Journal of Plant Biology, December 2002, 45(4) : 201-206

Effect of Plant Growth Regulators and Medium Composition on Cell Growth and Saponin Production during Cell-Suspension Culture of Mountain Ginseng (*Panax ginseng* C. A. Mayer)

Mei-Lan Lian[1,2], Debasis Chakrabarty[1], and Kee-Yoeup Paek[1]*
[1]Research Center for the Development of Advanced Horticultural Technology, Chungbuk National University, Cheongju 361-763, Korea
[2]Department of Horticulture, Yanbian University, Agriculture College, Longjing, China

We have established cell-suspension cultures of mountain ginseng (*Panax ginseng* C. A. Mayer), and have attempted to increase the yield of saponin by manipulating our processing method and culturing factors (e.g., media strengths; the presence of plant growth regulators or sucrose; ratios of NO_3^+/ NH_4^-). Maximum biomass yield was obtained in media containing 2,4-D. However, saponin productivity was much higher in a medium comprising either IBA or NAA; 7.0 mg/L IBA was optimal for promoting both cell growth (10.0 g/L dry weight) and saponin production (7.29 mg/g DW total ginsenoside). Although the addition of cytokinins (BA and kinetin) did not affect cell growth, the level of saponin (particularly in the Rb group) was enhanced when the media were supplemented with either 0.5 mg/L BA or 0.5 mg/L kinetin. Half- and full-strength MS media were equally suitable for inducing both biomass as well as saponin production. We also investigated the effect of various concentrations of sucrose and nitrogen, and found that 30 g/L sucrose enhanced biomass yield as well as saponin content. However, further increases (i.e., up to 70 g/L) led to a decrease in saponin accumulation and biomass production. Maximum growth and saponin productivity were reported from treatments with an initial nitrogen concentration of 30 mM. In general, the amount of saponin increased when the test media had high NO_3^+/ NH_4^- ratios; in fact, saponin production was greatest when nitrate was the sole nitrogen source.

Keywords: auxin, cytokinin, MS strength, nitrogen, sucrose

A number of physical and chemical factors have been identified that can influence secondary metabolism in plant cell cultures (Dornenburg and Knorr, 1995; Bourgaud et al., 2001). For example, optimizing the concentrations and combinations of hormones and nutrients is frequently effective. Although 2,4-D is most commonly used in routine culture maintenance (Wu and Zhong, 1999), this suspected carcinogen often presents concerns for health and safety. Altering environmental factors, such as light and temperature, can also increase productivity, while nitrogen source plays an important role in determining how much product is accumulated in the cells. For example, in *Lithospermum erythrorhizon*, shikonin synthesis is inhibited by ammonium ions, while cell growth is promoted by the same (Tabata and Fujita, 1985).

Ginseng (*Panax ginseng* C. A. Mayer; Araliaceae) is one of the most valuable oriental herbs. Wild (or mountain) ginseng was formerly found only in a few isolated areas of Korea and northwestern China. Now, these plants are even more rare, and the roots that are available in markets are collected mostly from farm cultivation. Demand is so high that a wild specimen may be sold for millions of dollars (Yu et al., 2000). Since ancient times, its dried roots have been used as a drug and health tonic in China, Japan, and Korea (Tang and Eisenbrand, 1992). This species contains saponins, antioxidants, peptides, polysaccharides, fatty acids, alcohols, and vitamins (Huang, 1993). Those saponins, known as ginsenosides, are widely believed to be a major bioactive compound.

The production of secondary metabolites in plant cells has been researched extensively (Bourgaud et al., 2001). Cell culturing can provide a viable alternative for the efficient production of ginseng's active ingredients, in terms of product quality and quantity (Choi et al., 1994a, 1994b; Ding, 1988; Hibino and Ushiyama, 1998; Wu and Zhong, 1999). However, productivity from this tissue-culture process must still be improved in order to be economically competitive with field cultivation of ginseng. Therefore, in this study, our objectives were to 1) establish an efficient protocol for cell-suspension culture of mountain ginseng; and 2) increase saponin yield by manipulating several culturing variables.

*Corresponding author;fax +82-431-275-7467
e-mail paekky@chungbuk.ac.kr

MATERIALS AND METHODS

Plant Material and Culture Conditions

Fresh roots of mountain ginseng were collected in Korea. Samples were washed with a detergent solution for 5 to 10 min, and rinsed with running tap water for another 5 to 10 min. They were then soaked in 70% aqueous EtOH for 0.5 to 3.0 min (under reduced pressure), further sterilized with 1% sodium hypochloride for 10 to 30 min, then rinsed repeatedly with sterile distilled water. Afterward, the sterilized roots were cut into 2- to 10-mm sections and inoculated into an MS solid medium (Murashige and Skoog, 1962) that contained 30 g/L sucrose, 1 mg/L 2,4-D (2,4-diphenoxyacetic acid), and 0.1 mg/L kinetin. Calli were induced after one month, then subcultured every 20 d for proliferation in the above MS medium..After being subcultured 10 times into solid media, the calli were inoculated into a liquid medium (same MS medium as above). These cells were cultivated in the dark at 25°C in 250-mL conical flasks (working volume of 50 mL) on a rotary shaker at 100 rpm. Cultivation continued for three weeks, and the inoculum size was controlled at 4 g/flask by wet weight (202 mg/flask, dry weight).

For our first experiments to investigate the effects of various plant hormones on cell growth and saponin production, we grew cell-suspension cultures of mountain ginseng in MS media supplemented with 1 mg/L 2,4-D or different concentrations of indolebutyric acid (IBA; 1, 3, 5, 7, or 9 mg/L) or α-napthalenacetic acid (NAA; 1, 3, 5, 7, or 9 mg/L). The second test involved combining 7 mg/L IBA with different concentrations of 6-benzylaminopurine (BA; 0.1, 0.5, or 1.0 mg/L) or kinetin (0.1, 0.5, or 1.0 mg/L). In the third set of experiments, the cell suspensions were cultured on 1) different strengths of MS media (0.5, 1.0, 1.5, or 2.0); 2) an MS medium supplemented with a range of nitrogen concentrations (30, 60, 90, or 120 mM); 3) MS media containing different NH_4^+/NO_3^- ratios (0:30, 5:25, 10:20, 15:15, 20:10, 25:5, or 30:0); or 4) various levels of sucrose (10, 30, 50, or 70 g/L). NH_4Cl and KNO_3 were used as the sole source of NH_4^+ and NO_3^-, respectively, in these experiments. All experimental media were autoclaved at 121°C, with pH adjusted to 5.8, and the cultures were maintained for three weeks.

Sampling and Analysis of Cell Weights

Three flasks were sacrificed at each sampling period. Their cell suspensions were filtered and washed three to four times with sufficient distilled water before fresh cell weights were measured. To determine dry weights, the cells were dried at 40°C for 2 d.

Extraction and Determination of Ginsenoside Content

Our procedure for extracting and determining the content of ginsenosides was modified from that of Furuya and Yoshikawa (1987) and William et al. (1996). The ginsenoside fraction was analyzed using an HPLC system (Waters 2690 87 Separation Module, Waters 996 Photodiode Array Detector, and Waters Millenium 2010 Chromatography Manager; Waters Co., Milford, MA, USA) on an Al-tec Platinum C18 column (ϕ1.5_m, 33 mm × 7 mm), with water and acetonitrile as the mobile phase. The ratios of water to acetonitrile for the first 10 min and the last 25 min were 75:25 and 63:37, respectively. Flow rate of the mobile phase was 1.2 mL per min, and ginsenoside was detected at 203 nm. Total ginsenoside content was calculated as the sum of the ginsenoside fractions; content from the adventitious roots was determined as described by Furuya and Yoshikawa (1987).

Determination of Ions in Media

Samples were collected following three weeks of suspension culture. After filtering through 0.2-μm membranes, all media were diluted 10-fold, and 1 mL of this was used for the ion analysis. Cations were analyzed via HPLC, with a Waters Conductivity Detector, on a Waters IC-Par CM/D column (3.9 × 50 mm; eluent: 0.5 mM EDTA per 2 mM HNO_3). Anions also were measured by HPLC, with a suppressed conductivity detector on an IC-Par Anion HR column (4.6 × 75 mm; eluent: 1.6 mM $NaHCO_3$ per 1.4 mM Na_2CO_3).

Experimental Design and Data Analysis

All experiments were repeated three times, with three replicates each. The data were subjected to Duncan's Multiple Range Test, using a SAS program (Version 6.12, SAS Institute Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

In optimizing the culturing technique for suspended mountain ginseng cells in a shaker flask, we focused our efforts on the effects of plant growth regulators (2,4-D, IBA, NAA, BA, or kinetin), nitrogen, and sucrose on cell

growth, saponin production, and nutrient utilization. The maximum biomass yield was obtained in the medium containing 2,4-D. However, saponin production was much higher in media supplemented with either IBA or NAA (Table 1). Based on these results, we determined that 7.0 mg/L IBA was the best for promoting both cell growth (10.0 g/L dry weight) and saponin production (7.29 mg/g DW total ginsenoside). Although the addition of cytokinins did not affect cell growth, saponin productivity (particularly in the Rb group) was increased when the medium was supplemented with either 0.5 mg/L BA or 0.5 mg/L kinetin (Table 2). These results are quite interesting because, until now, almost all reports from research on suspension cultures of ginseng cells have stated that 2,4-D is an absolutely critical component, even though this compound is a potent herbicide and possible carcinogen, and, thus, is unsuitable for pharmaceutical and food industries (Choi et al., 1994b). Therefore, we have demonstrated here that our system can be used successfully for commercial saponin production without the addition of 2,4-D.

Figure 1 summarizes the effects of Na^+, NH_4^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, NO_3^-, $H_2PO_3^-$, and SO_4^{2-} when different concentrations of auxins were added to our media. Ion utilization was much greater in cultures that had relatively rapid growth rates and high saponin productivity (i.e., at 8 mg/L IBA), except for $H_2PO_3^-$ or SO_4^{2-}. These results indicate that auxin not only influences cell growth and secondary metabolite production, but also affects the exploitation of media nutrients by the cultured cells. We also observed a correlation or interaction between nutrient utilization and secondary metabolite production. Therefore, the information gained from this experiment will be helpful in further improving media formulations and process efficiency.

The effects of different MS media strengths on biomass and saponin production are shown in Table 3. Half- and full-strength media were equally suitable, whereas the 2.0 medium, with a higher salt strength, inhibited both cell growth and ginsenoside productivity. Such a phenomenon has also been described for cultures of adventitious roots from mountain ginseng (Yu

Table 1. Effect of different auxins on cell growth and ginsenoside production in mountain ginseng after 3 weeks of cell-suspension culture.

Auxin (mg/L)		Fresh wt. (g/L)	Dry wt. (g/L)	Ginsenoside (mg/g DW)			Ginsenoside productivity (mg/L)		
				Rg	Rb	Total	Rg	Rb	Total
2,4-D	1	328.5 a[z]	11.9 a	1.81	2.35	4.16	21.6	28.1	49.7
IBA	1	144.5 d	7.5 d	2.16	3.05	5.21	16.2	22.9	39.1
	3	170.0 c	8.8 cd	2.09	3.49	5.58	18.4	30.7	49.1
	5	178.0 c	9.1 c	2.43	3.31	5.74	22.1	30.1	52.2
	7	216.5 b	10.0 b	2.60	4.69	7.29	26.0	46.9	72.9
	9	226.5 b	10.5 b	1.42	4.22	5.64	14.9	44.3	59.2
NAA	1	132.0 d	7.0 e	2.85	5.33	8.18	20.0	37.3	57.3
	3	134.0 d	7.3 de	3.28	5.48	8.76	23.9	40.0	63.9
	5	152.5 cd	7.8 d	2.61	4.83	7.44	20.4	37.7	58.0
	7	164.5 c	7.6 cd	2.16	4.08	6.24	16.4	31.0	47.4
	9	188.5 c	9.7 b	2.16	2.45	4.61	21.0	23.8	44.7

[z]Mean separation by Duncan's multiple range test at $P \leq 0.05$. Values followed by the same letter within a column are not significantly different.

Table 2. Effect of different cytokinins (plus 7 mg/L IBA) on cell growth and ginsenoside production in mountain ginseng after 3 weeks of cell-suspension culture.

Cytokinin (mg/L)		Fresh wt. (g/L)	Dry wt. (g/L)	Ginsenoside (mg/g DW)			Ginsenoside productivity (mg/L)		
				Rg	Rb	Total	Rg	Rb	Total
	0	192.0 b[z]	10.0 b	1.88	2.78	4.65	18.7	27.7	46.4
BA	0.1	230.5 a	11.0 ab	1.81	2.75	4.56	20.2	30.7	50.9
	0.5	252.0 a	11.5 a	1.75	5.33	7.08	20.5	62.2	82.7
	1.0	242.0 a	11.0 b	1.79	3.53	5.33	20.4	40.1	60.5
Kinetin	0.1	224.0 ab	11.1 ab	1.16	4.61	5.76	12.7	50.5	63.2
	0.5	240.5 a	11.7 a	1.49	5.85	7.34	17.3	67.5	84.8
	1.0	242.0 a	11.4 a	1.56	3.51	5.07	17.2	38.7	55.8

[z]Mean separation by Duncan's multiple range test at $P \leq 0.05$. Values followed by the same letter within a column are not significantly different.

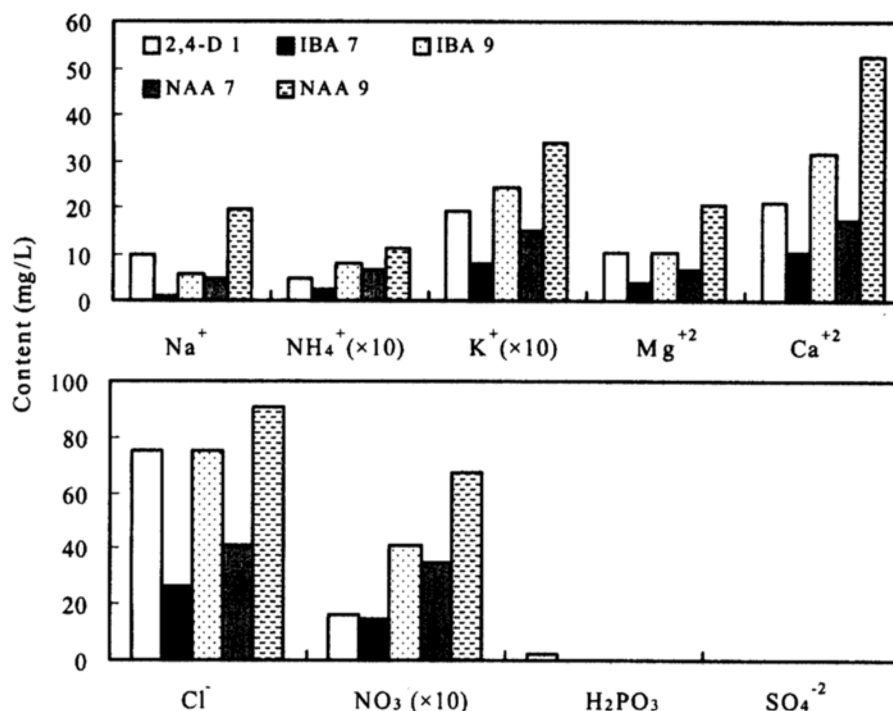

Figure 1. Changes in mineral nutrients in the medium as affected by different auxin treatments of mountain ginseng after 3 weeks of cell-suspension culture.

et al., 2000).

The culture medium that was supplemented with 30 g/L sucrose showed enhanced biomass yield (fresh weight 180.6 g/L; dry weight, 30 g/L) and saponin content (total ginsenoside productivity of up to 70.8 mg/L). However, further increases in concentration (i.e., up to 70g/L) led to decreases in both saponin accumulation and biomass production (Table 4). In

Table 3. Effect of different sucrose concentrations (MS + 7 mg/L IBA + 0.5 mg/L kinetin) on cell growth and ginsenoside production in mountain ginseng after 3 weeks of cell-suspension culture.

Sucrose concentration (g/L)	Fresh wt. (g/L)	Dry wt. (g/L)	Ginsenoside (mg/g DW)			Ginsenoside productivity (mg/L)		
			Rg	Rb	Total	Rg	Rb	Total
10	26.6 d[z]	2.9 d	0.32	0.62	0.92	0.9	1.8	2.7
30	180.6 a	10.8 a	2.17	4.39	6.56	23.4	47.4	70.8
50	98.2 b	8.4 b	1.06	2.23	3.29	8.9	18.7	27.6
70	52.0 c	5.7 c	0.08	1.56	1.64	0.5	8.9	9.3

[z]Mean separation by Duncan's multiple range test at $P \leq 0.05$.

Table 4. Effect of different strengths of MS media (7 mg/L IBA + 0.5 mg/L kinetin) on cell growth and ginsenoside production in mountain ginseng after 3 weeks of cell-suspension culture.

MS medium strength	Fresh wt. (g/L)	Dry wt. (g/L)	Ginsenoside (mg/g DW)			Ginsenoside productivity (mg/L)		
			Rg	Rb	Total	Rg	Rb	Total
0.5	224.3 a[z]	9.9 a	1.49	4.83	6.32	14.7	47.7	62.4
1	184.0 b	10.1 a	2.25	4.44	6.69	22.7	44.8	67.5
1.5	150.1 c	9.4 a	0.94	3.71	4.65	8.8	34.7	43.5
2	96.2 d	6.8 b	0.43	3.41	3.84	2.9	23.2	26.1

[z]Mean separation by Duncan's multiple range test at $P \leq 0.05$. Values followed by the same letter within a column are not significantly different.

Table 5. Effect of different nitrogen concentrations in MS media (7 mg/L IBA + 0.5 mg/L kinetin) on cell growth and ginsenoside production in mountain ginseng after 3 weeks of cell-suspension culture.

Nitrogen concentration in MS medium (mM)	Fresh wt. (g/L)	Dry wt. (g/L)	Ginsenoside (mg/g DW)			Ginsenoside productivity (mg/L)		
			Rg	Rb	Total	Rg	Rb	Total
30	206.1 a[z]	11.6 a	2.49	8.24	10.73	28.9	95.6	124.5
60	180.6 b	10.8 a	2.17	4.48	6.64	23.4	48.4	71.8
90	112.4 c	8.1 b	0.60	4.11	4.71	4.9	33.3	38.2
120	75.8 d	6.1 d	0.03	4.52	4.55	0.2	25.6	27.8

[z]Mean separation by Duncan's multiple range test at P ≤ 0.05. Values followed by the same letter within a column are not significantly different.

Table 6. Effect of different NH_4^+/NO_3^- ratios in MS media (7 mg/L IBA + 0.5 mg/L kinetin) on cell growth and ginsenoside production in mountain ginseng after 3 weeks of cell-suspension culture.

NH_4^+/NO_3^- ratio in MS medium (mM)	Fresh wt. (g/L)	Dry wt. (g/L)	Ginsenoside (mg/g DW)			Ginsenoside productivity (mg/L)		
			Rg	Rb	Total	Rg	Rb	Total
0/30	150.5 d[z]	11.3 a	3.40	10.66	14.06	38.4	120.5	158.9
5/25	176.2 c	10.5 a	3.47	8.90	12.37	36.4	93.5	129.9
10/20	238.0 b	10.9 a	2.48	7.75	10.23	27.0	84.5	111.5
15/15	292.1 a	11.2 a	0.30	6.65	6.95	3.4	74.5	77.8
20/10	236.0 b	10.8 a	0.30	6.06	6.36	3.2	65.4	68.7
25/ 5	162.5 d	7.6 b	0.10	1.63	1.73	0.8	12.4	13.1
30/ 0	94.3 e	4.8 d	0.08	1.62	1.70	0.4	7.8	8.2

[z]Mean separation by Duncan's multiple range test at $P \leq 0.05$. Values followed by the same letter within a column are not significantly different.

contrast to these results, other research has indicated that a relatively high sucrose level is beneficial to the synthesis of secondary metabolites (Akalezi et al., 1999). Likewise, Weselake et al. (1998) have reported that the triacylglycerol content of oilseed rape cells could be increased about 8-fold (fresh-weight basis) when the sucrose concentration in the growth medium was raised from 2 to 22% (w/v). Choi et al. (1994a, 1994b) also found that the optimum concentration was 30 to 50 g/L, but that 70 g/L sucrose inhibited cell growth. In that same study, saponin content steadily increased with sucrose levels of up to 60 g/L. Therefore, we conclude that these conflicting results suggest that the increased production of secondary metabolites due to sucrose is not a general phenomenon, and that this response depends upon plant species.

We also studied the effect of total initial nitrogen (30, 60, 90, or 120 mM) on cell culture, using an NH_4^-/NO_3^+ ratio of 2:1 (Table 5). Based on the kinetics (i.e., fresh and dry weights), it is apparent that growth was inhibited at the high initial concentration of 120 mM. Growth and saponin productivity were maximized by an initial N concentration of 30 mM. Therefore, we propose that nitrogen significantly affects cell growth and the accumulation of saponin in suspension cultures of mountain ginseng.

The effect of the NH_4^-/NO_3^+ ratio was investigated, using a total initial N level of 30 mM (Table 6). Final dry cell weights were similar overall, except for relatively low levels at ratios of 25:5 or 30:0. In fact, when ammonium was the sole N source (i.e., 30:0), the cells scarcely grew; high ammonium concentrations also had an inhibitory effect. Cell growth was best at a ratio of 1:1, whereas saponin production generally was improved in media with high NH_4^-/NO_3^+ ratios. When nitrate was the sole N source, saponin production was the highest, but the use of ammonium as the sole source was unfavorable to saponin biosynthesis. Similar results have been found in suspension-culture studies of *Panax quinquefolium* (Zhong et al., 1996; Zhong and Wang, 1998).

ACKNOWLEDGEMENTS

This work was supported by the Korea Science and Engineering Foundation (KOSEF), through the Research Center for the Development of Advanced Horticultural Technology at Chungbuk National University, Cheongju, 361-763, Korea.

Received July 18, 2002; accepted September 24, 2002.

LITERATURE CITED

Akalezi CO, Liu S, Li QS, Yu JT, Zhong JJ (1999) Combined effects of initial sucrose concentration and inoculum size on cell growth and ginseng saponin production by suspension cultures of *Panax ginseng*. Pro Biochem 34: 639-642

Bourgaud F, Gravot A, Milesi S, Gontier E (2001) Production of plant secondary metabolites: A historical perspective. Plant Sci 161: 839-851

Choi KT, Ahn IO, Park JC (1994a) Production of ginseng saponin in tissue culture of ginseng (*Panax ginseng* C.A. Mayer). Russ J Plant Physiol 40: 784-788

Choi KT, Lee CH, Ahn IO, Lee JH, Park JC (1994b) Characteristics of the growth and ginsenosides in the suspension-culture cells of Korean ginseng (*Panax ginseng* C. A. Mayer), *In* WG Bailey, C Whitehead, JTA Proctor, JT Kyle, eds, Proc Intl Ginseng Conf, Vancouver, BC, Canada, pp 259-268

Ding J (1998) Suspension culture of ginseng cells. Acta Plant Physiol I: 76-78

Dornenburg H, Knorr D (1995) Strategies for the improvement of secondary metabolite production in plant cell cultures. Enzyme Microb Technol 17: 674-684

Furuya, T, Yoshikawa T (1987) Saponin production by cultures of *Panax ginseng* transformed with *Agrobacterium rhizogenes*. Plant Cell Rep 6: 449-453

Hibino K, Ushiyama K (1998) Commercial production of ginseng by plant cell culture technology, *In* TJ Fu, G Singh, WR Curtis, eds, Plant Cell Culture for the Production of Food Ingredients, Proc ACS Symp, San Francisco, CA, USA, Plenum, New York, pp 13-17

Huang KC (1993) The Pharmacology of Chinese Herbs. CRC Press, Boca Raton, FL, USA, pp 21-45

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue. Physiol Plant 15: 473-497

Tabata M, Fujita T (1985) Producing of shikonin by plant cell cultures, *In* M Zaitlin, P Day, A Hollaender, eds, Biotechnology in Plant Science: Relevance to Agriculture in the Eighties. Academic Press, New York, pp 207-218

Tang W, Eisenbrand G (1992) *Panax ginseng* C. A. Mayer, Chinese Drugs of Plant Origin. Springer-Verlag, Berlin, pp 710-737

Weselake RJ, Byers SD, Davoren JM, Laroche A, Hodges DM, Pomeroy MK, Furukawa-Stoffer TL (1998) Triacylglycerol biosynthesis and gene expression in microspore-derived cell suspension cultures of oilseed rape. J Exp Bot 49: 33-39

William A, John G, Hendel J (1996) Reversed-phase high-performance liquid chromatographic determination of ginsenosides of *Panax quinquefolium*. J Chromatogr 775: 11-17

Wu JY, Zhong JJ (1999) Production of ginseng and its bioactive components in plant cell culture: Current technological and applied aspects. J Biotech 68: 89-99

Yu KW, Hahn EJ, Paek KY (2000) Production of adventitious ginseng roots using bioreactors. Kor J Plant Tiss Cult 27: 309-315

Zhong JJ, Bai Y, Wang SJ (1996) Effects of plant growth regulators on cell growth and ginsenoside saponin production by suspension cultures of *Panax quinquefolium*. J Biotech 45: 227-234

Zhong JJ, Wang SJ (1998) Effects of nitrogen source on the production of ginseng saponin and polysaccharide by cell cultures of *Panax quinquefolium*. Pro Biochem 33: 671-675