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Plant Regeneration from Callus and Adventitious Root Segments of *Pulsatilla Koreana* Nakai

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ABSTRACT Plant regeneration of *Pulsatilla koreana* was achieved via adventitious shoot formation indirectly from callus and directly from adventitious root segments. For the callus induction from leaf or petiole explants, combination of 2,4- dichlorophenoxyaceticacid (2,4-D) with 2.22 μM 6-benzyladenine (BA) was effective. Adventitious shoot induction from callus was enhanced by the combined treatment with 0.1 μM polyvinylpyrrolidone (PVP) compared to cytokinin treatment alone. Adventitious roots were induced from the petiole segments on 1/2 MS medium with 4.93 μM IBA. High frequency direct adventitious shoot formation from the segments of adventitious roots was achieved on medium with 4.92 μM 2-isopentenyladenine (2-ip). Elongated shoots were rooted on half-strength MS medium containing 5.71 μM indole acetic acid (IAA). Regenerated plantlets with well-developed shoots and roots were successfully transferred to soil. This *in vitro* propagation protocol might be useful for mass propagation as well as conservation of this plant.

Introduction

Pulsatilla koreana Nakai belongs to the family Ranunculaceae and is an endemic species in Korea. *P. koreana* is one of the important traditional medicinal plants in China and Korea (Hsu et al. 1986). The extracts of these plants are attributed with anti-tumor, anticoccidial, and anti-protozoal effects (Kim et al. 2002, Youn and Noh 2001, Youn et al. 2003). The roots of this plant have been widely used in traditional medicine for the treatment of several diseases, particular malaria and amoebic dysentery (Bae 1999). The root of these plants contains several medically active constituents mainly oleanane and lupane-type saponin (Kang 1989, Bang et al. 2005) and anemonin (Martin et al. 1990).

During the past decade a dramatic increase in exports of medicinal plants attests to worldwide interest and most of these plants being taken from the wild. Hundreds of species are now threatened with extinction because of overexploitation, destructive collection techniques, and conversion of natural habitats to crop based agriculture. *P. koreana* is one such overexploited plant in Korea.

The propagation of *P. koreana* through seed is held back by a low germination rate of seeds, and scanty and delayed rooting of seedlings. In seeds of *Pulsatilla cernua* var. koreana can maintain germination capability only 6 weeks under natural condition (Sang et al. 1993). The development of an efficient method for rapid clonal propagation is important to meet the pharmaceutical needs and for conservation of this valuable medicinal plant. Consequently, cell and tissue culture technology has allowed development of alternative approaches for the production of useful biomass and secondary metabolites. Since there is little information on *in vitro* propagation of this important herb (Lee and Oh 1993, Yoon 1996), we herein report an efficient

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plant regeneration protocol from callus and adventitious root segments of *P. koreana* Nakai.

Materials and Methods

Plant Material

Plants of *Palsatilla koreana* Nakai were purchased from National Horticultural Research Institute, Suwon, Korea and maintained in the greenhouse. Young leaves along with petiole (2 cm in length) were collected from mature plants and surface sterilized in 2% sodium hypochloride for 15 min followed by washing with sterile distilled water.

Callus Induction and Adventitious Shoot Regeneration

Leaf (5 x 5 mm) and petiole (5 mm) sections were inoculated on MS (Murashige and Skoog 1962) medium supplemented with 3% sucrose and 2,4-D (0.45 and 4.52 μM) or Naphthaleneacetic acid (NAA, 0.54 and 5.37 μM) alone or in combination with BA (0.44 and 2.22) for callus induction. Observations on the number of explants developing callus was recorded after six weeks of culture. For shoot regeneration from leaf and petiole derived callus was cultured on MS medium supplemented with 3% sucrose and BA (2.22 and 4.44 μM) or kinetin (2.32 and 4.65 μM) and PVP (0 and 0.1 μM) (PVP10, MW 10,000, Sigma, USA). The medium pH was adjusted to 5.8 prior to the addition of 0.2% (w/v) agar (Phyta agar, Duchefa, Zaandam, The Netherlands). Medium was distributed into either 15 x 140-mm petri dishes (15 ml of medium) or in 110 x 35 mm-polystyrene containers and autoclaved at 104 KPa at 121°C for 20 min. All cultures were incubated at 25 ± 2°C under a 16-h photoperiod using cool, white fluorescent lights of 40 μmol $m^{-2}s^{-1}$. During shoot regeneration the number of callus developing shoots, and number of shoots per callus was recorded after six of culture.

Adventitious Root Induction from Petiole Segments

Petiole segments (10 mm in length) were cultured onto 1/2 MS medium with 4.93 μM IBA, 3% sucrose and solidified with 0.25% gelrite. After 5 weeks of culture, induced adventitious roots were cut to segments and cultured onto different kinds of auxin (5.71 μM IAA, 4.93 μM IBA, or 5.37 μM NAA), supplemented with 3% sucrose and solidified with 0.25% gelrite. After 6 weeks of culture, frequency and number of adventitious root formation were examined.

Adventitious Shoot Formation from the Segments of Adventitious Roots

To induce adventitious shoots from the segments of adventitious root, adventitious root segments were cultured onto MS medium with different concentrations and kinds of cytokinin (2.46 μM and 4.92 μM 2-ip, 2.22 and 3.44 μM BA, 2.32, 4.65 μM kinetin), supplemented with 3% (w/v) sucrose and solidified by 0.27% gelrite. After 6 weeks of culture, frequency, number and length of adventitious roots were recorded. The culture room was maintained at 24 ± 2°C under a 16-h (light) / 8-h (dark) photoperiod with light being supplied by white fluorescent tubes at an intensity of 24 μmol $m^{-2}s^{-1}$.

Rooting of Shoots and Transplantation of Plantlets

Excised micro-shoots (about 1 cm) were separated individually and transferred to rooting medium consisted of half strength MS basal salts supplemented with 2% sucrose (w/v) and IAA (0.57, 1.14, 2.85 and 5.71 μM). During rooting of shoots, number of shoots developing roots and number of roots per shoot were recorded after four weeks of culture. Well rooted shoots were rinsed with sterile water to remove residual rooting media and transferred to 50 x 35 x 9 cm plastic trays containing vermiculite and perlite (3:1) and kept in a growth chamber under a day / night temperature regime of 25 / 15°C and 16 h photoperiod of 200 μmol $m^{-2}s^{-1}$. After two weeks the plants were transferred to soil and grown in a greenhouse.

Data Collection and Analysis

Experiments were set up in completely randomized design and repeated three times. Each treatment had five replications (culture vessels) and there were seven explants per culture vessel. Data were subjected to analysis of variance by Duncan's multiple range test.

Results and Discussion

Callus Induction and Adventitious Shoot Formation

Leaf and petiole segments were cultured on MS medium supplemented with 2,4-D or NAA alone and in combination with BA. Callus was developed from the cut edges of the explants in four weeks on all growth regulator supplemented medium and callus spread all over the explants in another two weeks. Growth and morphology of the callus varied with different levels of 2,4-D and NAA. On medium supplemented with 0.45 μM 2,4-D and 2,4-D (0.45 μM) in combination with BA (0.44 and 2.22 μM) induced callus from petiole and leaf explants in lesser frequencies (Table 1). Morphology of the callus was yellow and friable. Optimal frequencies of explants developed callus on the medium supplemented with 4.52 μM 2,4-D and in combination with 2.22 μM BA (Table 1 and Figure 1A). Similarly, medium supplemented with NAA (0.54 and 5.37 μM) alone and NAA (0.54 and 5.37 μM) in combination with BA (0.44 and 2.22 μM) also induced callus, but callus morphology was compact, yellow and nodular. On increasing the concentration of NAA from 0.54 to 5.37 μM increase in percentage of cultures forming callus was noticed. Since, medium supplemented with 4.52 μM 2,4-D and 2.22 μM BA has induced optimal callus, the callus regenerated from leaf and petiole explants on different media was sub-cultured and maintained on this medium once in four weeks. Similar to the present study, 2, 4-D or NAA alone and 2,4-D or NAA in combination with BA has used for the induction of callus from leaf explants of *Aegle* marmelos and *Hypercum perforatum* (Arumugam et al. 2003, Pretoo and Santarem 2000).

For shoot regeneration the callus was cultured on medium supplemented with BA (2.22 and 4.44 μM) or kinetin (2.32 and 4.65 μM) with or without PVP (0.1 μM). Adventitious shoot buds were differentiated from the surface of the callus within four weeks on all media (Table 2, Figure 1B).

Table1. Effect of growth regulators supplemented to MS medium on callus induction from ole and leaf explants of *Palsatilla koareana* Nakai after 6 weeks of culture.

MS + Growth regulators (μM)			Percentage of explants developing callus	
2,4-D	NAA	BA	Petiole	Leaf
0	0	0	0g	Oh[z]
0.45	0	0	10.2f	5.6g
0.45	0	0.44	13.4f	5.8g
0.45	0	2.22	35.2c	18.0e
4.52	0	0	20.4e	30.4cd
4.52	0	0.44	28.2d	34.6c
4.52	0	2.22	75.4a	85.6a
0	0.54	0	8.8f	6.4g
0	0.54	0.44	12.4f	10.2fg
0	0.54	2.22	28.8d	18.6e
0	5.37	0	19.6e	14.4ef
0	5.37	0.44	26.8d	25.6d
0	5.37	2.22	65.6b	57.8b

[z]In each column, the mean values with different alphabetical letters are significantly different according to Duncan's multiple range test at $P<0.05$.

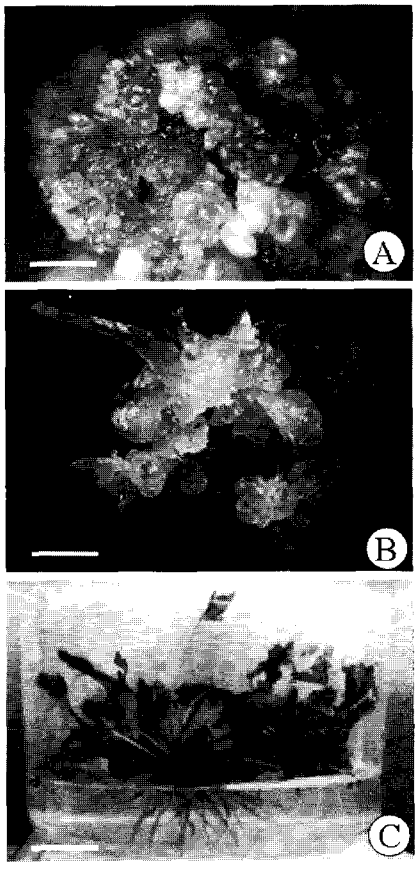

Figure 1. Plant regeneration through adventitious shoot formation from leaf-derived callus of Pulsatilla koreana Nakai. A, Adventitious bud induction from callus (bar 3 mm); B, Shoot growth from adventitious buds (bar 6 mm); C, Plantlets with shoots and roots in the plastic culture jar (bar 2 cm).

Percentage of regeneration varied between 0.9 to 42.4% and was dependent upon concentration of BA / kinetin and PVP. The best shoot regeneration response was found on the medium containing 4.44 μM BA and 0.1 μM PVP each. On this medium 4.5 shoots developed from each callus. Similar to the present results BA or kinetin was found to be more efficient in induction of adventitious shoot from callus derived from leaf and petiole explants (Fisal and Anis 2003, Thao et al. 2003). In the present studies, regeneration was drastically reduced if PVP removed from the medium. PVP is an antioxidant and prevents browning of the medium and helps in proliferation of developing shoots (Vengadesan et al. 2002). Enhanced shoot regeneration with the supplementation of PVP is also reported in *Acacia auriculiformis and Eucalyptus tereticornis* (Ranga Rao and Prasd 1991, Das and Mitra 1990).

Table 2. Effect of growth regulators and PVP supplemented to MS mediumon adventitious shoot regeneration from petiole and leaf derived callus of *Palsatilla koreana* Nakai after six weeks of culture.

MS + Growth regulators (μM)			Percentage of regeneration	Mean number of shoots / callus
BA	Kinetin	PVP		
0		0	0.9d	0.4f[z]
0		0.1	2.2cd	0.8ef
2.22		0	4.0cd	2.3bcd
2.22		0.1	13.3b	3.2b
4.44		0	11.7b	4.5a
4.44		0.1	42.4a	5.2a
0	2.32	0	2.6cd	1.2de
0	2.32	0.1	5.3c	1.8cde
0	4.65	0	4.5cd	2.1cd
0	4.65	0.1	10.3b	2.3bc

[z]In each column, the mean values with different alphabetical letters are significantly different according to Duncan's multiple range test at $P<0.05$.

Table 2. Effect of cytokinin on adventitious shoot formation from the culture of root segments after 6 weeks of culture.

Auxin (μM)		Frequency of adventitious root formation (%)	No. of root / explant
Control	0	0	0c[z]
IAA	5.71	89.3	3.2b
IBA	4.93	100	6.7a
NAA	5.37	92.1	7.25a

[z]In each column, the mean values with different alphabetical letters are significantly different according to Duncan's multiple range test at $P<0.05$.

Induction of Adventitious Roots from Petiole Segments

Petiole segments were cultured onto 1/2 MS with different kinds of auxin (IAA, IBA or NAA). After 2 weeks of culture, adventitious roots were induced from both excised region and/or the surface of explants. After induction of roots, roots were elongated further until 6 weeks of culture. Highest frequency of adventitious root formation was achieved on medium with 4.93 μM IBA and the frequency of adventitious root formation was 100% (Table 3). The number of roots was similar in between IBA and NAA treatment but low in IAA treatment. About 6-7 numbers of adventitious roots formed per segment on medium with IBA or NAA. In *P. koreana*, adventitious roots were slender and elongated rapidly in the presence of IBA (Figure 1A). Whereas, thick and short adventitious roots formed in the presence of NAA

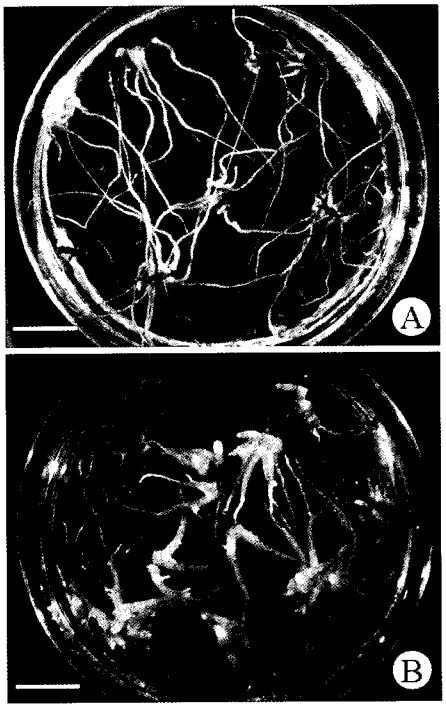

Figure 2. Adventitious root induction from petiol-derived adventitious root segments of P. koreana. A, Adventitious roots from root segments on 1/2 medium with 4.93 μM IBA (bar 15 mm); B, Adventitious roots from root segments on 1/2 medium with 5.37 μM NAA (bar 15 mm).

(Figure 1B). In control without auxin treatment, no adventitious roots formed.

Recently, adventitious root culture system can be applied practically for commercial large scale bioreactor production of plant materials when the root material have important medicinal values and contains rich valuable secondary compounds. Large scale bioreactor production of ginseng adventitious roots is the well-established production system (Choi et al. 2000). In *P. koreana*, the root contains several medicinally active constituents including 9%, mainly oleanane and lupane-type saponin (Kang 1989, Bang et al. 2005) and anemonin (Martin et al. 1990). The roots of *P. koreana* are antiinflammatory and antiparasitic (Martin et al. 1990). It has been used for the treatment of leucorrhoea, dysentery, scrofula and also as a contraceptive in Korea (Bae 1999). Thus adventitious root culture system of *P. koreana* can be valuable strategy for the production of medicinal plant materials.

Table 3. Effect of cytokinin on adventitious shoot formation from the culture of root segments after 6 weeks of culture.

Cytokinin (μM)		Frequency of adventitious hoot formation (%)	No. of shoot / explant
Control	0	91.3	1.3d[z]
2-ip	2.46	96.5	2.3c
	4.92	97.3	4.6a
BA	2.22	91.5	2.8bc
	3.44	92.5	2.5c
Kinetin	2.32	95.8	3.2b
	4.65	92.5	3.5b

[z]In each column, the mean values with different alphabetical letters are significantly different according to Duncan's multiple range test at $P<0.05$.

Table 4. Effect of IAA supplemented to half strength MS medium on rooting of shoots of *Palsatilla koreana* Nakai after four weeks of culture.

IAA Concentration (μM)	Percentage of rooting	Mean number of roots / shoot
0	42	2.6d[z]
0.57	45	3.2cd
1.14	80	5.9c
2.85	89	11.1b
5.71	100	16.5a

[z]In each column, the mean values with different alphabetical letters are significantly different according to Duncan's multiple range test at $P<0.05$.

Adventitious Shoot Formation from the Segments of Adventitious Roots

Adventitious root segments were cultured onto MS medium with different concentrations and kinds of cytokinin as shown in Table 4. More than 90% of root segments produced adventitious shoots. The number of shoots was similar among different levels and kinds of cytokinin. Even in hormone-free medium, adventitious roots formed but the site of root development was restricted near the proximal excised portion of root segments. In hormone-free medium, adventitious shoots were developed directly near the cut surfaces of root segments (Figures 3A, 4A). Whereas, cytokinin treatment induced the adventitious shoots not only on excised portions of root segments but also on the surfaces of roots (Figures 3B- C, 4B).

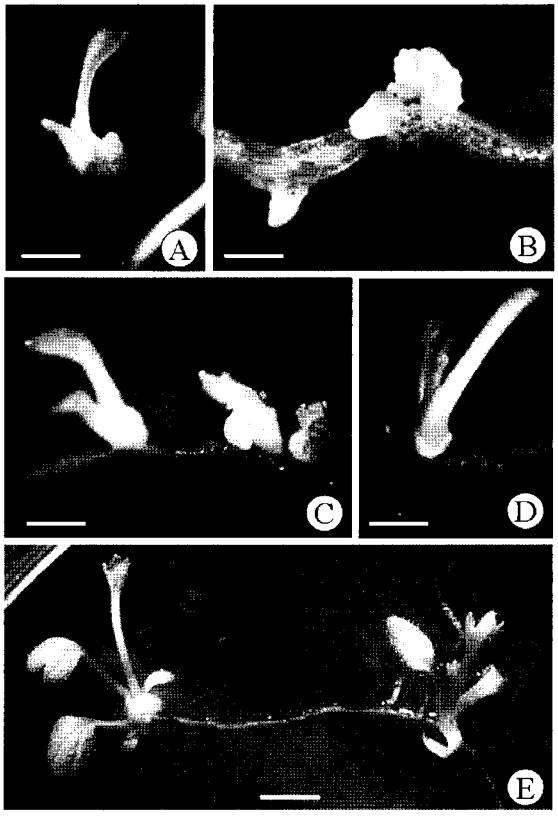

Figure 3. Direct adventitious shoot formation on the surfaces of adventitious root segments of P. koreana. A, Adventitious bud induction on the excised surface of root segment on hormone- free MS medium (bar 2 mm); B, Adventitious bud induction on the lateral surface of root segment on MS medium with 4.92 μM 2-ip (bar 2 mm); C-D, Shoot growth of adventitious buds (bar 6 mm); C, Advanced growth of adventitious shoots (bar 4 mm).

Rooting of Shoots

The regenerated shoots were transferred to rooting media supplemented with different concentrations of IAA (0.57 to 5.71 μM). Shoots did not develop roots on the full strength MS medium with or without IAA (data not shown here). However, shoots developed roots on half strength MS basal medium. The use of low salt MS medium for rooting of the *in vitro* induced shoots is a very common practice (Hiregoudar et al. 2003, Thomas and Sreejesh 2004). Half strength MS medium supplemented with 0.57 - 5.71 μM IAA induced higher frequency of rooting (Table 4). The maximum frequency of root formation was achieved on half strength MS medium supplemented with 5.71 μM IAA and an optimum of 16.5 roots were developed per each shoot (Figure 1C).

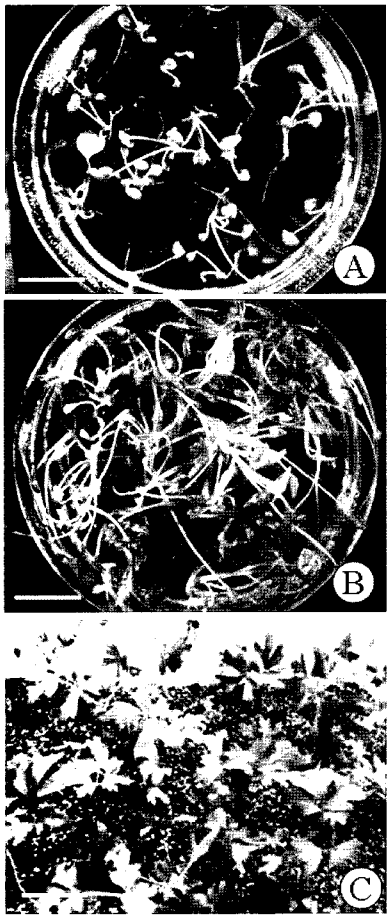

Figure 4. Adventitious shoot formation and soil transfer of plantlets of P. koreana. A, Adventitious bud induction form root segments on hormone-free MS medium (bar 20 mm); B, Adventitious bud induction from root segments on with 4.92 μM 2-ip (bar 20 mm); C, Acclimatized plantlets kept in plastic trays containing vermiculite and perlite (3:1).

Transfer to Soil

Regenerated plantlets were transferred to vermiculite and perlite (3:1) and reared in growth chambers for two weeks. After two weeks the plants were transferred to soil and grown in a greenhouse (Figure 4C). In conclusion, the success in raising plants through leaf and petiole derived callus has opened up the possibility for large-scale clonal propagation of *P. koreana.* This protocol is simple and efficient for the propagation of this important medicinal plant and may be useful for conservation of germplasm and might help in the genetic improvement of this species for commercial use.

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