Ginsenoside Production by Hairy Root Cultures of *Panax ginseng* C.A. Meyer in Bioreactors

K.W. Yu, E.J. Hahn and K.Y. Paek
Research Center for the Development of Advanced Horticultural Technology,
Chungbuk National University, Cheongju, Chungbuk 361-763, Korea

Keywords: *Agrobacterium rhizogenes*, elicitors, jasmonic acid, Rb group, Rg group

Abstract

Hairy roots infected with *Agrobacterium rhizogenes* were induced from roots, stems, and leaves of Korean ginseng (*Panax ginseng* C.A. Meyer) on half strength MS medium supplemented with 300 mg·L^{-1} Cefotaxim sodium. DNA extraction was carried out and PCR results confirmed that the hairy roots induced by *A. rhizogenes* KCTC 2703 have the *rol* C gene in their T-DNA. Selected root lines were propagated in 5-litre cone type bubble bioreactors containing MS media supplemented with 2.0 mg·L^{-1} NAA and 30 mg·L^{-1} sucrose. To increase ginsenoside content, jasmonic acid in various concentrations was added to the culture medium after 30 days of culture and the roots were then cultured for 7 more days until harvest. Total ginsenoside content increased with increasing jasmonic acid concentration, but high concentrations inhibited the root growth. Ginsenosid productivity was greatest at 2.0 mg·L^{-1} jasmonic acid. On the other hand, ginsenosides in the Rb group mainly increased, while those in the Rg group did not. Particularly, high concentrations (5 and 10 mg·L^{-1}) of jasmonic acid decreased Rg1 content to almost half of that in the control, but significantly increased the Rb1 content of the Rb group. Among the ginsenosides in the Rb group, the Rb1 content increased more than Rb2, Rc, and Rd. There were gradual increases in ginsenosides in the Rb group, but those in the Rg group fluctuated then slightly decreased at the end of the culture period. Further studies are required to raise the contents of ginsenosides in the Rg group and to determine the optimal type of elicitors as well as optimal time for the elicitor treatment for accelerating ginsenoside production.

INTRODUCTION

Panax ginseng C.A. Meyer belongs to the Araliaceae family and is traditionally considered one of the most potent medicinal plants in the Orient, where it has been used for centuries as a health tonic. The most important active component of ginseng roots is ginsenoside and more than 20 different ginsenosides have been identified (Lee et al., 1995). The demand for ginseng roots and its extracts has been increasing but cultivated ginseng roots are expensive since seeding is still a principal propagation method, which takes about 4 to 6 years to harvest. Disease control remains the central problem in commercial cultivation of ginseng. Consequently, the commercial cultivation of ginseng has required the application of pesticides, which resulted in the serious problem of pesticide residues (Yu and Ohh, 1995). Another major problem in ginseng cultivation is replant diseases. Due to the problems, traditional cultivation cannot meet the increasing demand of the ginseng market, especially that for ginsenosides.

There have been alternative approaches for ginsenoside production through cell culture and tissue culture technology and there are many reports on ginsenoside production in cell cultures by controlling growth regulators (Zhong et al., 1996), the nitrogen source (Zhong and Wang, 1998), sucrose concentration and inoculum cell size (Akalezi et al., 1999). From the view of biomass production, on the other hand, root culture is more efficient compared to cell culture due to fast growth and stable metabolite productivity (Carvalho and Curtis, 1998). For commercial production of ginsenoside, adventitious roots were cultured in large-scale bioreactors and the ginsenoside profiles of the adventitious roots were similar to those of field-grown ginseng roots (Seon et al., 1999; Son et al, 1999). Hairy roots infected by *Agrobacterium rhizogenes* also produce

the same secondary metabolite as those synthesized in intact parent plant roots with similar or higher yields (Zehra et al., 1999). However, the ginsenoside content of the roots remains low and should be increased (Yoshimatsu et al., 1996; Pinol et al., 1999).

A wide variety of elicitors has been employed to increase the content of secondary metabolites in plant cell cultures (Dornenburg and Knorr, 1995; Chang et al., 1998) and we found jasmonic acid to be efficient for ginsenoside production (Yu et al., 2000a). Apart from elicitors, there are many factors that affect the content of secondary metabolites, e.g., medium components, growth regulators, carbon source, medium pH, and air temperature. In this experiment, concentrations of sucrose, benzyl adenine (BA), and jasmonic acid were varied at different levels in ginseng hairy root cultures to determine their effects on biomass increase and ginsenoside production.

MATERIALS AND METHODS

Establishment of Hairy Root Culture

Roots, stems, and leaves of ginseng were sterilized, cut into 5 mm lengths and then placed on NS solid medium (Nutrient broth, DIFCO) infected with *Agrobacterium rhizogenes* (KCTC 2703, 2704, and 2743), where they were kept overnight. Explants were transferred to half strength MS (Murashige and Skoog, 1962) media supplemented with 300 $mg \cdot L^{-1}$ Cefotaxim sodium to induce roots. After root induction, DNA extraction was carried out by the method reported by White and Sinkar (1987). The PCR results were checked by agarose gel electrophoresis with marker Hind-ш-digested-λDNA, which confirmed that ginseng hairy roots induced by *A. rhizogenes* KCTC 2703 (ATCC 15383) had the *rol* C gene in its T-DNA. Root lines were selected according to root growth and ginsenoside content and suspension cultured in 400 mL conical flasks containing 100 mL half strength MS medium with 3% sucrose at 23±2 ºC in darkness.

Experiment on Sucrose Concentration

Two grams of hairy roots were inoculated in a 400 mL conical flask containing 100 mL of half strength MS medium, in which sucrose concentrations were varied at 1, 2, 3, 5, 7, and 9%. Cultures were maintained at 23±2 ºC in darkness. Biomass increase, ginsenoside content, and ginsenoside productivity were investigated after 5 weeks of culture.

Experiment on BA Concentration

Forty grams of hairy roots were inoculated in a 10 L drum type airlift bioreactor containing 8 L of half strength MS medium supplemented with 30 $mg \cdot L^{-1}$ sucrose. BA concentrations were varied at 0, 1, and 3 $mg \cdot L^{-1}$ and the volume of the input air was adjusted to 0.1 vvm. Culture conditions and the procedure used were the same as in the experiment on sucrose.

Experiment on Jasmonic Acid Concentration

Twenty grams of hairy roots were inoculated in a 5 L drum type airlift bioreactor containing 4 L of half strength MS medium supplemented with 30 $mg \cdot L^{-1}$ sucrose. The roots were treated with different jasmonic acid concentrations: 0, 1, 2, and 5 $mg \cdot L^{-1}$ 7 days before harvest. Culture conditions and the procedure used were the same as in the experiment on sucrose.

RESULTS AND DISCUSSION

Sucrose concentrations affected root growth and ginsenoside content in different ways. Dry weight and percentage dry weight increased with increasing sucrose concentration but ginsenoside production was promoted by low sucrose concentrations (2-5%), while high sucrose concentrations (7-9%) inhibited ginsenoside production (Table 1). One % sucrose, on the other hand, inhibited both root growth and ginsenoside production. The maximum ginsenoside content (8.01 $mg \cdot g^{-1} \cdot dw$) as well as the maximum

ginsenoside productivity was obtained with 2% sucrose (Table 1). Sucrose concentrations of 1, 2, and 3% were favorable for both ginsenoside Rg group and Rb group synthesis. Among ginsenosides, the contents of Rg, Rb1, Rb2, and Rd decreased more evidently than other ginsenosides at high sucrose concentrations (data not shown). These results indicated that a two stage culture system is necessary for ginseng hairy root culture: higher sucrose concentration during the growth stage and a relatively low concentration of sucrose during the ginsenoside production stage. Sucrose concentration also influenced the contents of reducing sugar, total sugar, and starch (Fig. 1). Increasing sucrose concentration resulted in higher contents of reducing sugar, total sugar, and starch in the hairy roots. The result suggested that the amount of sucrose absorbed and assimilated by hairy roots is closely related to sucrose concentration in the culture medium (Yu et al., 2000a).

Table 2 clearly shows that BA strongly inhibited hairy root growth and ginsenoside production. Hairy root biomass, growth rate, ginsenoside content, and ginsenoside productivity decreased with increasing BA concentration. Addition of BA in the culture medium resulted in thicker and yellow-coloured roots. Unlike adventitious roots, hairy roots have a hormone-synthesis gene and it can auto-synthesize auxins and cytokinins, which means the inate physiological condition of hairy roots is different from that of adventitious roots. The inhibitory role of growth regulators in secondary metabolite production has been demonstrated by the result of transgenic cell cultures of *Catharanthus roseus*: the presence of NAA during the production phase led to lower levels of alkaloid accumulation (Aerts et al., 1994) and 2,4-D in the culture medium also reduced culture aggregation and repressed secondary metabolism (Whitmer et al., 1998). In hairy root cultures of *Bupleurum falcatum*, IAA and IBA showed a potent inhibition effect on saikosaponin biosynthesis with treatments of 0.01 to 5 $mg \cdot L^{-1}$, while growth rate increased by 60% by treatment with 0.5 $mg \cdot L^{-1}$ IBA (Ahn et al., 1999). Plant growth regulators are an important factor affecting hairy root cultures since the relationship between exogenous and endogenous growth regulators is very complex, and therefore, further studies are required.

The effect of jasmonic acid on the accumulation of ginsenoside in hairy roots was highly significant. The higher the jasmonic acid concentration, the higher the ginsenoside content and ginsenoside productivity (Table 3). Ginsenoside productivity increased about 2 fold and total ginsenoside content about 4 times with the treatment of 5.0 $mg \cdot L^{-1}$ of jasmonic acid. Accumulation of the ginsenoside Rb group was more significant in comparison with the ginsenoside Rg group (Table 4). Unlike ginsenoside content, root growth was inhibited by jasmonic acid. Among ginsenosides, Rb1, Rb2, Rc, and Rd contents increased more significantly than other kinds of ginsenosides. Similar results were obtained in adventitious root cultures of *Panax ginseng* (Yu et al., 2000b). The effect of jasmonic acid on secondary metabolite synthesis has been confirmed by many reports. Activation of monoterpenic indole alkaloid biosynthesis by jasmonate was reported in cell suspension cultures of *Rauwolfia canescens* (Gundlach et al., 1992), and in *Catharanthus roseus* and *Cinchona ledgeriana* seedlings (Aerts et al., 1994). In *C. roseus* suspension cells cultured in 2,4-D-starved medium, exogenous jasmonic acid greatly increased alkaloid production (Gantet et al., 1998). Elicitor-induced accumulation of secondary metabolites has received much attention during the past decade. There are four groups of elicitors (Eiler, 1987), but the elicitors that have been used in ginseng root cultures belong mostly to the abiotic group. Therefore, further experiments are necessary with elicitors in other groups.

Literature Cited

Aerts, R.J., Gisi, D., De Carolis, E., De Luca, V. and Baumann, T.W. 1994. Methyl jasmonate vapor increases the developmentally controlled synthesis of alkaloids in *Catharanthus* and *Cinchona* seedlings. Plant J. 5:635-643.

Ahn, J.C., Kim, E.S., Lee, H.J. and Hwang, B. 1999. Effect of IAA, IBA, and media on growth and saikosaponin biosynthesis in *Bupleurum falcatum* hairy root culture. Kor.

J. Plant Tiss. Cult. 26:171-175.
Akalezi, C.O., Liu, S., Li, Q.S., Yu, J.T. and Zhong, J.J. 1999. Combined effects of initial sucrose concentration and inoculum size on cell growth and ginseng saponin production by suspension cultures of *Panax ginseng*. Process Biochemistry 34:639-642.
Carvalho, E.B. and Curtis, W.R. 1998. Characterization of fluid flow resistance in root cultures with a convective flow tubular bioreactor. Biotechol. Bioeng. 60:375-384.
Chang, J.H., Shin, J.H., Chung, I.S. and Lee, H.J. 1998. Improved menthol production from chitosan-elicited suspension culture of *Mentha piperita*. Biotech. Lett. 20:1097-1099.
Dornenburg, H. and Knorr, D. 1995. Strategies for the improvement of secondary metabolite production in plant cell cultures. Enzyme Microb. Technol. 17:674-684.
Eiler, U. 1987. Elicitation:methodology and aspects of application. In:Constabel F, Vasil IK (eds), Cell Culture and Somatic Cell Genetics of Plants, Academic, San Diego Vol. 4:153-196.
Gantet, P., Imbault, N., Thiersault, M. and Doireau, P. 1998. Necessity of a functional octadecanoic pathway for indole alkaloid synthesis by *Catharanthus roseus* cell suspensions cultured in an auxin-starved medium. Plant Cell Physiol. 39:220-225.
Lee, H.S., Kim, S.W., Lee, K.W., Eriksson, T. and Liu, J.R. 1995. *Agrobacterium*-mediated transformation of ginseng (*Panax ginseng*) and mitotic stability of the inserted beta-glucuronidase gene in regenerates from isolated protoplasts. Plant Cell Rep. 14:545-549.
Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with plant tissue cultures. Physiol. Plant. 15:473-497.
Pinol, M.T., Palazon, J., Cusido, R.M. and Ribo, M. 1999. Influence of calcium ion concentration in the medium on tropane alkaloid accumulation in *Datura stramonium* hairy roots. Plant Sci. 141:41-49.
Seon, J.H., Yu, K.W., Cui, Y.Y., Kim, M.H., Lee, S.J., Son, S.H. and Paek, K.Y. 1999. Application of bioreactor for the production of saponin by adventitious root cultures of *Panax ginseng*. In:Altman, A. (ed.), Plant biotechnology and *in vitro* biology in the 21st century. Dordrecht, The Netherlands, Kluwer Academic. pp 329-332.
Son, S.H., Choi, S.M., Kwon, S.R., Lee, Y.H. and Paek, K.Y. 1999. Large-scale culture of plant cell and tissue by bioreactor system. J. Plant Biotech. 1:1-8.
White, F.F. and Sinkar, V.P. 1987. Molecular analysis of root induction by *Agrobacterium rhizogenes*. In:Denberra ES, Hohn B, Hohn T, King PJ, Schell J, Verma DPS (eds), Plant Gene Research, Basic Knowledge and Application, Springer-Verlag, Wien, New York. pp 149-177.
Whitmer, S., Verpoorte, R. and Canel, C. 1998. Influence of auxins on alkaloid accumulation by a transgenic cell line of *Catharanthus roseus*. Plant Cell Tiss. Org. Cult. 53:135-141.
Yoshimatsu, K., Yamaguchi, H. and Shimomura, K. 1996. Traits of Panax ginseng hairy roots after cold storage and cryopreservation. Plant Cell Rep. 15:555-560.
Yu, K.W., Gao, W.Y., Son, S.H. and Paek, K.Y. 2000a. Improvement of ginsenoside production by jasmonic acid and some other elicitors in hairy root culture of ginseng (*Panax ginseng* C.A. Meyer). In Vitro Cell. Dev. Biol. 36:424-428.
Yu, K.W., Hahn, E.J. and Paek, K.Y. 2000b. Production of adventitious ginseng roots using bioreactors. J. Plant Biotech. 27:309-315.
Yu, Y.H. and Ohh, S.H. 1995. Problems and present status of research on ginseng diseases in Korea. In: Bailey, W.G., Whitehead, C., Proctor, J.T.A. and Kyle, J.T. (eds.). Proc Int Ginseng Conf Vancouver 1994, Canada. pp 120-130.
Zehra, M, Banerjee, S., Sharma, S. and Kumar, S. 1999. Influence of *Agrobacterium rhizogenes* strains on biomass and alkaloid productivity in hairy root lines of *Hyoscyamus muticus* and *H. albus*. Planta Medica. 65:60-63.
Zhong, J.J. and Wang, S.J. 1998. Effects of nitrogen source on the production of ginseng saponin and polysaccharide by cell cultures of *Panax quinquefolium*. Process

Biochemistry 33:671-675.
Zhong, J.J., Bai, Y. and Wang, D.J. 1996. Effect of plant growth regulators on cell growth and ginsenoside saponin production by suspension cultures of *Panax quinquefolium*. J. Biotech. 45:227-234.

Tables

Table 1. Effects of sucrose concentration on hairy root growth and ginsenoside production. Roots were cultured in 400 mL conical flasks for 5 weeks.

Sucrose (%)	Root growth			Ginsenoside (mg·g^{-1} DW)			Ginsenoside productivity (mg·L^{-1})
	Fresh weight (g)	Dry weight (g)	Growth yield	Rb group[z]	Rg group[y]	Total	
1	15.4±0.04	0.69±0.01	4.14	4.13 b	3.00 a	7.13 a	49.20
2	21.5±0.50	1.30±0.22	7.74	5.22 a	2.80 a	8.01 a	104.13
3	23.3±0.40	1.31±0.01	7.80	5.30 a	3.20 a	6.53 b	85.54
5	22.5±0.03	1.83±0.01	10.92	3.28 b	1.70 b	4.98 b	91.13
7	21.1±0.80	1.90±0.05	8.60	1.56 c	1.12 c	2.67 c	50.73
9	19.8±0.05	2.09±0.06	10.56	0.74 d	1.02 c	1.77 d	36.99

[z]The amount of Rb group was calculated as the total ginsenoside Rb1, Rb2, Rc, and Rd, having protopanaxadiol as saponins.
[y]The amount of Rg group was calculated as the total ginsenoside Rg1, Re, and Rf, having protopanaxadiol as saponins.

Table 2. Effects of benzyl adenine (BA) on hairy root growth and ginsenoside production. Roots were cultured in 10 L drum type airlift biorectors for 5 weeks.

BA (mg·L^{-1})	Root growth			Total ginsenoside content (mg·g^{-1} DW)	Ginsenoside productivity (mg·L^{-1})
	Fresh weight[z] (g)	Dry weight[y] (g)	Growth yield		
0	1670	109	76.8	14.65 a	199.6
1	1356	96	67.6	5.27 b	63.24
3	1200	81	57.0	5.33 b	53.97

[z,y]Not significant in Duncan's multiple range test.

Table 3. Effects of jasmonic acid on hairy root growth and ginsenoside production. Roots were cultured in 400 mL conical flasks for 5 weeks.

Jasmonic acid ($mg \cdot L^{-1}$)	Root growth			Ginsenoside ($mg \cdot g^{-1}$ DW)			Ginsenoside productivity ($mg \cdot L^{-1}$)
	Fresh weight (g)	Dry weight (g)	Growth yield	Rb group[z]	Rg group[y]	Total	
0	32.2 a[x]	1.52 a	7.12	10.31 d	5.51 a	15.85 d	240.92
1	24.5 b	1.31 b	6.12	30.08 c	5.87 a	35.98 c	471.34
2	20.0 c	1.08 c	5.04	41.59 b	6.05 a	47.69 b	515.05
5	14.1 d	0.86 d	4.04	52.98 a	5.60 a	58.65 a	504.39

[Z]The amount of Rb group was calculated as the total ginsenoside Rb1, Rb2, Rc, and Rd, having protopanaxadiol as saponins.
[y]The amount of Rg group was calculated as the total ginsenoside Rg1, Re, and Rf, having protopanaxadiol as saponins.
[X]mean separation within columns by Duncan's multiple range test, 5% level.

Table 4. Effects of jasmonic acid on ginsenoside Rb1, Rb 2, Rc, Rd, Rg1, Re, and Rf production in ginseng hairy roots after 5 weeks of culture.

Jasmonic acid ($mg \cdot L^{-1}$)	Ginsenoside ($mg \cdot g^{-1}$ DW)							Total
	Rb group				Rg group			
	Rb1	Rb2	Rc	Rd	Rg1	Re	Rf	
0	7.23 d[Z]	1.13 d	1.20 b	0.77c	1.63 ab	3.02 a	0.87 a	15.85 d
1	18.99 c	3.44 c	5.56 a	2.14 b	1.85 a	3.49 a	0.52 a	35.98 c
2	24.09 b	4.62 b	7.26 a	4.67 a	1.73 ab	3.69 a	0.63 a	47.69 b
5	33.70 a	8.80 a	6.19 a	4.31 a	1.34 b	3.74 a	0.61 a	58.65 a

[z]mean separation within columns by Duncan's multiple range test, 5% level.

Figures

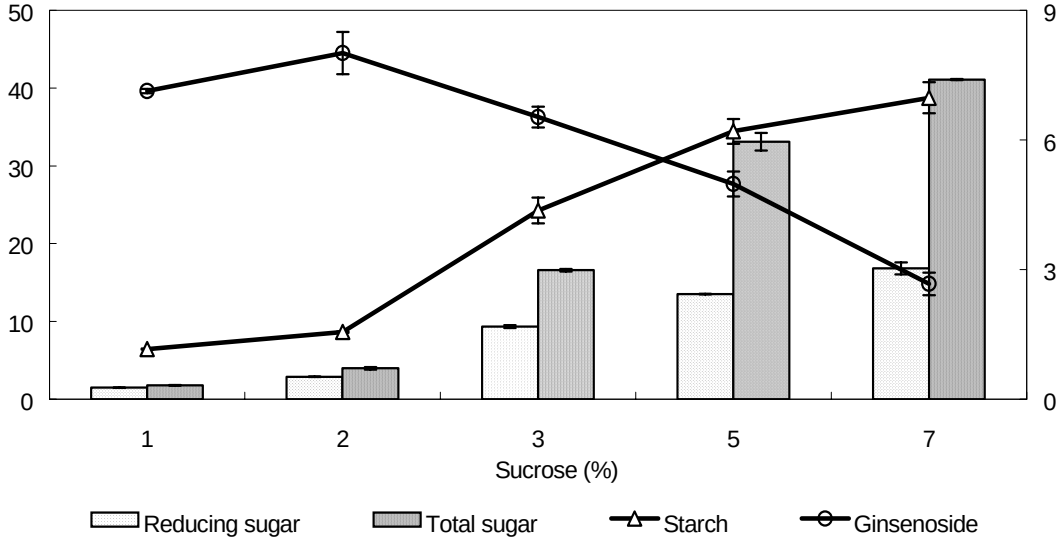

Fig. 1. Effects of sucrose concentration on ginsenoside ($mg \cdot g^{-1} \cdot dw$, right axis), starch (left axis), reducing sugar, and total sugar contents in hairy root culture of ginseng.