% ethanol for 60 s and rinsed five times with sterile distilled water. After sterilized with $HgCl_2$ for various durations respectively, the blocks (explants) were rinsed five times with sterile distilled water and inoculated on 25 ml of MS (Murashige and Skoog, 1962) medium supplemented with 4.5μM 2,4-D, 2.2μM BA and 30 g l^{-1} sucrose in 9-cm petri dishes (10 explants per plate) at 25±2ºC under a 12-h. light/12-h. dark photoperiod (60 μmol m^{-2} s^{-1} light intensity provided by white fluorescent tubes). All culture media were solidified with 2.6 g l^{-1} phytagel (Sigma) and adjusted to pH 5.8 prior to autoclaving for 20 min at 121ºC.

2. Effect of culture medium type and explant type on callus induction

Four culture media [MS, half-strength MS, N6 (Chu et al., 1975) and White's medium (White, 1943)] and three explant types (leaf, petiole and tuber) were assayed for their ability to proliferate callus. For analysising the effect of different media mentioned above, the tubers, after sterilized with 0.1% $HgCl_2$ for 10 min. followed by rinses with sterile distilled water five times, were peeled and cut into blocks of 2 mm^3 in volume and cultured in each of the four media containing 4.5 μM 2,4-D and 2.2 μM BA. Leaf, petiole and tuber in blocks of 2 mm^3 were separately cultured on MS medium supplemented with 4.5 μM 2,4-D and 2.2 μM BA after the same sterilizing treatment for testing the effect of different explants mentioned above. All the cultures were maintained at 25±2ºC under a 12-h light/12-h dark photoperiod (60 μmol m^{-2} s^{-1} light intensity provided by white fluorescent tubes). Thirty explants were used in each treatment and each treatment was done in triplicate. The explant with clumps of calli after 4 weeks of culture, was used as evaluation index.

3. Effect of plant growth regulators on callus induction

The explants in blocks of 2 mm^3 were cultured on MS basal medium containing different concentrations and combinations of plant growth regulators (NAA, KT, 2,4-D and BA) (Table 1). All the media contained 3% sucrose and the explants were cultured under a 12-h light/12-h dark photoperiod (60 μmol m^{-2} s^{-1} light intensity provided by white fluorescent tubes). Subculture was carried out at 2-week intervals and the

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Abbreviations : 2,4-D, 2,4-Dichlorophenoxyacetic acid; BA, 6-Benzylaminopurine; IAA, Indole-3-Acetic acid; IBA, Indole-3-Butyric acid; KT, Kinetin; MS, Murashige and Skoog culture medium; NAA, α-Naphthaleneacetic acid.

Table 1. Effect of plant growth regulators on callus induction of *P.cordatae* after 4 weeks in culture.

Combination and concentration (μM) of plant growth regulators	Callus induction frequency (%)	Multiplication rate of callus (FW)
0	0	–
2,4-D 2.3	0	–
2,4-D 4.5	0	–
2,4-D 9.1	0	–
2,4-D 4.5+KT 2.3	34.3 ± 2.3	2.7
2,4-D 4.5+KT 4.7	8.0 ± 1.7	1.6
2,4-D 9.1+KT 4.7	42.3 ± 5.0	2.4
2,4-D 4.5+BA 2.2	79.0 ± 8.5	3.7
2,4-D 4.5+BA 4.4	67.7 ± 5.0	2.7
2,4-D 9.1+BA 4.4	100 ± 0.0[a]	4.8[b]
NAA 5.4+KT 2.3	9.0 ± 1.7	2.1
NAA 5.4+KT 4.7	0	–
NAA 10.7+KT 4.7	10.0 ± 3.0	2.6
NAA 5.4+BA 2.2	11.0 ± 1.7	2.6
NAA 5.4+BA 4.4	4.7 ± 4.0	1.3
NAA 10.7+BA 4.4	15.7 ± 2.3	1.6

Data represent means±SD based on three replicates (30 explants per replicate and 90 explants were used). The tubers were cut in blocks (2 mm^3) and used as explants. They were cultured on MS basal medium containing different concentrations and combinations of plant growth regulators under a 12-h light/12-h dark photoperiod. Subculture was carried out at 2-week intervals and the Callus induction frequency was measured after 4 weeks. The calli over 6 weeks of subculture were tested for the multiplication rate (multiplication rate=fresh weight of calli one month later/initial fresh weight of calli) on the same hormone-containing media. [a]: Significant at $P<0.05$ for callus induction frequency compared to other treatments. [b]: Significant at $P<0.05$ for multiplication times of callus growth compared to those from other auxin and cytokinin combinations.

fresh weight of calli was measured after 4 weeks in culture.

4. Shoot regeneration

After 4 to 6 weeks of subculture, the calli of 4 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 solidified the second medium with gelling agent semi-reduced as that of the first medium after 10 days of culture (Tang et al., 2000). The callus was first transferred to regeneration medium solidified with 5.2 g l^{-1} phytagel for 10 d and then to the same medium, but with phytagel concentration of 2.6 g l^{-1}. The effects of different plant growth regulators on shoot regeneration were examined 1 month later.

5. Rooting, acclimation and transplanting of plantlets

Shoots (1 cm in height) were excised and transferred to flask containing different 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 at 25±2ºC for 3 d in the dark and then under a 16-h light/8-h dark photoperiod (60 μmol m^{-2} s^{-1} light intensity provided by white fluorescent tubes) for 20 d. Root number per shoot, percent of shoot rooting and time to rooting were measured. The lids of flask were removed when plants developed at least three roots. Two days later, the medium on the plantlet was washed off and the plantlets were transplanted into plastic vessels containing medium that was 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) four times per day. The light intensity in the greenhouse was 60 μmol m^{-2} s^{-1}.

Results and Discussion

Being an ideal explant type, the tuber is difficult to disinfect. Our preliminary results on *P. cordatae* showed that tubers peeled and cut into blocks of 2 mm^3, were much easier to disinfect than the whole tubers (data not shown), the cut blocks of tubers were used as explants in the present study. Culture medium and explant type were found to significantly influence callus induction and regeneration. Through mono-factor test, we identified optimal culture medium and explant type for callus induction respectively. Macro elements, gross and type of nitrogen source have

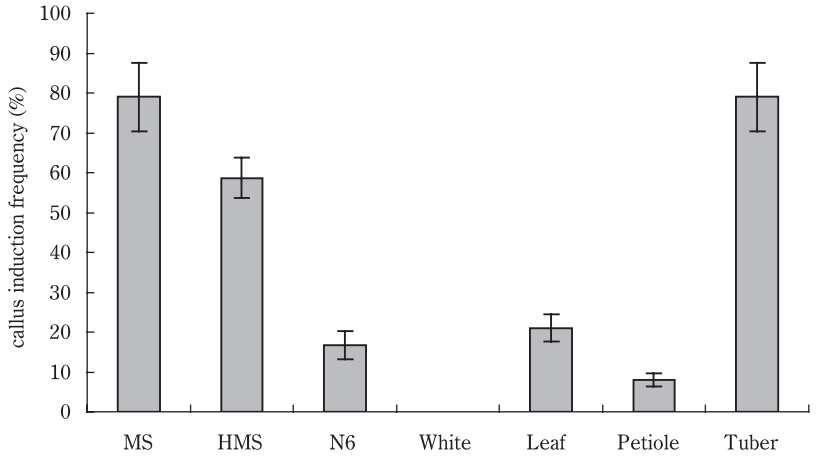

Fig. 1. Influence of medium type and explant type on callus induction frequency of *P.cordatae*. Data represent means ± SD based on three replicates (30 explants per replicate and 90 explants were used). Vertical bars represent SD of the means. The explants were cultured under a 12-h photoperiod and callus induction frequency was measured 4 weeks later. Callus induction frequency was significant at $P<0.05$ when tuber was cultured on MS medium as the explant for callus induction compared to those when leaf or petiole was cultured on MS medium or other culture media were used. 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.

potent regulating effects on the structure of tissue cells (Chen and Chang, 2002). Among four basal media (MS, half-strength MS, N6 and White's medium) used in this study, MS medium was the most favorable for the 20 explants proliferating callus while callus couldn't be induced in White's medium (Fig. 1). Callus induction frequency was also found to be significantly influenced by the source of explants used. In this present study, other two explant type (leaf and petiole) in addition to tuber explant were tested. In this test, the tuber explant had the highest callus induction frequency, followed by leaf explants, while the petiole was the most difficult to induce calli (Fig. 1).

Callus could be induced on MS basal medium supplied with both auxin and cytokinin (Table 1). When inoculated on media containing no plant hormones, no callus was induced whatever explants were used. 2,4-D alone showed no effect on callus induction, but the combined use of auxin and cytokinin significantly improved callus induction and growth. Among the hormone combinations, the use of 2,4-D and BA together were superior to the use of other auxin and cytokinin combinations for callus induction. The highest callus induction frequency (100%) was achieved from explants cultured on the medium containing 9.1 μM 2,4-D and 4.4 μM BA (Table 1). Maintenance of the calli was carried out by periodic subculture at 2-3w intervals. Calli still had the potential to regenerate plants after 18 months of subculture (data not shown).

Under the one-step procedure where one gelling agent concentration is used, no differentiation was found after 30 d and only few shoots, on less than 2% of the explants, appeared 40 d later (data not shown). The low shoot regeneration frequency led us to consider other methods of improving shoot regeneration. Based on the successful use of a two-step procedure (2 gelling agent concentration) on the plant regeneration from protoplast-derived calli of rice (Tang et al., 2000), we tested this method of plant regeneration from calli of *P. cordatae*. In the two-step regeneration procedure, the callus was placed in the basal MS medium supplied with certain plant hormone and double amount of phytagel (5.2 g l^{-1}) for 10 d and then transferred to the same medium with normal amount of phytagel (2.6 g l^{-1}). On the high-phytagel containing medium the callus hardened and, when transferred to the second medium, the hard and compact calli rapidly developed shoots. The earlier literature indicated that increased osmolarity in the medium enhanced plant regeneration of rice callus and suspension cells (Tsukahara and Hirosawa, 1992; Jain and Wu, 1996). The stimulatory effect of the two-step regeneration procedure may share the same mechanism.

In most cases, calli cultured on regeneration media became hard, turned green and subsequently the whole callus developed into a protuberant structure in high phytagel-containing (5.2 g l^{-1}) medium in the first step. Subsequent transfer of such callus to low phytagel-containing (2.6 g l^{-1}) medium in the second step led the protuberances to differentiate into shoots about 10 to 15 d later and leaves developed soon after shoot emergence. For a few relatively soft calli, many tiny green spots emerged on the surface of calli in the

Table 2. Effect of plant growth regulators on shoot regeneration of *P.cordatae*.

Combination and concentration of plant growth regulators (μM)	Shoot regeneration frequency (%)	No. of shoots per callus	Length of the longest shoot (cm)	The shortest time of shoot emergence (day)
KT 4.7+2,4-D 2.3	81.3±5.1[b]	3.8	0.8	22
KT 9.3+2,4-D 2.3	99.0±1.7[a, b]	4.3	2.3	18
KT 9.3+2,4-D 4.5	94.7±4.0[b]	1.0	1.8	21
BA 4.4+2,4-D 2.3	39.0±5.3	4.5	1.4	13
BA 8.9+2,4-D 2.3	40.3±2.5	7.3	0.6	16
BA 8.9+2,4-D 4.5	51.3±5.1	2.8	1.1	20
KT 4.7+NAA 2.7	0	–	–	–
KT 9.3+NAA 2.7	14.3±2.3	1.0	1.2	24
KT 9.3+NAA 5.4	0	–	–	–
BA 4.4+NAA 2.7	11.0±4.2	3.1	0.9	25
BA 8.9+NAA 2.7	24.3±5.1	6.7	1.0	19
BA 8.9+NAA 1.0	17.7±5.0	4.5	0.7	22

Data represent means±SD based on three replicates (30 calli per replicate and 90 calli were used). Calli were cultured on MS basal medium containing different concentrations and combinations of plant growth regulators under a 12-h light/12-h dark photoperiod using the two-step regeneration procedure. Shoot regeneration was measured 1 month later. [a]: Significant at $P<0.05$ for shoot regeneration frequency compared to other treatments. [b]: Significant at $P<0.05$ for shoot regeneration frequency compared to those from other auxin and cytokinin combinations.

Table 3. Effect of auxin on rooting of *P.cordatae* shoots.

Plant growth regulator (μM)	Rooting time (d)	Rooting rate (%)	No. of roots per shoot
IAA 0.6	15	52.3±5.0	4.3±1.4
IAA 1.1	10	75.0±4.4	4.4±2.0
IAA 2.9	12	33.3±3.5	3.3±1.8
NAA 0.5	4	100±0[a]	8.7±0.6
NAA 1.1	3	100±0[a]	7.3±1.0
NAA 2.7	8	73.0±3.0	6.7±1.5
IBA 0.5	6	78.7±5.1	7.5±2.5
IBA 1.0	6	100±0[a]	8.2±1.3
IBA 2.5	4	58.7±5.1	5.7±1.5
no hormone control	27	16.7±0.6	1.3±0.3

Data represent means±SD based on three replicates (10 shoots per replicate and 30 shoots were used). Shoots were cultured on half-strength MS basal medium containing different concentrations of plant growth regulators. Root time, rooting rate and the number of roots developed per plantlet were measured 20 d later. [a]: Significant at $P<0.05$ for rooting rate compared to other treatments.

first step, and some of them differentiated into little regeneration shoots about 10 d later in the second step, so several shoots derived from one callus (Table 2).

As has been shown with most plants, suitable combinations of auxin and cytokinin in the regeneration medium was important for regeneration of *P. cordatae* (Table 2). The use of KT and 2, 4-D together were superior to the other auxin and cytokinin 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 KT. The maximum number of shoots per callus (7.3) was achieved with the medium containing 8.9 μM BA+2.3 μM 2,4-D and the longest shoot (2.3 cm) with 9.3 μM KT+2.3 μM 2,4-D. The time of shoot emergence, varying from 13 d (4.4 μM BA+2.3 μM 2,4-D) to 25 d, was severely affected by combination and concentration of plant growth regulators too.

On regeneration medium, shoots developed roots very slowly. Rooting medium promoted root development. Although few roots developed from shoots on

the hormone-free medium, the rooting efficiency was greatly improved by the addition auxin to the medium (Table 3). The highest rooting rate (100%) was achieved from shoots cultured on half-strength MS medium containing low concentrations (0.5 or 1.1 μM) of NAA or 1.0 μM IBA.

After 1 month of acclimation, well-rooted plants were transplanted into pots with over 95% survival. The process of acclimation, by removing container lids, is necessary for the successful transplant of plants. The plants which were not acclimated died within 1 week after transferred to pots.

In this study, we determine the disinfector kind, sterilization time, explant type and culture medium for callus induction, shoot regeneration and root generation, and have established a stable and efficient system for plant *in vitro* regeneration of *P. cordatae*. Only 70 d were needed from callus induction to plantlet transplantation. No significant morphological variation was observed between the plants regenerated by this procedure and the wild plants. Chemical and genetic analysis on *in vitro* regenerated plants of *P. cordatae* awaits the further studies.

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