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Effect of Polyploidy Induction on Biomass and Ginsenoside Accumulations in Adventitious Roots of Ginseng

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Adventitious roots of *Panax ginseng* C.A. Meyer (a natural tetraploid) were treated with 50 or 100 mg L^{-1} colchicine for 12, 24, 36, 48, or 60 h to induce polyploid (octoploid) roots. The largest number of octoploid roots was obtained with a 100 mg L^{-1} colchicine treatment over 60 h. To verify that ginsenoside was being accumulated in the developing tissues, the tetraploid (control) and octoploid roots were cultured for 40 d in Murashige and Skoog media that lacked NH_4NO_3 but was supplemented with 2 mg L^{-1} naphthaleneacetic acid and 50 g L^{-1} sucrose. Levels of fresh and dry biomass were greater in the octoploid roots. Although total ginsenoside and Rb-group ginsenoside contents were less in the octoploid roots than in the tetraploids, the former had a higher amount of Rg-group ginsenosides (especially Rg1). These results demonstrate the benefit that polyploid adventitious roots provide in enhancing the production of secondary metabolites in ginseng.

Keywords: colchicine, octoploid, *Panax ginseng* C.A. Meyer, tetraploid

Ginsenoside, a triterpenoid saponin, is a principal bioactive ingredient in *Panax ginseng*, a species with various physiological and pharmacological properties. The major types of ginsenosides are found in the Rb and Rg groups, which are derived from the 20(S) protopanaxadiol and 20(S) protopanaxatriol structures, respectively. Their main components include Rb1, Rb2, Rc, and Rd, from the Rb group; and Re, Rf, and Rg1 from the Rg group (Palazon et al., 2003).

As alternatives to field cultivation, ginseng cell and root cultures are used to produce ginsenosides because of their faster growth and stable metabolite production. However, ginsenoside contents in those cultured tissues are usually only about 30% of those derived from field-grown plants (Yu et al., 2002). Polyploidy is responsible for increases in cell size, a characteristic that leads to larger vegetative and reproductive organs. Polyploids are also responsible for improved secondary metabolite production (Dhawan and Lavania, 1996). Recently, this attribute has been induced in *Chamomilla recutita*, *Petunia*, and *Salvia miltiorrhiza*, where they are able to induce more flavonoid and terpenoid development than in their diploid counterparts (Gao et al., 1996; Griesbach and Kamo, 1996; Svehlikova and Repcak, 2000). Therefore, the objective of this study was to use colchicine treatments to induce polyploidy (octoploidy) in the adventitious roots of *P. ginseng* in order to enhance root biomass and ginsenoside accumulation. We also compared root growth and ginsenoside contents between octoploid and naturally occurring tetraploid adventitious roots.

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MATERIALS AND METHODS

Establishment of Adventitious Root Cultures and Induction of Polyploidy

Ginseng (*P. ginseng* C.A. Meyer) adventitious roots were induced and proliferated in an MS liquid medium (Murashige and Skoog, 1962) that lacked NH_4NO_3 but which was supplemented with 2 mg L^{-1} naphthaleneacetic acid (NAA) and 50 g L^{-1} sucrose (Kim et al., 2003). Cultures were maintained by shaking (100 rpm) at 25°C in the dark. To induce polyploidy (i.e., octoploidy), we inoculated 30 adventitious roots (3 cm long each) in 100-mL Erlenmeyer flasks containing 50 mL of culture media supplemented with 50 or 100 mg L^{-1} of 0.2 μm filtered and sterilized colchicine (Sigma-Aldrich, USA). Each experiment lasted 12, 24, 36, 48, or 60 h. Following this colchicine treatment, the root tips were rinsed twice

with 10 mL of MS media, transferred to fresh media, and maintained at 25°C in the dark. Subsequent root lines were initiated from lateral root tips grown from the original colchicine-treated roots, which were examined on a monthly basis for the stability of their ploidy level. The tetraploid and octoploid roots were cultured for 40 d, after which we recorded their root growth, morphology, and ginsenoside contents.

Preparation of Chromosomes for Ploidy-Level Determination

Root tips were collected, pretreated with a 1-bromonapthalene solution for 2 h, and fixed in a 3:1 (v/v) mixture of ethyl alcohol and acetic acid at 4°C for 24 h. These pretreated roots were then hydrolyzed for 3 min in 1 N HCl at 60°C, rinsed with distilled water, and stained with a Feulgen solution for 30 min. Stained root-tip meristems (about 2 mm long) were removed, placed on a clean slide, and squashed in 1% (w/v) aceto-orcein solution. The preparations were observed under an optical microscope (1000X), and the chromosomes were counted.

Analysis of Ploidy Level by Flow Cytometry

The ploidy analysis was performed with a PA flow cytometer (Partec, Germany), according to the manufacturer's instructions, and using barley cells (nuclei) as the internal standard.

Determination of Ginsenoside Content

Ginsenosides were extracted and analyzed by the method of Yu et al. (2002). The ginsenoside fraction was assessed using an HPLC system (Waters 2690 separation module, Waters 996 photodiode array detector, and Waters millennium 2010 chromatography manager, USA) on an Altec Platinum C18 column (Φ1.5 μm, 33 mm × 7 mm). The ratio of water and acetonitrile (mobile phase) was fixed at 75:25 for the first 10 min, and at 63:37 for the last 25 min. Flow rate of the mobile phase was 1.2 mL min^{-1}, and ginsenoside was detected at 203 nm. Authentic samples of ginsenosides (Chromadex, USA) were compared with the accumulation pattern of the adventitious roots. Total ginsenoside contents were defined as the sum of those fractions. Its content in the roots was calculated as:

Ginsenoside (mg g^{-1} DW) = sample ginsenoside concentration (mg L^{-1}; from HPLC) × sample volume (L)/cell dry weight (g).

RESULTS AND DISCUSSION

Effect of Colchicine Concentration on Polyploidy Induction

P. ginseng is a natural tetraploid, and its chromosome number exists within various populations (Grushvitskii, 1961; Bulgakov et al., 2000). We confirmed that chromosome number in the adventitious roots as 2n = 4x = 48, and used it in the present study. Here, 50 mg L^{-1} colchicine was not sufficient to induce polyploidy in those roots, although all polyploidy roots survived. In fact, only 2 out of 30 roots were involved in doubling (to 96 chromosomes) when

Table 1. Number of regenerated roots, percentage survival, and occurrence of chromosome doubling in ginseng adventitious roots as influenced by colchicine concentration and treatment period.

Colchicine (mg L^{-1})	Treatment period (h)	No. treated roots	No. regenerated roots	Root survival (%)	No. octoploid roots[z]
50	12	30	30	100	0
	24	30	30	100	0
	36	30	30	100	0
	48	30	30	100	0
	60	30	30	100	2
100	12	30	30	100	7
	24	30	30	100	5
	36	30	27	90	8
	48	30	25	83	8
	60	30	22	73	12

[z]Status in each root was assessed by ploidy analyzer (Partec).

tissues were treated with 50 mg L^{-1} colchicine for 60 h. In contrast, 100 mg L^{-1} colchicine induced chromosome doubling regardless of the treatment period, although the percent root survival decreased with increasing duration. For the 100 mg L^{-1} colchicine treatment over 60 h, 73% of the roots showed doubling (Table 1).

Roots that had been determined to be octoploids (by chromosome count) were selected and sub-cultured in fresh culture media that lacked colchicine. Their ploidy level was confirmed by flow cytometry. The cells of barley, used as the internal standard, as well as those of the experimental materials were stained together and fed to the flow cytometer (Fig. 1). The internal standard (barley) showed two peaks (Peaks 1 and 3, Fig. 1A; Peak 1 and 2, Fig. 1B) whereas the tetraploid cells (control: natural ginseng roots) produced a histogram with only one peak (Peak 2, Fig. 1A). Compared with that tetraploid peak, the octoploid cells (from colchicine-treated roots) exhibited a peak twice the size of the channel position (Peak 3 in Fig. 1B), thereby confirming chromosome doubling as a result of colchicine application.

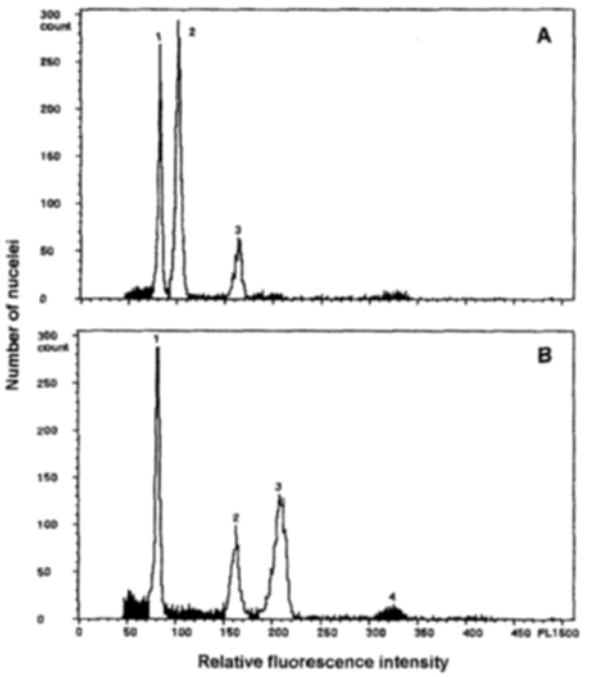

Figure 1. Comparison of histograms obtained after flow cytometric analysis of tetraploid and octoploid adventitious roots of ginseng. **A**, Peaks 1 and 3 represent 2x and 4x nuclei of barley (internal standard); Peak 2 represents 4x nuclei of ginseng adventitious roots (tetraploid, without colchine treatment). **B**, Peaks 1 and 2 represent 2x and 4x nuclei of barley (internal standard); Peak 3 represents 8x nuclei of ginseng adventitious roots (octoploid, after colchicine treatment).

Growth and Ginsenoside Accumulation in Tetraploid and Octoploid Adventitious Roots

The octoploid adventitious roots were thick and bore fewer lateral branches (Fig. 2B), while the tetraploid adventitious roots were thin and had more laterals (Fig. 2A, Table 2). Fresh and dry biomasses of the octoploid adventitious roots were much greater (164.8 ± 11.5 FW and 13.8 ± 0.69 DW mg/root) compared with the naturally tetraploid roots (106.5 ± 3.9 FW and 11.7 ± 0.84 DW mg/root; Table 2). A similar increase in biomass has been reported in the polyploid (tetraploid) hairy roots of *Artemisia annua* when compared with its diploid roots (De Jesus-Gonzalez and Weathers, 2003).

Ginsenoside contents in both tetraploid and octoploid roots were analyzed by HPLC (Table 3). The amounts of total and Rb-group ginsenosides in the octoploid roots were lower (3.3 and 1.4 mg g^{-1} DW,

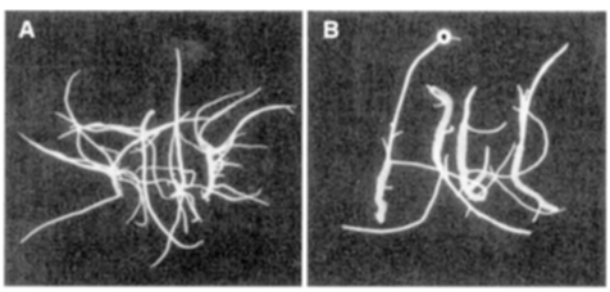

Figure 2. Morphology of tetraploid (**A**) and octoploid (**B**) adventitious roots of ginseng, grown in MS medium (without NH_4NO_3) and supplemented with 2 mg L^{-1} NAA and 50 g L^{-1} sucrose.

Table 2. Growth and proliferation of tetraploid (4x) and octoploid (8x) adventitious roots after 40 d of culture.

Ploidy level	Fresh weight (mg)/ vessel	Dry weight (mg)/vessel	Dry weight/ fresh weight (%)	No. lateral roots/root
4x[z]	106.5 ± 3.9[y]	11.7 ± 0.84	10.9	11.9 ± 0.77
8x	164.8 ± 11.5	13.8 ± 0.69	8.4	4.4 ± 0.61

[z]represents chromosome set per nucleus.
[y]Data are mean values ± SD from 30 adventitious roots.

Table 3. Ginsenoside accumulation in tetraploid (4x) and octoploid (8x) adventitious roots after 40 d of culture.

Ploidy level	Ginsenosides (mg g^{-1} dry wt)										
	Rb group				Rg group			TRb	TRg	Total	Rb/Rg
	Rb1	Rb2	Rc	Rd	Re	Rf	Rg1				
4x	0.6 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.4 ± 0.0	0.6 ± 0.2	0.5 ± 0.0	0.7 ± 0.2	1.9 ± 0.2	1.8 ± 0.3	3.7 ± 0.3	1.1
8x	0.4 ± 0.1	0.3 ± 0.2	0.3 ± 0.0	0.4 ± 0.1	0.5 ± 0.1	0.3 ± 0.1	1.1 ± 0.3	1.4 ± 0.2	1.9 ± 0.1	3.3 ± 0.2	0.7

[y]Data are mean values ± SD from 3 replicates.

respectively) than in the tetraploid roots (3.7 and 1.7 mg g^{-1} DW, respectively). However, Rg-group ginsenosides increased by at least 2%, with Rg1 ginsenoside, in particular, rising by 22% in the octoploid tissue. The overall ratio for the Rb:Rg group was 1.1 in octoploid roots and 0.7 in the tetraploids.

The various ginsenosides have different biological roles, e.g., the Rb group (protopanaxadiol saponins) shows anti-tumor activity (Zheng and Yang, 1994), while the Rg group (protopanaxatriol saponins) affects the motility of the intestine (Shin et al., 1997). Among the Rg-group ginsenosides, Rg1 (an active ingredient in ginseng), may have potential as a neuroprotective drug against Parkinson's disease (Chen et al., 2003) as well as being a hypertension, anti-fatigue medication. Therefore, the significantly enhanced level of Rg1 ginsenoside (up by 22%) in our octoploid roots demonstrates that this compound would be very advantageous for the intentional manipulation of the heterogeneity of ginsenosides in ginseng cell and root cultures. Quantitative differences in metabolites have been reported following polyploidy induction, for example, in the tetraploids of *Petunia*, *Salvia*, and *Artemisia*, which produce more secondary metabolites per gram of tissue than their diploid counterparts (Greisbach and Kamo, 1996; Svehlikova and Repcak, 2000; De Jesus-Gonzalez and Weathers, 2003). This enhanced accumulation of Rg1 ginsenoside in our octoploid adventitious roots of ginseng might also be exploited to develop clones for the production of higher amounts from the Rg1 group.

In conclusion, we have successfully induced octoploid ginseng adventitious roots via treatment with colchicine. To our knowledge, this is the first report on polyploid clones in that species. Here, the octoploid roots were responsible for higher accumulations of fresh and dry biomass. Although the total and Rb-group ginsenoside contents in the octoploid clones were lower than those measured in the tetraploids, we were able to obtain a greater amount of Rg-group ginsenosides, especially Rg1. These results indicate that induction of polyploidy, followed by screening for increased accumulations of individual ginsenosides, could be beneficial to the production of secondary metabolites from ginseng cultures.

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