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Ginsenoside Production and Morphological Characterization of Wild Ginseng (*Panax ginseng* Meyer) Mutant Lines Induced by γ-irradiation (^{60}Co) of Adventitious Roots

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With the purpose of improving ginsenoside content in adventitious root cultures of Korean wild ginseng (*Panax ginseng* Meyer), the roots were treated with different dosages of γ-ray (5, 10, 25, 50, 75, 100, and 200 Gy). The growth of adventitious roots was inhibited at over 100 Gy. The irradiated adventitious roots showed significant variation in the morphological parameters and crude saponin content at 50 to100 Gy. Therefore, four mutant cell lines out of the propagation of 35 cell lines treated with 50 Gy and 100 Gy were selected on the basis of phenotypic morphology and crude saponin contents relative to the wild type control. The contents of 7 major ginsenosides (Rg_1, Re, Rb_1, Rb_2, Rc, Rf, and Rd) were determined for cell lines 1 and 3 from 100 Gy and lines 2 and 4 from 50 Gy treatments. Cell line 2 showed more secondary roots, longer length and superior growth rate than the root controls in flasks and bioreactors. Cell line 1 showed larger average diameter and the growth rate in the bioreactor was comparable with that of the control but greater in the flask cultured roots. Cell lines 1 and 2, especially the former, showed much more ginsenoside contents than the control in flasks and bioreactors. Therefore, we chose cell line 1 for further study of ginsenoside contents. The crude saponin content of line 1 in flask and bioreactor cultures increased by 1.4 and 1.8-fold, respectively, compared to the control. Total contents of 7 ginsenoside types (Rg_1, Re, Rb_1, Rb_2, Rc, Rf, and Rd) increased by 1.8 and 2.3-fold, respectively compared to the control. Crude saponin and ginsenoside contents in the bioreactor culture increased by about 1.4-fold compared to that the flask culture.

Keywords: *Panax ginseng*, Adventitious root, γ-irradiation, Mutant, Ginsenosides

INTRODUCTION

There are several medicinal Panax species identified from all over the world. From a number of species, *P. ginseng* is one of the oldest and the most widely used herbal medicine and widely cultivated under shade conditions in Korea, China, Japan, and several countries in North America and Europe [1-4]. Ginseng is claimed to be effective for a wide range of health conditions, such as cancer [5], the immune system [6], blood pressure

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[7], postmenopausal symptoms [8] and improved liver function [9]. *P. ginseng* (Ginseng) and *P. quinquefolius* (American ginseng) are characterized well by phytochemistry [10,11] and more than 30 ginsenosides are identified from this genus. They can be classified into three groups based on their aglycones: the protopanaxadiol-type, protopanaxatriol-type, and oleanane type saponins. The major groups of ginsenosides are Rb and Rg groups derived from the 20(S)-protopanaxadiol and 20(S)-protopanxatriol structures, respectively. Among these, ginsenosides, Rb_1, Rb_2, Rc, and Rd from the Rb group, and Re, Rf and Rg_1 from the Rg group are the main components [12,13].

Due to certain limitations in genus *Panax* breeding, it is difficult to produce large amounts of roots under field conditions [14]. Hence, some scientists followed different biotechnological methods such as root culture, *Agrobacterium*-mediated hairy root production, and bioreactor-scale production. The use of precursors for enhanced production of ginsenosides has also been successful. The positive effect of methyl jasmonate on ginsenoside production from ginseng cell suspension, hairy root and adventitious root cultures has been previously documented [15-18]. Mutation breeding is considered as one of the virtual plant breeding methods for improved variety of crop production. Among several methods, γ-irradiation is used in several species for crop improvement program. Recent reports showed that mutagenesis by γ-irradiation enhanced ginsenoside production in *P. ginseng* at dosage 30 Gy-treatment, and shikonin content in callus cultures of *Lithospermum erythrorhizon* S. significantly increased at dosage 16 Gy-treatment [19,20]. γ-irradiation in other species also showed a significant effect [21,22]. The use of suspension culture methods resulted in significant responses in plant secondary metabolite production. In particular, bioreactor-based cultures displayed a significant improvement in the secondary metabolite production [23-25].

Our current work was designed to induce the formation of mutants by applying γ-irradiation of *P. ginseng* adventitious roots. There are two types of root cultures maintained in the bioreactor and flask to obtain valuable information in adventitious root cultures of *P. ginseng*.

MATERIALS AND METHODS

Establishment of adventitious root cultures

Ginseng adventitious roots from wild ginseng (about 100 years) were provided by Sunchon National University and cultured in Murashige and Skoog (MS) medium supplemented with 1-naphthaleneacetic acid (NAA), indole-3-acetic acid (IAA) and 5% sucrose [26]. We investigated the effects of different growth regulators on the biomass production of adventitious roots. The adventitious roots were cultivated on MS solid medium containing growth regulators (NAA and IAA) at 23±2°C. Root inducing efficiency was tested on MS solid medium on petridishes in presents of NAA from 0, 0.5, 1, 2, 4 mg/L and IAA from 0, 0.25, 0.5, 1, 2 mg/L. 5 Pieces of adventitious roots 1-2 cm in length were cultured on petridishes and each petridish supplied with 50 mL MS solid medium and cultured under dark condition for 30 d. Optimal concentrations of NAA and IAA on petridishes were estimated based on the higher number of secondary roots on MS solid medium, and these concentrations were used further to investigate the optimal concentration in MS liquid culture medium. Root inducing efficiency was also estimated by measuring secondary root number every 30 d. Then, the optimal concentration was used for *P. ginseng* adventitious root culture system (Fig. 1).

For petridish cultures, 10 pieces of adventitious roots 1-2 cm in length were cultured on petridishes and each petridish with 50 mL MS solid medium under dark condition at 23±2°C. For flask cultures, 0.8 g fresh roots were inoculated into a 100 mL Erlenmeyer flask with 50 mL MS liquid on a gyratory shaker (110 rpm) in the light. For bioreactor cultures, Fresh weight (15 g) of adventitious roots was inoculated into 15 L bioreactors (Biopia, Yongin, Korea) with 5 L MS medium for proliferation and the bioreactors were maintained at 23±2°C in the light until harvest. After 30 d, the proliferated adventitious roots were used as explants for further experiments. The growth of adventitious roots was estimated by measuring root dry weight (g) every 30 d.

γ-Irradiation and survival rate determination

Adventitious roots (1-2 cm) were placed on plastic petridishes (10 pieces adventitious roots per petridish), cultured for 5 d on MS solid medium with NAA, IAA and 5% sucrose. They were exposed to γ-radiation from cobalt (^{60}Co) source using an γ-radiation apparatus at the Applied Radiological Science Research Institute, Jeju National University. Irradiation dosages were 0 (non-irradiated), 5, 10, 25, 50, 75, 100, and 200 Gy. Each samples of 90 individual adventitious roots were taken from every treatment. An effect of gamma irradiation on adventitious root survival rate was evaluated by measuring the amount of survival adventitious roots after 5 wk growth.

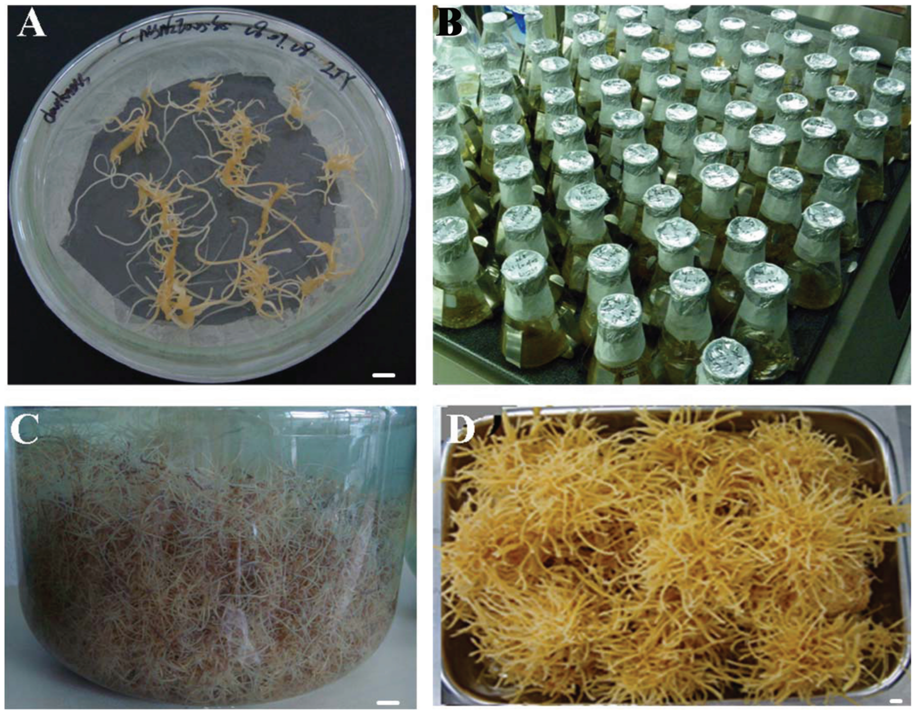

Fig. 1. Culture system of *Panax ginseng* adventitious roots. (A) Adventitious root formation on Murashige and Skoog (MS) solid medium on petridishes. (B) Proliferation of the adventitious root in MS liquid medium in flasks. (C) Production of the adventitious root in bioreactors. (D) Harvest adventitious roots. Bar=1 cm.

Cell line selection in suspension culture

The survival roots from each dosage were transferred into 50 mL MS suspension culture medium with NAA and IAA in the flask. In the case of the flask culture, it is very difficult to modify the culture environment within flasks and is used for only small-scale cultures due to the limited air supply. The bioreactor fitted with a controller for air supply. Progress in tissue automation will depend on the use of liquid cultures in bioreactors. Possibly the growth condition and the ginsenoside content in bioreactors were different from those of flask cultures. So, selected cell lines were cultured in a 15 L bioreactor with 5 L MS medium. Cell lines selected according to the phenotypes of secondary root number, length, diameter and crude saponin content. Secondary root number, length and diameter were measured from 40 pieces of adventitious and secondary roots in flasks and bioreactors.

Determination of root weight, growth ratio, secondary root number, length and diameter

Fresh weight (FW) and dry weight (DW) were measured after 10, 20, and 30 d of growth in flask and bioreactor cultures. Root FW and DW were determined as follows. Roots were separated from the medium by passing through a 1 mm stainless steel sieve. FW was measured after washing three times with tap water and blotting away surface water. DW was recorded after roots were dried at 75°C for three d [27]. Root growth ratio was calculated by using the following formula [28]:

$$\text{Growth ratio} = \frac{\text{Harvested DW (g)}}{\text{Inoculated DW (g)}}$$

After 10, 20 and 30 d growth, the length of secondary root (cm) was measured by using the calipers. Secondary root number (N) was counted from the adventitious root. Secondary root diameter (mm) was measured with a microscope after treatment by acetocarmine solution.

Extraction and determination of crude saponin

Extraction and determination of crude saponin were carried out by modifying the method of Kwon *et al*. [29] and Shin *et al*. [30]. Extraction of crude saponin from adventitious roots of different cell lines was conducted as follows. Dried sample powder (1 g) was extracted by sonication (Branson, Danbury, CT, USA) at 40°C for 1 h with 30 mL of 80% (v/v) methanol-water. The extract obtained was evaporated using a rotary evaporator under vacuum at 55°C. The evaporated residue (total extract yield) was dissolved in 20 mL of distilled water and washed twice with 20 mL of diethyl ether to remove the fat contents using a separating funnel. The aqueous layer was extracted four times with 20 mL of water-saturated n-butanol. The butanol solution was washed twice with 30 mL of distilled water to remove the impurities. The remaining butanolic solution was transferred to the tarred round bottom flask for the evaporation using a rotary evaporator under vacuum at 55°C. After evaporation, the residue was dissolved in 1.5 mL 100% methanol for HPLC analysis. Crude sapopnin content was calculated as follows,

Crude saponin content (mg/g ginseng) = (W2-W1)/W3

where, W1 is the weight of empty flask, W2 is the weight of the flask with dried residue and W3 is the weight of ginseng powder.

Determination of ginsenoside Rg_1, Re, Rb_1, Rb_2, Rc, Rf and Rd

The crude saponins were dissolved in methanol and the amounts of ginsenosides in each sample was determined by HPLC. The HPLC conditions for ginsenoside isolation were modified from Park *et al*. [31]. Quantitative determinations were achieved by HPLC using a Capcell-pak C^{18} MG (4.6×250 mm) column (Shiseido, Tokyo, Japan), Waters 2998 Photodiode Array Detector, Waters 2690 Separations Module and Empower Program. The solvents used were of HPLC grade (Fisher, Pittsburgh, PA, USA). The water used was treated with a Milli-Q water purification system (Millipore, Bedford, MA, USA). Ginsenosides Rg_1, Re, Rb_1, Rb_2, Rc, Rf and Rd standards were purchased from BTGin Co., Ltd (Daejeon, Korea). Digoxin was used as the internal standard. The HPLC conditions for ginsenoside isolation were as follows: mobile phase, water and acetonitrile; gradient elution, the eluents being 0 to 22 min, 18% acetonitrile; 22 to 32 min, 30% to 45%; 32 to 50 min, 45% to 50%; 50 to 55 min, 50% to 18%; 55 to 60 min 18%; flow rate, 1 mL/min; column temperature, 35°C; detector wavelength, 203 nm; injection volume, 10 μL. Analysis of ginseng ginsenosides was modified according to Yu *et al*. [32]. The total ginsenoside content was calculated as the sum of individual ginsenoside fractions.

The ginsenoside content of ginseng adventitious roots was calculated as:

$$\text{GC (mg/g)} = \frac{\text{SGC (from HPLC) (mg/L)} \times \text{SV (L)}}{\text{AR (g)}}$$

(GC, ginsenoside content; SGC, sample ginsenoside concentration; SV, sample volume; AR, adventitious root)

The ginsenoside productivity of ginseng adventitious roots was calculated as:

$$\text{GP (mg/L)} = \frac{\text{TGC (mg/g)} \times \text{HR (g)}}{\text{MV (L)}}$$

(GP, ginsenoside productivity; TGC, total ginsenoside concentration; HR, harvested root; MV, medium volume)

Method validation and statistical analysis

Stock solutions for the 7 ginsenosides were prepared separately in 100% methanol. Digoxin stock solution was prepared in 80% methanol. Working solutions were prepared in 100% methanol by mixing known amounts of all the compounds. The linear range, limit of detection (LOD) and limit of quantification (LOQ) were studied for the developed method. The linearity of calibration curve was tested by standard analysis. The calibration curves of individual ginsenosides were constructed using a range of five concentrations of the standard, and LOD and LOQ for each analyte were evaluated at signal-to-noise ratios (S/N) of 3:1 and 10:1, respectively.

Statistical analysis was performed according to the SPSS ver. 13.0 (SPSS Inc., Chicago, IL, USA). Mean and standard errors were used throughout, and statistical significance between the mean values was assessed by applying a Duncan's multiple range test. A *p*-value of less than 0.05 was taken to indicate statistical significance.

RESULTS AND DISCUSSION

Effects of plant growth regulators on adventitious root suspension culture

The role of auxin is crucial for *P. ginseng* secondary root induction and elongation. The number of secondary roots increased considerably at the concentrations of 2 mg/L NAA and 1 mg/L IAA on MS solid medium on petridishes (Table 1). The growth rate of the cultures increased, reaching a maximum in MS liquid medium in flasks containing the concentrations of 2 mg/L NAA and 0.25 mg/L IAA (Fig. 2). Usually natural auxin IAA and synthetic auxin NAA and indole-3-butyric acid (IBA) are used for rooting. As previously reported, in adventitious root cultures of *Eleutherococcus sessiliflorus*, the frequency of adventitious root induction was highest on MS medium with 0.5 mg/L NAA [33]. In *P. ginseng* adventitious root cultures, the IBA treatment was more effective for lateral root induction and root growth compared to NAA [34]. IAA and NAA significantly increased the number of root in *Lycopersicon esculentum* root cultures

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Table 1. Efficiency of secondary root induction of *Panax ginseng* adventitious root at various NAA and IAA concentration combinations

NAA mg/L	IAA mg/L	No. of secondary root
0	0	0±0
	0.25	0±0
	0.5	0±0
	1	0.8±0.5
	2	1.0±0.8
0.5	0	2.4±0.5
	0.25	3.2±0.7
	0.5	5.4±0.7
	1	1.9±0.3
	2	1.4±0.9
1	0	2.6±0.8
	0.25	2.6±0.5
	0.5	3.4±0.3
	1	2.5±0.7
	2	2.6±0.5
2	0	3.4±0.5
	0.25	3.2±0.5
	0.5	3.3±0.7
	1	8.2±0.9
	2	6.1±1.0
4	0	0.5±0.6
	0.25	0.5±0.6
	0.5	1.3±1.0
	1	2.3±0.6
	2	0.9±1.0

Ginseng adventitious root was chopped in each 1-2 cm fragment and imbedded 5 fragments of root per plate.
Means±SE of 6 independent experiments.
NAA, 1-naphthaleneacetic acid; IAA, indole-3-acetic acid.

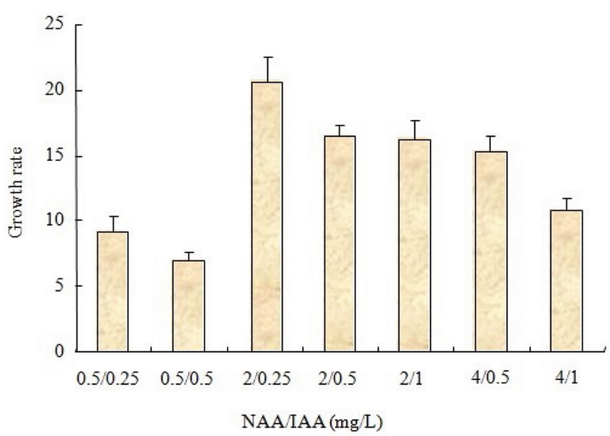

Fig. 2. Efficiency of secondary root induction of *Panax ginseng* adventitious root at various concentration 1-naphthaleneacetic acid (NAA) and indole-3-acetic acid (IAA) combination in the flask test. Bar shown are means±standard errors of three replicates.

[35]. Therefore, NAA and IAA were used for further experiments with *P. ginseng* adventitious root cultures.

Adventitious root survival rate and cell line selection

Initially, we determined the survival rates of adventitious roots at different dosages of γ-irradiation. Data presented in Figs. 3 and 4 showed the percentage of adventitious root survival rate after treated with various

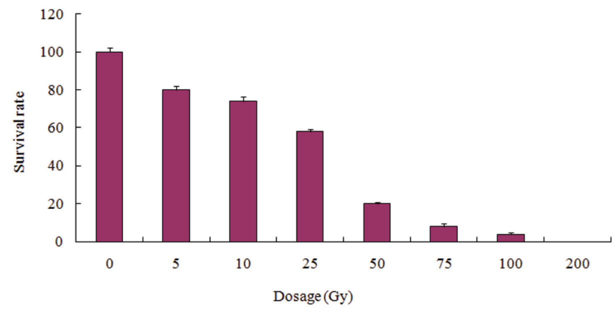

Fig. 3. Survival rate of *Panax ginseng* adventitious roots with 7 different dosages, growth 5 weeks on petridish on Murashige and Skoog solid medium with 2 mg/L 1-naphthaleneacetic acid, 1 mg/L indole-3-acetic acid and 5% sucrose at 23°C in light condition after irradiation. Bar shown are means±standard errors of three replicates.

dosages of γ-rays (0, 5, 10, 25, 50, 75, 100, and 200 Gy). There was a surpassing response for 5 Gy which gave the highest percentage (80%) of adventitious root survival rate, but 100 Gy dosage gave the lowest one (4%). Therefore it may be concluded that survival rate differed in their ability to different dosages of γ-rays depending on the adventitious root survival number. The low dosages of γ-ray caused a higher adventitious root survival rate, while higher dosages reduced it. Similar results were obtained by Abdullah *et al*. [36]. The survival rate of roots completely stopped at 200 Gy. The growth of adventitious roots was inhibited at γ-ray dosages over 50 Gy treatment. The LD_{50} (median lethal dose) was established at about 40 Gy based on the survival of adventitious roots.

In *P. ginseng* hairy root cultures, hairy roots were irradiated by ^{60}Co γ-ray and the growth was inhibited at over 30 Gy [37]. Usually, based on the highest growth rate, the optimal γ-irradiation dosage was selected for continuing studies [20]. In our case, we selected 4 root cell lines which survived at γ-ray dosages between 50 and 100 Gy for further studies. Because among these dosage, the diameter of some cell lines are larger compared with the control. The growth ratio in some cell lines was higher than the control and in some other cell lines was lower than the control. Crude saponin contents in some cell lines were also different compared to the control. Cell lines 1 and 3 were selected from 100 Gy treatment. Cell lines 2 and 4 were selected from 50 Gy treatment.

Phenotypic characteristics of selected cell lines

After the dosage of 50 Gy to 100 Gy treatment, 4 cell lines were isolated at last. Characteristics of these cell lines were determined after a few months of growth in MS liquid medium with NAA and IAA in flask and bioreactor cultures. After 30 d of flask culture, the number

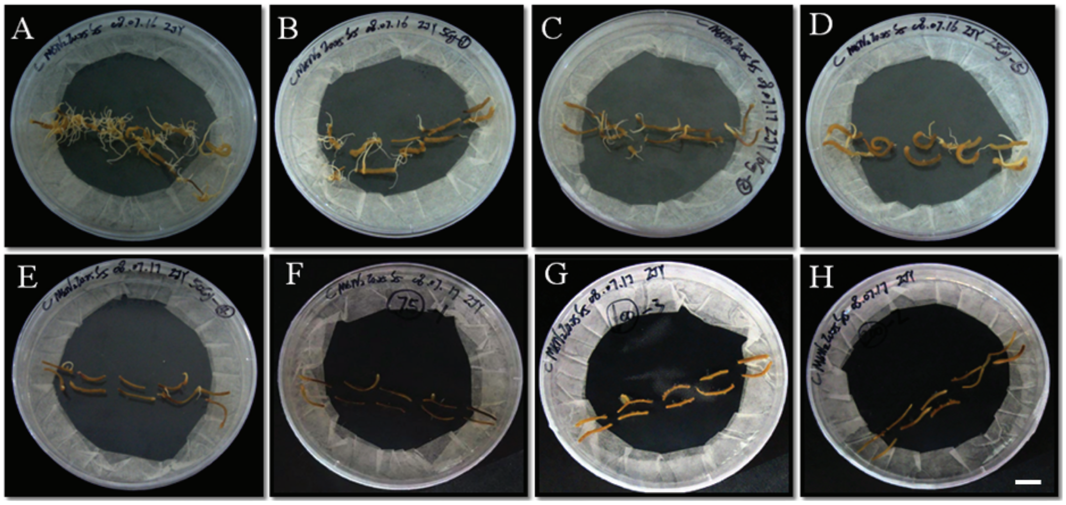

Fig. 4. Effect of γ-rays on adventitious root survival number on Murashige and Skoog solid medium with 2 mg/L 1-naphthaleneacetic acid, 1 mg/L indole-3-acetic acid and 5% sucrose at 23°C in light condition after irradiation. (A) Control, (B-H) irradiated with 5, 10, 25, 50, 75, 100, and 200 Gy γ-radiation. Bar=1 cm.

Table 2. Secondary root number, length and diameter of the control and cell lines of *Panax ginseng* adventitious root

Cell lines		No. of secondary root			Length of secondary root (cm)			Diameter of secondary root (mm)		
		10 day	20 day	30 day	10 day	20 day	30 day	10 day	20 day	30 day
Flask	Control	221±8^{b}	314±6^{b}	372±7^{b}	1.73±0.12^{a}	2.52±0.20^{a}	3.51±0.15^{a}	0.181±0.013^{c}	0.251±0.012^{d}	0.313±0.011^{c}
	CL1	158±7^{d}	185±10^{d}	244±8^{d}	1.17±0.08^{b}	1.81±0.11^{b}	2.09±0.08^{b}	0.196±0.012^{c}	0.404±0.013^{a}	0.411±0.014^{a}
	CL2	292±5^{a}	389±3^{a}	408±8^{a}	1.38±0.06^{b}	2.78±0.10^{a}	3.22±0.08^{a}	0.274±0.013^{b}	0.329±0.015^{bc}	0.363±0.013^{b}
	CL3	188±12^{c}	254±17^{c}	286±20^{c}	0.81±0.06^{c}	1.43±0.10^{b}	2.03±0.08^{b}	0.129±0.007^{d}	0.299±0.013^{c}	0.376±0.009^{ab}
	CL4	286±5^{a}	329±6^{b}	318±4^{b}	1.89±0.08^{a}	2.61±0.15^{a}	2.61±0.15^{a}	0.309±0.015^{a}	0.342±0.015^{b}	0.380±0.017^{ab}
Bioreactor	Control	940±32^{a}	1107±56^{b}	1472±110^{b}	1.72±0.10^{a}	2.57±0.18^{a}	3.52±0.16^{a}	0.201±0.019^{b}	0.263±0.016^{b}	0.339±0.018^{b}
	CL1	570±21^{b}	683±27^{d}	1051±56^{c}	1.15±0.09^{b}	1.87±0.13^{b}	2.20±0.08^{b}	0.194±0.017^{b}	0.400±0.02^{a}	0.416±0.017^{a}
	CL2	545±28^{bc}	1333±38^{a}	1965±36^{a}	2.87±0.15^{a}	2.87±0.15^{a}	3.19±0.18^{a}	0.311±0.016^{a}	0.376±0.016^{a}	0.388±0.02^{ab}
	CL3	490±9^{c}	671±27^{d}	962±6^{c}	1.45±0.07^{c}	1.45±0.07^{c}	2.07±0.09^{b}	0.143±0.013^{c}	0.294±0.016^{b}	0.377±0.011^{ab}
	CL4	884±12^{a}	980±10^{c}	1328±109^{b}	2.80±0.16^{a}	2.80±0.16^{a}	3.4±0.14^{a}	0.329±0.019^{a}	0.363±0.02^{a}	0.414±0.02^{a}

The data were collected after 30 days of culture in the 100 mL flask with 50 mL Murashige and Skoog (MS) medium and in the 15 L bioreactor with 5 L MS medium.
The results represent the means±SE of values obtained from three experiments.
Different corresponding letters within a column are significant different at $p<0.05$ by Duncan's multiple range test.
CL, cell line.

of secondary roots was higher (408) in cell line 2 compared to other cell lines and the control (372). Cell line 1 showed a minimum number of secondary roots. In bioreactors, the number of secondary root was 1,472 in the control. The value of secondary root number of cell line 2 increased compared to the control, but cell lines 1 and 3 had a decrease in the number of secondary roots, compared to the control, and the cell line 4 showed the result similar to the control (Table 2 and Fig. 5). In the case of secondary root length in flasks and bioreactors, cell lines 2 and 4 showed lengths similar to the control. Distinctly, cell lines 1 and 3 showed shorter root lengths than other cell lines and the control (Table 2 and Fig. 5). Interestingly, in flasks and bioreactors, the diameter of secondary root increased in all four selected cell lines compared to the control (Table 2 and Fig. 5).

The growth ratios of adventitious roots were determined depending on the formation of lateral roots and the increase in root diameter [38]. After 30 days of growth in flasks, the growth ratios of cell lines were 4.6 in the control, and 3.9, 6.1, 3.5 and 5.5 for cell lines 1, 2, 3 and 4, respectively (Fig 6A). The growth ratios in cell lines 2 and 4 were 33% and 20% higher than the control, but in cell lines 1 and 3 the ratio declined relative to the control. Interestingly, in the bioreactor cell lines, the values of growth ratio varied; 14.8 in the control cell line, and 14.4, 17.2, 8.3, and 14.5, respectively, in cell lines 1, 2, 3, and 4 (Fig. 6B). The values of growth ratio in cell line 2 was 16% higher compared to the control. On the contrary, cell line 3 had a lower ratio than the control, whereas cell

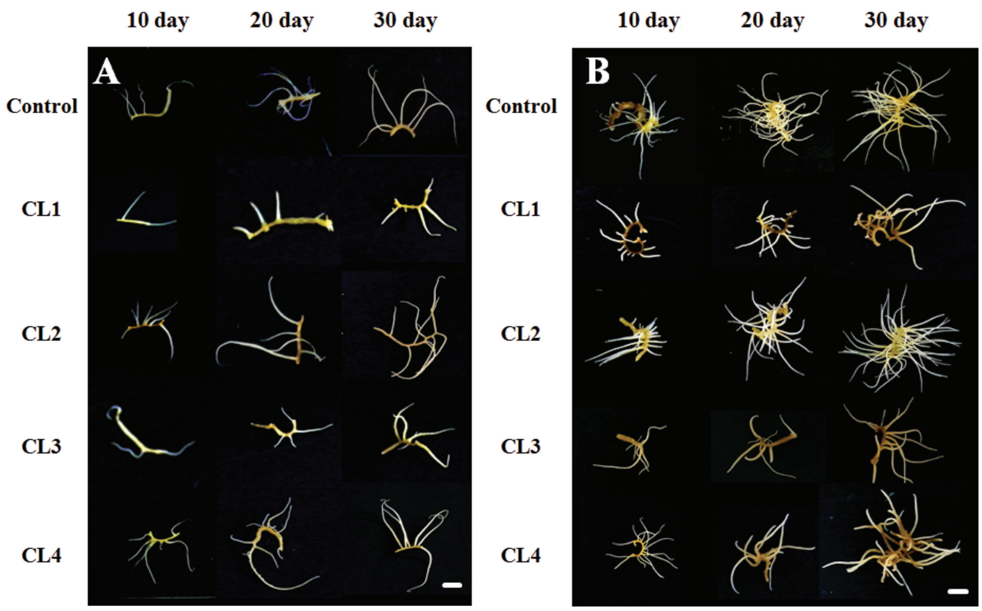

Fig. 5. Phenotypic characteristics of secondary roots in the control and cell lines growth in the flask with 50 mL Murashige and Skoog (MS) medium (A) and bioreactor with 5 L MS medium (B). Bar=1 cm. CL, cell line.

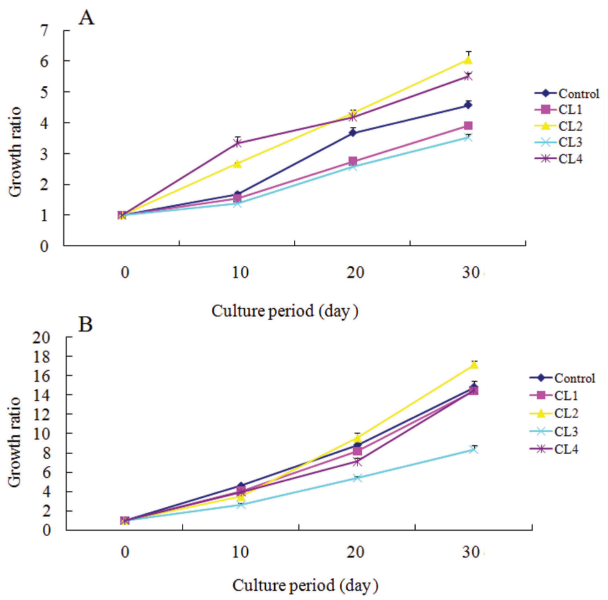

Fig. 6. Determinated growth ratio of *Panax ginseng* adventitious root in the control and cell lines after 10, 20, and 30 days of culture in the flask with 50 mL Murashige and Skoog (MS) medium (A) and bioreactor with 5 L MS (B). Bar shown are means±standard errors of three replicates. CL, cell line.

lines 1 and 4 showed the ratios similar to the control.

In our study the bioreactor culture system was preferable to the culture adventitious root because of higher growth ratios and more biomass production. Aeration and medium currency could be regulated in using the bioreactor. The bubbles in a bubble column create less shear stress, so that it is useful for organized culturing structures. In *Artemisia annua* transformed hairy root cultures, root biomass significantly increased in an acoustic mist bioreactor [39]. In the cultivation of *Salvia officinialis* L. (sage) hairy root, biomass of the roots grown in the bioreactor was about 1.5-fold higher than that achieved for roots cultured in shake flasks. The biomass concentration of *Stizolobium hassjoo* hairy root in the shake flask on the 16th day was 61.2% of that in the mist trickling reactor [40]. In *P. ginseng* hairy roots cultures, growth was enhanced about three times in both the bubble column and the stirred bioreactor compared with the flask cultures [41]. An improved method of adventitious roots culture system through the use of a bioreactor seems to be a reliable method for the commercialization of *P. ginseng*.

Calibration curve, limit of detection and limit of quantification

The calibration curves and the LOD for the ginsenosides are shown in Table 3. The correlation coefficients are all better than 0.99, which show good linearity. The LODs, which are in the range from 0.08 to 2.32 mg/L for the 7 ginsenosides, were determined as the lowest concentrations injected that yielded a S/N of 3. The LOQs were determined as the concentrations that yielded a S/N of 10 and are in the range from 0.27 to 7.71 mg/L

Table 3. Calibration curve, limit of detection (LOD) and limit of quantification (LOQ) for seven ginsenosides

Compound	Calibration curve[1)]	r^2	Linear range (mg/L)	LOD (mg/L)	LOQ (mg/L)
Rg_1	y = 3762.2x - 719.38	0.9908	25-150	2.32	7.71
Re	y = 2452.5x + 22171	0.9979	30-180	1.0	3.06
Rb_1	y = 2343.7x - 1448.4	0.9967	30-150	0.84	2.81
Rb_2	y = 1978.3x + 27044	0.9933	50-200	1.30	4.32
Rc	y = 6048.9x - 12290	0.9984	10-50	0.5	1.67
Rf	y = 9588.1x - 10035	0.9995	10-50	0.45	1.51
Rd	y = 6263x + 1238.8	1	10-50	0.08	0.27

[1)]y is the peak area and x is the concentration of analyte.

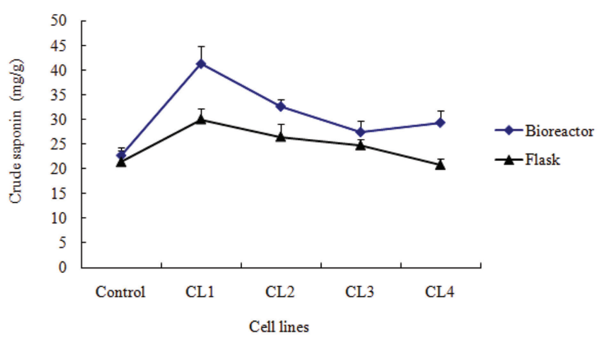

Fig. 7. Effect of γ-ray on crude saponin content in the control and cell lines of *Panax ginseng* adventitious root after 30 days growth in the flask with 50 mL Murashige and Skoog (MS) medium (♦) and bioreactor with 5 L MS medium (▲). Bar shown are means±standard errors of three replicates. CL, cell line.

Crude saponin and ginsenoside production of cell lines

Optimal cell line is important in order to maximize the final metabolite concentration in the cultures. Crude saponins and contents of 7 ginsenoside types of the Rg_1, Re, Rb_1, Rb_2, Rc, Rf, and Rd from the control and 4 cell lines were compared. In flasks, the crude saponin content was highest (29.96 mg/g) in the cell line 1, which increased by 1.4-fold above that of the control. Cell lines 2 and 3 showed higher crude saponin contents than cell line 4 and the control. In bioreactors, the value of crude saponin content was 41.19 mg/g in cell line 1, which represents a 1.8-fold increase over the control. Other three cell lines had 1.2 to 1.4 fold increases over the control (Fig. 7). We also analyzed the cell lines by using HPLC. Typical chromatograms were shown in Fig. 8. The total contents of the 7 ginsenoside types were significantly enriched, especially in cell line 1, showing a 1.8-fold increase over the control (Table 4). Next to the cell line 1, cell line 2 showed higher total contents of the 7 ginsenoside types than cell lines 3 and 4. Cell line 4 showed lower total contents of the 7 ginsenoside types than the control and other cell lines. Overall, the highest ginsenoside productivity (31.20 mg/L) was obtained with cell line 1. To analyze the amounts of 7 ginsenosides in the bioreactor cultures, we used the same HPLC conditions. The total contents of the 7 ginsenoside types obtained were 3.62 mg/g, 8.22 mg/g, 5.03 mg/g, 3.49 mg/g, 4.22 mg/g, respectively, for the control and the cell lines. The total contents of the 7 ginsenoside types in cell line 1 were 2.3-fold higher than the control (Table 4), and about 1.6-fold higher in cell line 2 relative to the control. In cell lines 3 and 4, the total ginsenoside contents were similar to those of the control (Table 4). On the whole, the highest ginsenoside productivity (28.66 mg/L) was obtained with cell line 1.

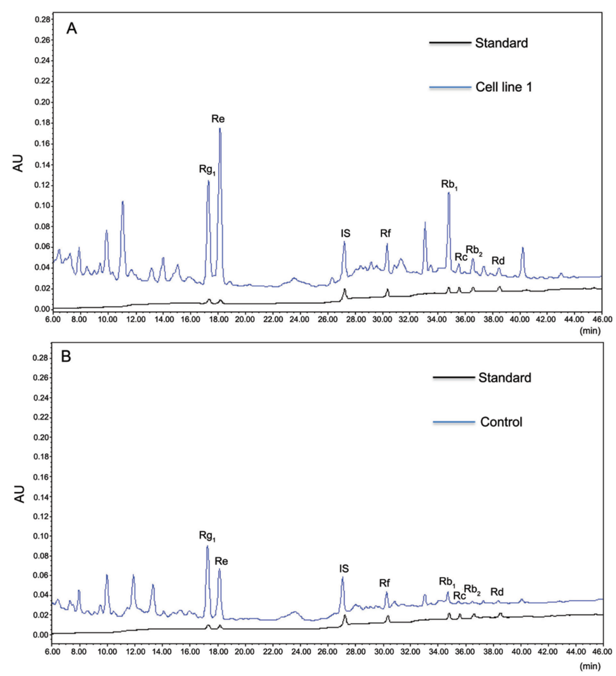

Fig. 8. Typical chromatograms obtained from the standard solution, cell line 1 (A) and control (B) solution extracted from bioreactor by UV detection at 203 nm. IS, internal standard of digoxin.

In this study, we observed that crude saponin content was lower in the flask cultures than in the bioreactor cultures. Thus, the ginsenoside content was greater with the bioreactor cultures than with the flask cultures. Similarly,

Table 4. Ginsenoside production of the control and cell lines of *Panax ginseng* adventitious root

Cell lines		Ginsenoside (mg/g DW)							Ginsenoside productivity (mg/L)
		Rg_1+Re	Rb_1	Rb_2	Rc	Rf	Rd	7 Ginsenosides contents	
Flask	Control	2.31±0.11^{b}	0.62±0.03^{bc}	0.21±0.03^{a}	0.03±0.002^{b}	0.10±0.005^{c}	0.017±0.003^{c}	3.29±0.38^{b}	21.00±2.33^{b}
	CL1	4.07±0.24^{a}	0.99±0.11^{a}	0.35±0.01^{a}	0.06±0.003^{a}	0.30±0.002^{a}	0.043±0.002^{a}	5.83±0.19^{a}	31.20±1.26^{a}
	CL2	2.43±0.13^{b}	0.71±0.10^{b}	0.20±0.05^{a}	0.03±0.001^{b}	0.23±0.01^{b}	0.03±0.003^{b}	3.64±0.08^{b}	30.97±1.20^{a}
	CL3	2.28±0.21^{b}	0.47±0.04^{cd}	0.20±0.02^{a}	0.027±0.001^{b}	0.06±0.005^{d}	0.013±0.001^{c}	3.06±0.28^{c}	15.17±1.81^{b}
	CL4	1.72±0.12^{bc}	0.36±0.02^{d}	0.16±0.10^{a}	0.03±0.002^{b}	0.11±0.003^{c}	0.013±0.002^{c}	2.36±0.3^{c}	18.26±2.45^{b}
Bioreactor	Control	2.30±0.26^{c}	0.89±0.19^{b}	0.25±0.02^{b}	0.034±0.003^{b}	0.13±0.003^{b}	0.017±0.001^{b}	3.62±0.39^{c}	13.91±1.10^{c}
	CL1	5.64±0.06^{a}	1.62±0.11^{a}	0.49±0.01^{a}	0.08±0.005^{a}	0.33±0.01^{a}	0.05±0.002^{a}	8.22±0.12^{a}	28.66±1.61^{a}
	CL2	3.39±0.13^{b}	0.91±0.03^{b}	0.34±0.05^{b}	0.04±0.002^{b}	0.31±0.005^{a}	0.04±0.003^{a}	5.03±0.13^{b}	19.63±0.05^{b}
	CL3	2.39±0.19^{c}	0.59±0.02^{b}	0.39±0.05^{ab}	0.03±0.001^{b}	0.07±0.006^{c}	0.017±0.001^{b}	3.49±0.16^{c}	8.06±0.52^{d}
	CL4	2.85±0.28^{bc}	0.77±0.06^{b}	0.2±0.06^{b}	0.04±0.003^{b}	0.15±0.008^{b}	0.013±0.003^{b}	4.22±0.37^{c}	19.2±2.13^{b}

The data were collected after 30 days of culture in the 100 mL flask with 50 mL Murashige and Skoog (MS) medium and in the 15 L bioreactor with 5 L MS medium.
The results represent the means±SE of values obtained from three experiments.
Different corresponding letters within a column are significant different at $p<0.05$ by Duncan's multiple range test.
DW, dry weight; CL, cell line.

in the Devil's claw hairy root cultures (*Harpagophytum procumbens*), the harpagide content in bioreactors was more than that of the flask cultures and had higher levels of sugars and amino acids, probably due to their nutrient status and low-stress environments [23]. In the hairy root cultures of *Pueraria phaseoloides*, the puerarin content in a 2.5-L bioreactor was 200 times as much as in 250 ml flask cultures [24]. *Artemisia annua* hairy root cultures were examined in shake flasks and compared with cultures grown in two types of bioreactors, a mist bioreactor and a bubble column reactor. Mist reactors produced significantly more artemisinin, while bubble column reactors provided greater biomass and the roots grown in shake flasks contained a minimal amount of artemisinin [25]. In our study, we observed that cell line 1 yielded about 1.4-fold increase in crude saponin and ginsenoside contents from the bioreactor culture as compared with those from the flask culture. Cell line 1 was the best cell line because of the highest crude saponin and ginsenoside contents when cultured in the bioreactor.

In conclusion, results showed that mutant lines induced by γ-ray irradiation (^{60}Co) were characterized by superior morphological qualities and efficient production of secondary metabolites such as ginsenoside. According to our observations, the highest ginsenoside content was achieved with the mutant cell line 1. Hence, we plan to use the cell line 1 to carry out additional studies on the identification of the critical genes that relate to the biosynthetic pathway of the ginsenosides in *P. ginseng*.

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