In vitro Induction of Tetraploid Roots by Various Pretreatments from Anther of Panax ginseng C. A. Meyer


*Korean Ginseng Center for Most Valuable Products & Ginseng Genetic Resource Bank, Kyung Hee University, Seocheon-dong, Kiheung-gu Yongin, Kyunggi-do 446-701, Korea.
**Chungbuk Agricultural Research and Extension Services, Cheongwon, Korea
(Received March 6, 2009; Accepted March 12, 2009)

Abstract: This experiment was done to determine the optimum conditions for the induction of tetraploidy in Panax ginseng C. A. Meyer using bud length, temperature and plant growth regulator pretreatments. Highest callus formation was obtained when the medium was inoculated with flower bud in the size of 2-3 mm in length. The optimum temperature for the callus formation was high when treated at 4°C for 4-5 days. Among the treatments of growth regulators and different concentration, highest callus formation was observed in combination of 5 mg/L 2,4-D and 1 mg/L kinetin for P. ginseng. As a result of flow cytometer analysis, all 7 adventitious roots were confirmed as tetraploids. Cytological analysis revealed that the chromosome number of tetraploid roots was 96, while that of diploid roots was 48. Tetraploid ginseng roots were inoculated to flower bud size of 2-3 mm in length. The callus formation was optimum when treated with 1 mg/L 2,4-D at 4°C for 5 days. Compared with control roots, tetraploid roots were thicker and longer and had few lateral branches. Fresh weight of tetraploid roots was relatively higher than the control roots.

Key words: Anther, chromosome, Panax ginseng, polyploidy, pretreatment

INTRODUCTION

Panax ginseng C. A. Meyer is one of the most widely used general tonic herb that has many bioactive effects such as adaptogen, antiaging, antistress, antitumor, immunoenhancement, etc.1,2 It has been reported that P. ginseng contains various polysaccharides, saponins, antioxidants, peptides, alkaloids,3,4 Currently, for this reasons, a great deal of P. ginseng is made into and consumed for healthy functional foods. But, studies on various improvements of P. ginseng are not many. It would be very desirable to have higher contents of pharmaceutical components as well as higher yield of this species. One of the ways to achieve this goal is the production of polyploidy of ginseng that may have more pharmaceutical effects than the existing diploid (2n=2x=48) ginseng. As the breeding strategy, polyploidisation has been used one of available method to induce genetic variation.5,6

In many plant species, polyploidy has been shown to increase the size of the cells, leading to thicker leaves, stems and roots, increased width-to-length ratio of leaves, a deeper green color, a more compact growth habit, and a longer and delayed flowering period.7,8 In addition, polyplloid plants have been reported to increase the production of secondary metabolites in many plants compared to their diploid plants. Tetraploidp plants of Atropa belladonna had about 1.5 times higher alkaloid content as compared with diploid plants.9 Tetraploidy's of Salvia miltiorrhiza Bge produced more flavonoids and terpenoids than its diploidy.8

The technique of in vitro polyploidy induction has been widely used by artificial treatment of colchicine.10 Colchicine is known to disturb the formation of spindle, thus preventing the normal chromosome movements of congression and separation at first and second meiotic divisions, and inducing restitution monads instead of tetrads.11 It has been reported from many plants such as Tradescantia paludosa,12 Triticum aestivum,13 Vaccinium darrowi,14 Cyclamen persicum,15 Punica granatum,16 and Zizyphus jujube.17 But colchicine is cancerogenic substance and is known to cause side effects, such as sterility, abnormal growth, morphology, and to induce chi-
meric plants, due to asynchrony of cell divisions in many species.\textsuperscript{18)} Therefore, it needs to explore the possibilities about other methods for induction of polyploidy.

To date, anther culture technology has been used one of methods for haploid plant production.\textsuperscript{19)} In case of \textit{P. ginseng}, Du \textit{et al.} (1987)\textsuperscript{20)} induced the haploid plantlet from anthers by various induction media using plant growth regulators and cold pretreatment. But until now, there was no report about polyploidy induction from anther of \textit{P. ginseng}. In this study, we attempted to produce the polyploidy of \textit{P. ginseng} roots from anther by using various pretreatments.

**MATERIALS AND METHODS**

1. **Plant material and temperature pretreatment of umbel of \textit{Panax ginseng}**

For anther culture, Yun-poong umbels of \textit{Panax ginseng} C.A. Meyer were collected from Pochoen in Gyeonggi-do, Korea (provided by Ginseng Genetic Resource Bank). The umbels from \textit{P. ginseng} were pretreated with chilling and heating conditions. Chilling pretreatment was carried out at 4°C for 1, 2, 3, 4, 5, 7, 10 and 15 days prior to anther culture. And heating pretreatment was pretreated at 37°C for 1, 2 days prior to anther culture. Control was used at 25°C.

2. **Surface sterilization and anther separation by bud size**

The umbels were immersed for 30 seconds in 70% ethyl alcohol and sterilized for 15 minutes in a 1% solution of sodium hypochlorite. Then they washed 4-5 times in sterile water. Buds picked from umbel and individual anthers were separated carefully. Anthers were placed horizontally on the medium, respectively. To assess appropriate culture conditions for obtaining morphological criteria for recognition of flower buds at a responsive anther stage, we divided into two bud size groups of 1-2, 2-3 mm in diameter.

3. **Induction of callus in \textit{P. ginseng} anthers**

MS medium\textsuperscript{21)} with 3% sucrose and 0.7% agarose was used for anther culture. The medium was supplemented with 2,4-D, IAA or NAA alone or the combination of 2,4-D, kinetin, IAA, and BA (Table 2). The media were adjusted to the pH 5.7 before autoclaving and were autoclaved at 121°C for 15 minutes. The cultures were maintained at 25 ± 2°C in dark for 40-50 days. When the white-yellow callus grew to 4-5 mm, they transferred to differentiation media for induction of root.

4. **Root induction**

The media, a Gamborg’s B\textsubscript{5} medium\textsuperscript{22)} supplemented with 2 mg/L NAA was used. The medium was supplemented with 3% sucrose and 0.7% agarose. The pH of the media was adjusted to 5.7 before autoclaving at 121°C for 15 minutes. The cultures were subcultured at 25 ± 2°C in dark for 30-40 days.

5. **Ploidy measurements**

The DNA means data of ploidy were analyzed by flow cytometer (Patec PA-, Germany), using a procedure modified from Arumuganathan and Earle (1991).\textsuperscript{23)} Samples were prepared for young roots including the growing points about 2 cm from callus-derived roots of \textit{P. ginseng}. Adventitious root of Yun-poong, one of specific kinds of \textit{P. ginseng} was used.

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Days of Pretreatment</th>
<th>No. of Anther Inoculated</th>
<th>Frequency of calli produced (%)</th>
<th>Mean of calli per anthers inoculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cold</td>
<td>0</td>
<td>540</td>
<td>37.0</td>
<td>5.56 ± 0.247</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>540</td>
<td>59.3</td>
<td>9.06 ± 0.591ab</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>540</td>
<td>56.7</td>
<td>8.50 ± 0.379ab</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>540</td>
<td>60.9</td>
<td>9.14 ± 0.412b</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>540</td>
<td>69.8</td>
<td>10.47 ± 0.448c</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>540</td>
<td>69.6</td>
<td>10.44 ± 0.579c</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>540</td>
<td>54.3</td>
<td>8.14 ± 0.697ab</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>540</td>
<td>51.1</td>
<td>7.67 ± 0.465a</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>540</td>
<td>50.9</td>
<td>7.81 ± 0.504ab</td>
</tr>
<tr>
<td>Hot</td>
<td>1</td>
<td>540</td>
<td>0</td>
<td>0.00 ± 0.00d</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>540</td>
<td>0</td>
<td>0.00 ± 0.00d</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SE of two repeat experiments with 15 replicates each.

The numbers in a column followed by same letter are not significantly different at \( P < 0.05 \) by Dunndan’s multiple range test.
ginseng, were used as internal standards. Callus-derived roots and internal standard roots were put into a petri-dish and chopped finely with a sharp razor blade in 500 µl nuclei extraction buffer (solution A of the High Resolution Kit for Plant DNA, Pactec, Germany) for 30-60 seconds. After filtration through a 30 µM Cell-Trics disposable filter (Partec, Germany), 2 ml staining solution containing the dye 4,6-diamidino-2-phenylindole (DAPI; solution B of the kit, Pactec, Germany) was added. Routinely, 8,000-10,000 nuclei were measured per sample and histograms of DNA content were generated using the Partec software package.24)

6. Chromosome verification

The root tips were cold-treated in ice water for 24 hrs and fixed in the ratio of 3 : 1 (95% ethanol : glacial acetic acid) for 24 hrs and then stored in distilled water for 5 minutes. Next, the tips were hydrolyzed with 1 N HCl solution at 60°C for 5 minutes, stained with feulgen solution for 30 minutes. For mitotic examination, the root tips were cut and squashed in 1% (v/v) aceto-carmine solution. The preparations were examined under light microscope (1000X) and the chromosomes were counted.

7. Biomass analysis and statistical analysis

Fresh weight was measured after the roots were washed with distilled water every fifth day. The data from all experiments were analyzed using an SPSS statistical analysis package (Version 12.0). Mean values of calli produced per 15 anthers under different regimens were determined and analyzed. Statistically significant different values (P < 0.05) are labeled with different superscripts.

RESULTS

1. Root induction from anthers of Panax ginseng

The P. ginseng anthers were cultured on various media

Table 2. Effects of different supplemented compositions on callus induction media from anthers of Panax ginseng

<table>
<thead>
<tr>
<th>IAA</th>
<th>NAA</th>
<th>2,4-D</th>
<th>BA</th>
<th>kinetin</th>
<th>No. of Anther Inoculated</th>
<th>Frequency of calli produced (%)</th>
<th>Mean of calli per anthers inoculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>600</td>
<td>31.8</td>
<td>4.34 ± 0.395a</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>600</td>
<td>47.7</td>
<td>6.50 ± 0.622bc</td>
</tr>
<tr>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>600</td>
<td>46.0</td>
<td>6.20 ± 0.610ab</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>600</td>
<td>61.7</td>
<td>8.32 ± 0.709cd</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>0.5</td>
<td>-</td>
<td>600</td>
<td>53.5</td>
<td>7.43 ± 0.687bcd</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.5</td>
<td>600</td>
<td>43.7</td>
<td>5.95 ± 0.593ab</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>0.5</td>
<td>600</td>
<td>60.5</td>
<td>8.20 ± 0.731cd</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>5</td>
<td>-</td>
<td>1</td>
<td>600</td>
<td>49.3</td>
<td>6.82 ± 0.617bc</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>5</td>
<td>-</td>
<td>1</td>
<td>600</td>
<td>66.3</td>
<td>9.05 ± 0.740d</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SE of two repeat experiments with 15 replicates each. The numbers in a column followed by different letters (a, b, c, d) are not significantly different at P < 0.05 by Dundan’s multiple range test.

Fig. 1. Anther culture and root induction of Panax ginseng. A, anthers plated on medium; B, callus development from anther; C, anther (including filament of anther)-derived callus; D, callus-derived root; E, Root on induction medium of root; F, degenerated anther.
in dark at 25 ± 2°C (Fig. 1A). There were two general responses noted after 4 weeks in all media. One was that anthers initially showed slight swelling on various media and during incubation, they started callusing (Fig. 1B), then white friable calli (approximately 4-5mm diameter) were formed on over 50% of the cultured anthers (Fig. 1C). 6 weeks after initial anther culture, anthers-derived calli grew well on B5 medium supplemented with 2 mg/L NAA and 3 % sucrose. After transfer to media, adventitious roots developed from calli on various media (Fig. 1D, E) and the anthers with < 2 mm diameter turned brown and degenerated (Fig. 1F).

2. Effect of various factors

1) Effect by bud size

Bud size was used as a guide to the optimum stage of anther development for culture. Two bud sizes within the range of 1 to 2 mm, 2 to 3 mm were selected. As no interaction could be detected between the influence of bud size and callus production, the means of all replicates of all pretreatments were calculated. Analysis of anthers cultured showed that bud size did not have a significant effect on callus induction. 1-2mm bud size groups produced 49 % of callus, but the 2-3mm size produced 52% (not shown).

2) Effect of temperature pretreatment

The effect of temperature pretreatment of umbels on anther culture for the production of polyploidy of *P. ginseng* was examined. The umbels was incubated at 4oC for 1, 2, 3, 4, 5, 7, 10 and 15 days and at 37oC for 1 to 2 days. In Table 1, it showed that pretreatment temperature and duration had a significant effect on anther response and callus yield. Chilling pretreatment of umbels was more effective than unpretreated control that incubated at 25oC. At 25oC, calli were formed at a rate of about 37% when anthers were not pretreated, while at 4°C, calli were formed at a rate of more 50% when anthers were pretreated for 1-15 days. The best treatment for the induction of callus was a temperature of 4°C for 4 and 5 days by 69.8% and 69.6%. However, heating pretreatment of umbels was not better than the unpretreated control. No callus was formed on anthers pretreated at 37°C.

3) Effects of medium by plant growth regulator

In the present experiment, anthers from *P. ginseng* were used to study their callus ability on different combinations and of IAA, NAA, 2,4-D, BA and Kinetin. Nine different combinations of these five growth regulators were studied to determine the most suitable combination and concentration for callus induction. The results on callus induction are presented in Table 2. It was observed that an average frequency of callus induction in control medium without plant growth regulator was lower than that in media with various combination of plant growth regulator. Except control, callus induction frequencies were variable and ranged from 43.7 to 66.3%, respectively. However, highest frequency (66.3%) of callus induction was obtained on the medium that supplemented with 5 mg/L 2,4 D and 1 mg/L Kinetin. Induced calli were transferred to B5 medium supplemented with 2 mg/L NAA, then successfully differentiated to roots.

3. The result of ploidy measurements and morphological analysis

Ploidy levels were determined using a flow cytometer (Partec PAS I) and chromosome counting. Callus-derived roots were subjected to flow cytometer to determine their ploidy. As the internal standard, “Yun-poong” adventitious roots were used. Individual roots were analyzed as diploidy and polyploidy according to the peaks obtained by flow cytometer (Fig. 2). The roots with approximately equal numbers of diploid and tetraploid nuclei were regarded mixoploid roots (Fig. 2B). As a result, seven roots were confirmed tetraploidy. The DNA means of

![Fig. 2](image-url)
control roots revealed about 200 (Fig. 2A), whereas the DNA means of tetraploid roots exhibited about 400 (Fig. 2C). Cytological analysis established that the chromosome number of diploid roots was 48 and tetraploid roots was 96 (Fig. 3A and B).

4. Morphological characteristics and growth analysis

Induced roots from callus were grown in Gamborg’s B5 medium supplemented with 2 mg NAA/L 3% sucrose. Compared with diploid roots, tetraploid roots were thicker and longer and had few lateral branches (Fig. 4A and B). There was no significant difference in relative growth rates between diploid and tetraploid roots. However, relatively, fresh weight of the tetraploid roots was higher than diploid roots (Fig. 5).

DISCUSSION

We investigated the polyploidy induction of Panax ginseng via anther culture by various pretreatments such as temperature and plant growth regulators. The P. ginseng anthers which cultured on various media were two general responses. One is that calli (approximately 4-5 mm diameter) were formed of the cultured anthers, another response was anthers with no growth or with small (< 2 mm diameter) and it was turned brown and degenerated. 5 weeks after initial anther culture, the calli grew well on B5 medium supplied with 2 mg/L NAA and 3% sucrose. After transfer to media, adventitious roots developed from calli on various media.

With regard to the effect of various pretreatment, first analysis of anthers cultured showed that bud size did not have a significant effect on callus induction in our results. But the bud size of anther has been correlated with the developmental stage of anther.20) In case of P. ginseng, frequency of callus induction induced to the highest when anthers at the middle uninucleate stage were inoculated.20) It needs the future plan for step-by-step cell observation in our study. For effect of temperature, we pre-treated umbels at 4°C and at 37°C during a few days. Our studies showed that chilling pretreatment of umbels was more effective than unpretreated control (25°C), while heating pretreatment of umbels was not better than the control. Murthy et al. (2000)25) reported that pretreatment of excised flower buds can help to improve anther response in culture. Similar analysis for anthers cultured at 4°C showed that the cold pretreatment of flower buds was necessary for subsequent callus induction from anthers,
and 48 h of cold (4°C) pretreatment proved to be optimal as it gave the best callus induction in *Eriobotrya japonica*. However, in contrast with our results, the high temperature treatment for a few days had reported that it tended to be effective in inducing embryo. Upon the heat pre-treatment of anthers with liquid medium, induction of embryo increased up to 4.2% in papaya. It means that high auxin concentrations were deleterious. In this study, we confirmed that a combination of 5 mg/L 2,4-D and 1 mg/L kinetin in the induction medium is necessary for callus development from anthers.28,29) Addition of low levels of cytokinin to the induction medium may be beneficial for obtaining callus of high morphogenetic potential, although higher concentrations were deleterious.29) In this study, we obtained some tetraploid roots from anthers. The morphological traits of tetraploid roots were thicker and longer and had few lateral branches than diploid controls. Similar results were reported in tetraploidy of *P. ginseng* via colchicine treatments and also the biomass of the polyploid roots was higher than control roots.30)

This study favored potentiality of polyploidy as well as haploidy created by anther culture. Polyploidy is widely used in plant breeding. Polyploid plants are known that they are superior to diploids with respect to genetic adaptability, pest resistant and disease tolerance to environmental stresses. In horticulture, polyploid plants are known that they are superior to diploids with respect to genetic adaptability, pest resistant and disease tolerance to environmental stresses. Therefore, we will continue to go on study further about characteristics of polyploidy of *P. ginseng*, comparing with control adventitious roots.

ACKNOWLEDGEMENTS

This study was supported by KGCMVP for Technology Development Program of Agriculture and Forestry, Ministry of Agriculture and Forestry, Republic of Korea.

REFERENCES

18. Wan, Y., Petolino, J. F. and Widholm, J. M.: Efficient production of doubled haploid plants through colchicine treat-


