PRODUCTION OF GINSENOSIDES THROUGH *IN VITRO* CULTURE OF GINSENG(*Panax ginseng* C.A. MEYER)

K.T. Choi, I.O. Ahn and J.C. Park

Korea Ginseng and Tobacco Research Institute, Science Town, Taejon 304 - 345, Korea

ABSTRACTS

Ginseng root explants and calli induced from selected cell lines were cultured on modified Murashige and Skoog's media supplemented with different concentrations of organic or inorganic compounds and plant growth requlators to clarify the effects of chemical composition and plant growth regulators in the medium on the growth of ginseng calli and the production of ginseng saponin. For optimum growth of calli, the concentrations of 2, 4 - D and sucrose were the range of 1 to 3 ㎎/ℓ and 1 to 3%, respectively. And it was clarified that sucrose, nitrogen, phosphate, calcium, magmesian plant growth regulators and their concentrations influcenced the relative biosynthesis of saponin in tissue cultures of *Panax ginseng*. The patterns of ginsenosides, pharmacologically useful component, were different among the cell lines and contents of ginsenosides were much higher in selected cell lines than in original cell line.

INTRODUCTION

Ginseng is a perennial medicinal plant belonging to Araliaceae family and *Panax* genus, Ginseng seedlings grown on the nursery bed for about 17 months are transplanted during late in March and early in April in Korea. 4 - or 6 - year old roots are harvested and cultivation of the ginseng plant in the field requires 5 to 7 years. As the ginseng cultivation through the conventional methods is carried out in the field, it is subjected to the climatic condition. And ginseng cultivation works also require large facilities and manpower besides needing unusually long duration of years. Therefore, the production of one of the active components, ginseng saponin, by *in vitro* grown culture of *Panax ginseng* has been studied by a number of investigators[11-15,18].

Callus and cell suspension cultures have been established from somatic tissues of *Panax ginseng* C. A. Meyer. Tissues amenable to such culture include root[2-10,17,18], stem[2,7], cotyledon[7], hypocotyl and epicotyl[7]. Butenko *et al.*[2] first found conditions for sterilizing them, and determined the composition of nutrient medium, such as inorganic salts, carbohydrates, and physiologically active compounds, which fulfilled the requirements of ginseng root for growth in solid and liquid media in darkness and in light. In general, the growth of ginseng calli on solid agar medium is rapid. From a piece of tissue weighing 100 - 120 ㎎, one obtains 1 - 2 g of tissue after 1 month of culture. The productivity of ginseng calli cultures is considerably higher than that of cultivated plants. Furuya *et al.*[11-15] isolated the sapogenin, panaxatriol, from *Panax ginseng* callus and in 1973 isolated panaxadiol and oleanolic acid from *Panax ginseng* callus cultures. It is interesting that the kind and amount of saponins in ginseng callus are about the same as those in the ginseng root.

Therefore, this study was undertaken to establish sen seng tissue culture for the production of ginseng saponin.

MATERIALS AND METHODS

1. Induction of callus

Six - year old ginseng roots were surface - sterilized for 20 minutes in 2% sodium hypochlorite solution and rinsed 4 times with sterile distilled water. For the callus growth, the segments of root with cambial cells and two cell lines(PG1 and PG2) were cultured on the basal medium with different concentrations of plant growth regulators for 40 days. The basal medium used was a modified formulation from Murashige and Skoog and hereafter designated as MS medium. The modifications were : 0.5 ㎎ ℓ nicotinic acid, 0.5 ㎎ ℓ pyridoxine HCl, 0.1 ㎎ ℓ thiamine HCl, 100 ㎎ ℓ myo - inositol, 2 g ℓ glycine. The pH was adjusted to 5.8 before autoclaving. To clarify the effect of individual constituents on the growth of callus and the production of saponin, ammonium nitrate and potassium nitrate as nitrogen sources, potassium dihydrogen phosphate as phosphate source, magnesium sulfate as magnesium source, calcium chloride as calcium source, 2, 4 - D and kinetin as growth regulators, and sucrose as carbon source were tested in combination and in different concentrations. On 40 days after culture, the average fresh weight of callus in 100 ㎖ Erlenmyer flasks was determined. The callus harvested was dried in drying oven at 60℃.

2. Determination of saponin and ginsenosides

Saponin contents in dried callus were determined by the method of Ando *et al.*[1]. The last butanol layer was evaporated *in vacuo* to dryness until no butanol smell was detecta ble. The residue was weighed as crude saponin. And ginsenoside content was determined quantitatively by HPLC.

RESULTS AND DISCUSSION

1. Formation and growth of callus

Ginseng calli were usually obtained by culturing explants on a solid medium containing a high concentration of salts, organic constituents and high auxin, About 15 days after

culture, ginseng calli were visiblely arising at the injured cambial cells of the cut surface of explant. Regardless of the parts they arose, calli were formed more profusely on the media containing 2, 4-D. The tissue were soft and friable and comprised a wide variety of cell shapes and sizes. In general, a temperature of 25 to 27℃ is employed for *in vitro* culture. However, the ginseng callus grew vigorously and fast at 20℃, while at 15 and 25℃ it grew slowly.

Plant growth regulators, in intact plant, act to regulate and coordinate processes which lead to normal development. Growth, as well as differentiation of tissue and cells is affected by plant growth regulators. The addition of plant growth regulators to tissue culture medium is necessary for ginseng callus culture[7, 13]. The commonly used auxins are 2, 4-dichlorophenoxyacetic acid(2, 4-D), naphthaleneacetic acid (NAA), and indole-3-acetic acid(IAA). In *Panax ginseng* C.A. Meyer, 2, 4-D is the most effective auxin in both induction and growth of callus.

In order to determine the optimum levels of 2, 4-D for the induction of callus, the segments of root were inoculated on the Murashige and Skoog's basic medium supplemented with different concentrations of 2, 4-D.

The concentration of 2, 4-D for optimum growth of ginseng callus was in the range of 1 to 3 ㎎/ℓ(Fig. 1).

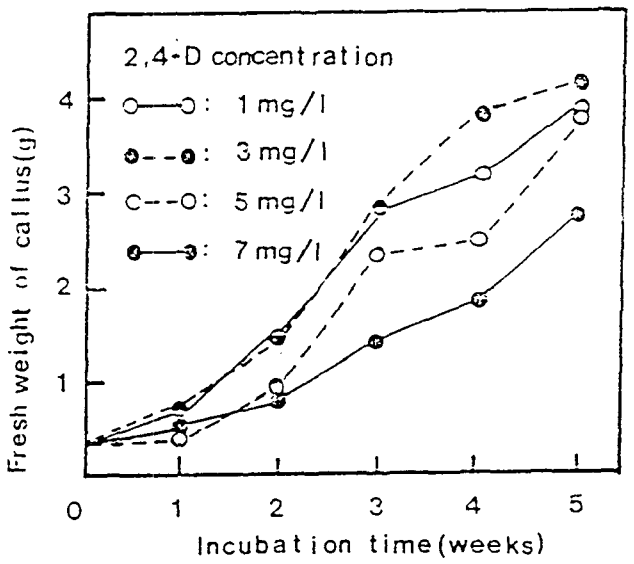

Fig. 1. Effect of 2, 4-D on the growth of ginseng callus.

The addition of an organic carbon source, such as sucrose, to ginseng tissue culture media is absolutely necessary for all tissues. Therefore, the requirement of sucrose for the growth of callus was tested in the presence of different concentrations of sucrose. Fig. 2 shows the fresh weights of calli cultured on the media supplemented with different concentrations of sucrose.

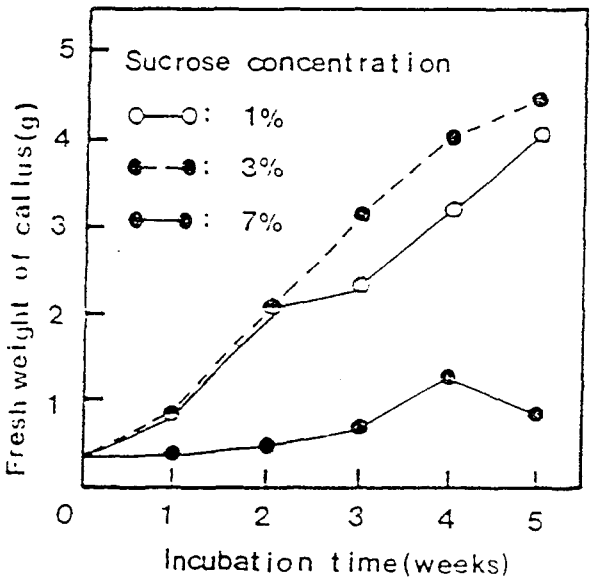

Fig. 2. Effect of sucrose on the growth of ginseng callus.

The concentration of sucrose for optimum growth of ginseng callus was in the range of 1 to 3%. Whereas higher concentration, 7% sucrose, caused growth retardation, the dry weights were greater than those where the concentration of sugar was lower.

Several medium formulations such as White, Murashige and Skoog, and Veliky proved to be successful in culture of ginseng somatic tissues. However, the most vigorous callus growth was obtained on MS medium(Table 1).

Table 1. Effects of basal media on the growth of ginseng callus at 25℃ in the darkness.

Media	Growth rate(%) a
White	1,150
Murashige & Skoog	1,297
Veliky	1,268

a : Gorwth rate $= \frac{\text{(final} - \text{initial) fresh wight}}{\text{initial fresh weight}} \times 100$

2. Production of saponin from ginseng callus cultures

During approximately two decades of study it has been demonstrated that plant tissue culture can produce many useful metabolites ; that cell strains can be selected for the high production of some alkaloids and pigments ; that some plant tissue cultures can be stored for long periods ; and that plant cells can be grown in fermentors. However, it remains to be solve that some compounds of interest are not produced in detectable or adequated amounts ; selected strains are not

always stable ; growth may be undesirably long and costly : and some compounds may be affected by culture conditions.

Therefore the effects of macro-elements, sucrose and plant growth regulators on the production of saponin through ginseng callus culture was tested in the presence of different concentrations of macro-elements and sucrose. Fig. 3 showed saponin contents of ginseng calli cultured on MS media containing different concentrations of nitrogen.

The saponin contents of callus was increased by raising nitrogen source in the media(Fig. 3) On the other hand, phosphorus and magnesium decreased the saponin contents. Namely saponin contents decreased when phosphorus and magnesium in the medium were raised from 0.5-fold to 1.5-fold of MS medium(Fig. 4 and 5) . Further increases in the total phosphorus and magnesium increased the formation of this compound. Fig. 6 shows the effect of calcium concentrations on the saponin synthesis of ginseng calli. The formation of saponin was inhibited when the concentration of calcium in the medium was less or greater than 1.5-fold of MS medium(Fig. 6).

The effects of the sucrose concentration on the yield of secondary products in plants cell cultures has been examined by several groups. For example, increasing sucrose 2 to 4% increased the polyphenols per culture in *Rosa sp.* suspension cultures[10], while increasing sucrose from 2 to 5% decreased

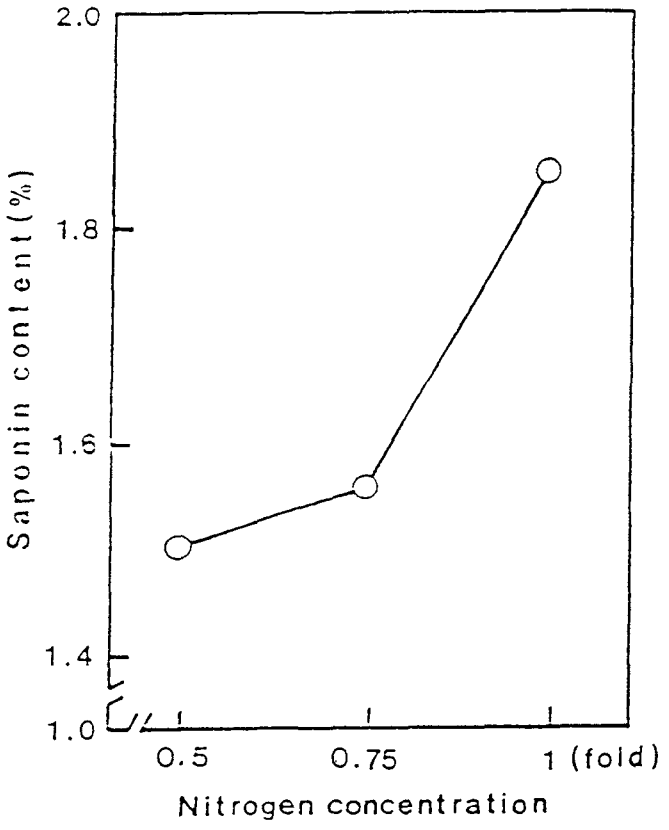

Fig. 3. Effect of nitrogen on the saponin content of ginseng callus.

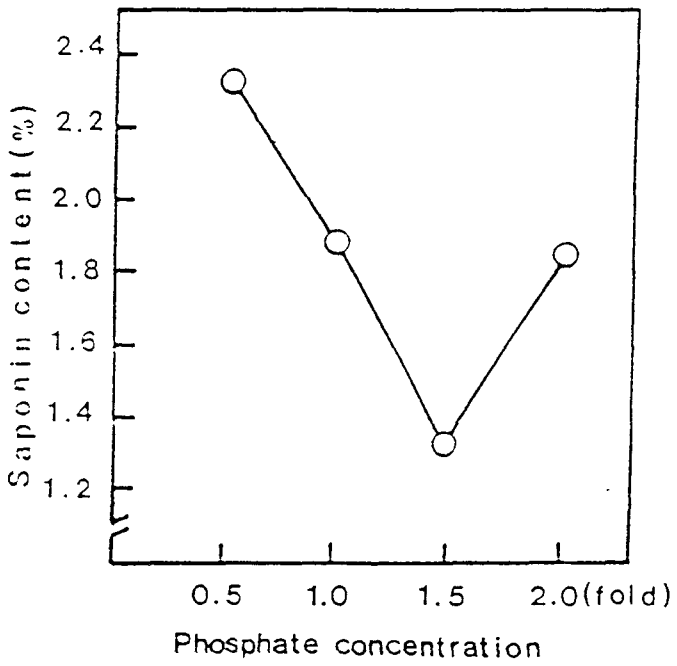

Fig. 4. Effect of phosphate on the saponin content of ginseng callus.

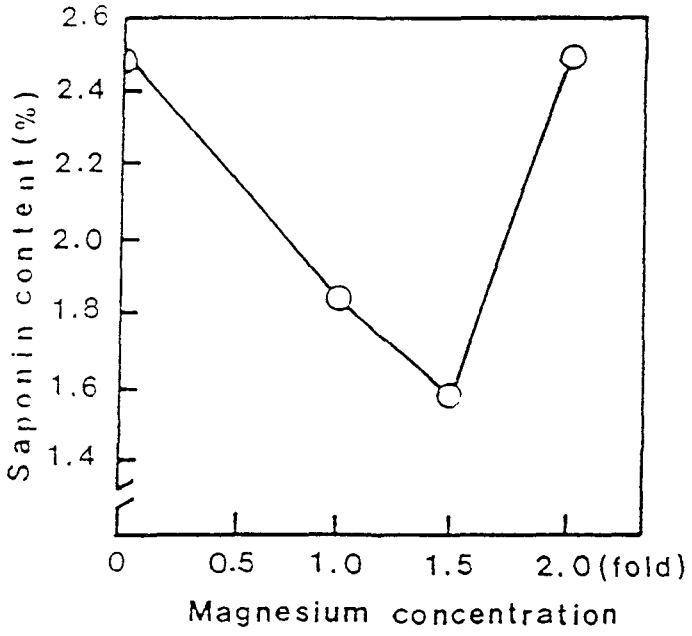

Fig. 5. Effect of magnesium on the saponin content of ginseng callus.

the ubiquinone per gram of dry cells in *Nicotiana tabacum* L. cv. BY-2[16].

Therefore, ginseng calli were cultured on the media containing different concentrations of sucrose to determine optimal concentration for the production of saponin from ginseng callus tissues. The yield of saponin of ginseng callus in-

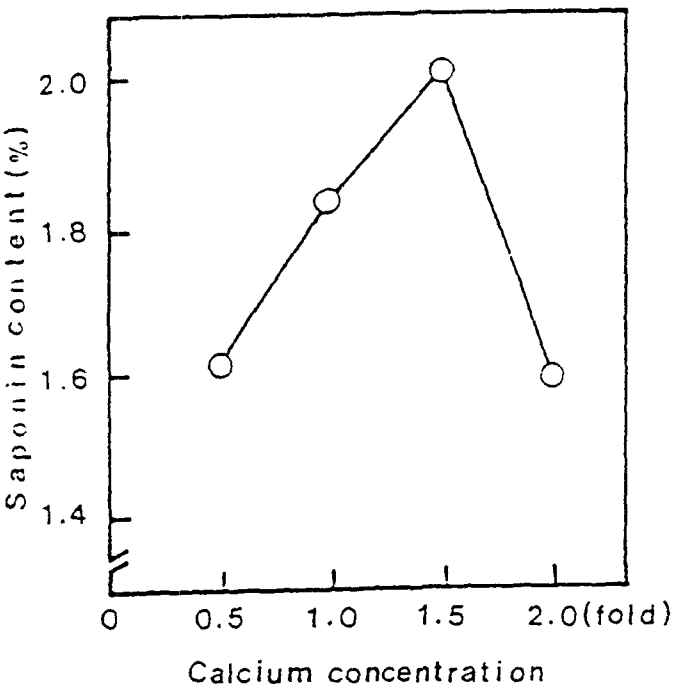

Fig. 6. Effect of calcium on the saponin content of ginseng callus.

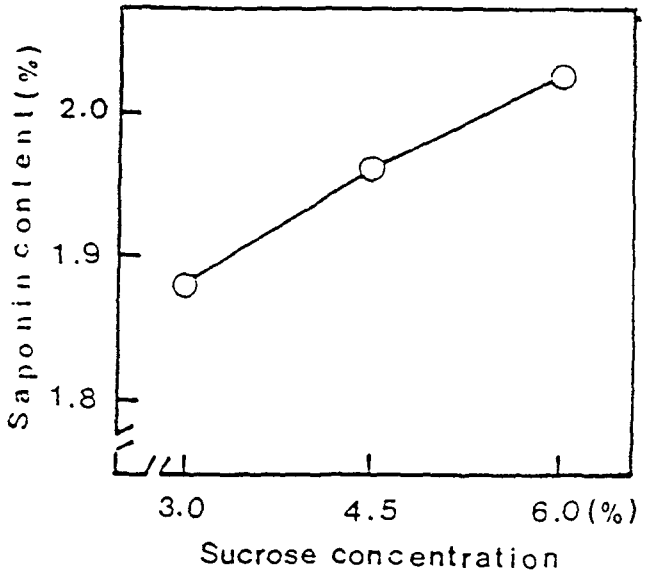

Fig. 7. Effect of sucrose on the saponin content of ginseng callus.

creased with increasing sucrose from 3 to 6% (Fig. 7).

The majority of plant cell parts, when excised and placed in tissue culture, require an exogenous supply of auxins and/or cytokinins for growth and cell division. The greatest difference between tissues from different species in terms of their behavior in tissue culture lies in the levels of auxins and/or cytokinins required for the growth and secondary product formation. Furuya et al.[13-15] reported that the content of saponin in ginseng callus cultured on the meida containing 2, 4-D, IBA or kinetin was low(0.08-1.49%).

And it was also confirmed that ginsenosides Rb_1 and Rg_1 among ginseng saponins are the most active principles. Therefore, normal calli were cultured on the medium containing 2, 4-D in order to determine the ginsenoside content of ginseng callus. Table 2 shows the ginsenoside contents in ginseng callus and root. Ginsenosides Rb_1, Re and Rg_1 were found in a low yield in the callus but were found in a high yield in the root. Ginsenoside Rc was not found in the callus. Ginsenoside Rg_2 was found in a high yield in the callus. but was found in a low yield in the root. Total ginsenosides were 3.18 mg/g D.W. for callus and 9.08 mg/g D.W. for root.

In these experiments, we found that macro-elements, sucorse and plant growth regulators influenced the relative biosynthesis of saponin in the ginseng tissue culture and that the ability of ginseng tissue culture to produce medicinal compound was apparently lower than that of the intact plants.

Table 2. Ginsenoside contents in ginseng callus and root.

	Total ginsenosides (mg/g)	Ginsenosides(mg/g D.W.)							
		Rb_1	Rb_2	Rc	Rd	Re	Rf	Rg_1	Rg_2
Callus	3.18	0.33	0.08	–	0.08	0.32	0.06	0.86	1.46
Root	9.08	2.55	0.86	0.91	–	1.70	0.79	1.89	0.40

3. Characteristics of Ginseng Cell Lines Selected

When plant cell cultures are considered as a new source for pharmacologically active compounds, the criterion is whether the product levels and production rates are sufficiently competitive and if not, what the prognoses are for achieving this goal by applying new approaches, The production of pharmacologically active compounds can often be further improved by the better culture conditions and development of high-producing cell lines. Therefore, we obtained two cell lines(PG_1 and PG_2) by the cloning method.

In general, the induction and growth of ginseng normal callus can be affected both in light and in darkness. Therefore, the callus tissues of PG_1 and PG_2 cell lines were cultured on the medium containing 2, 4-D in the dim light or in the darkness in order to clarify the effects of light or darkness on the growth and ginsenoside production in callus cultures of ginseng cell lines selected, The growth of PG_1 and PG_2 calli was not affected both in light and in darkness (Table 3). On the other hand, light affects the ginsenoside contents of calli in both PG_1 and PG_2 cell lines(Table 4). When the calli were cultured under the light, total ginsenosides were 9.96 mg/g D.W. for PG_1 cell line, 10.40 mg/g D.W. for PG_2cell line, and 3.18 mg/g D.W. for original cell line(Table 4). In comparison to the dark culture, these showed high concentrations of total ginsenosides in both PG_1 and PG_2 cell lines. And PG_1 and PG_2 cell lines also showed a threefold increase in total ginsenosides as compared with original cell line.

Table 3. Growth of calli induced from ginseng cell line cultured under light and dark conditions.

Culture condition	Dry weight of callus(g/100 ml flask)	
	PG_1	PG_2
Light	0.396	0.240
Darkness	0.375	0.250

Table 4. Total ginsenoside contents of calli induced from ginseng cell lines cultured under light and dark conditions.

Culture condition	Total ginsenoside contents(mg/g D.W.)		
	PG_1	PG_2	Original
Light	9.96	10.40	3.18
Darkness	5.03	7.97	

Growth, as well as differentiation of tissues and cells, is affected by plant growth regulators. The commonly used auxins are 2, 4-D, IAA, and IBA. Among them 2, 4-D is the most effective auxin in both induction and growth of ginseng callus. Therefore, tissues of PG_1 and PG_2 cell lines were cultured on the media supplemented with different concentrations of 2, 4-D in order to clarify the effect of 2, 4-D on the growth of calli of selected ginseng cell lines. Table 5 showed the effect of 2, 4-D on the growth of $PG_1$and PG_2 cell lines. The growth of callus was increased by raising 2, 4-D concentration in both PG_1 and PG_2 cell lines(Table 5). At 3 mg/l 2, 4-D, the growth of PG_2 callus was better than that of PG_1 callus.

There have been numerous reports on the conditions of plant growth regulators to enhance the yields of secondary metabolites, but it is obvious that there is no general rule in most cases of plant cell cultures. Since 2, 4-D was the best for the growth of ginseng callus, experiments were done in the concentration of 0.1 and 3 mg/l 2, 4-D in oredr to clarify the effects of 2, 4-D on the ginsenoside contents of PG_1 and PG_2 calli. Table 6 showed the ginsenoside contents of PG_1 and PG_2 calli cultured on the media containing different concentrations of 2, 4-D. As the calli were cultured on the medium containing 3 mg/l 2, 4-D, total ginsenosides were 2.31 mg/g D.W. for PG_1cell line and 3.26 mg/g D.W. for PG_2cell line(Table 6). In comparison to the low concentration of 2, 4-D(0.1 mg/l), these showed very low concentrations of total ginsenosides in both PG_1 and PG_2 cell lines. In most cases 2, 4-D inhibited the secondary metabolite production, even though it supported good growth, In *Catharanthus roseus* culture, 2, 4-D and NAA suppressed alkaloid formation while IAA gave high cell and alkaloid yield[21]. Koul *et al.*[19] also reported similar results in suspension cultures of *Hyoscyamus*. On the other hand, Ikeda *et al.*[16] reported that addition of 2, 4-D was remarkably effective on ubiquinone formation in cell suspension culture of

Table 5. Effect of 2, 4-D on the growth of calli induced from ginseng cell lines.

Concentration of 2,4-D(mg/l)	Dry weight of callus(g/100 ml flask)	
	PG_1	PG_2
0.1	0.39	0.24
3.0	0.41	0.44

Table 6. Effects of 2, 4-D on the total ginsenoside contents of calli induced from ginseng cell lines.

Concentration of 2, 4-D(mg/ℓ)	Total ginsenoside of callus(mg/g D.W.)		
	PG_1	PG_2	Original
0.1	9.96	10.40	3.18
3.0	2.31	3.26	–

tobacco plant and a higher ubiquinone content was observed with a higher 2, 4-D concentration. Berberine-producing activity was remarkably enhanced by simultaneous administration of auxin and cytokinin in cell suspension cultures of *Thalictrum minus*[20]. In these experiments, we found that 2, 4-D strongly inhibited ginsenoside formation in the callus cultures of selected cell lines, PG_1 and PG_2

Table 7 showed ginsenoside contents and pattern in ginseng cell lines. Ginsenoside Rb_1 was found in a high yield in PG_1 cell line but was found in a low yield in PG_2 and original cell lines. Ginsenoside Rb_2 was found in a low yield in PG_2 and original cell but was not found in PG_1 cell line. Ginsenoside Rc was found only in PG_2 line. Ginsenoside Rd was found in a low yield in PG_2 and original cell lines but was not found

Table 7. Ginsenoside contents and pattern of calli induced from ginseng cell lines.

Cell line	Ginsenosides(mg/g D.W.)							
	Rb_1	Rb_2	Rc	Rd	Re	Rf	Rg_1	Rg_2
PG_1	1.28	–	–	–	1.23	0.69	6.09	0.66
PG_2	0.87	0.16	0.07	0.18	4.82	0.04	3.60	0.68
Original	0.33	0.08	–	0.08	0.32	0.06	0.86	1.46

in PG_1 line. Ginsenoside Re was found in a much higher yield in PG_2 line and in a low yield in PG_1 and original cell lines. Ginsenoside Rf was found in a low yield throughout all lines. Ginsenoside Rg_1 was found in an much higher yield in PG_1 and PG_2 cell lines but was found in a low yield in original cell line. Especially, PG_1 line contained a significantly higher quantity of ginsenoside Rg_1, and showed a sevenfold increase in ginsenoside Rg_1 in comparison with the original cell line, Ginsenoside Rg_2 in PG_1 and PG_2 cell lines was relatively lower than in original cell line.

In conclusion, we found that the ability of ginseng cell lines to produce medicinal compound by cultured tissue was apparently higher than that of original cell line. Our data suggest that the plant growth regulators influence the ginsenoside contents of the calli induced from ginseng cell lines. Therefore, the ability of ginseng to produce a medicinal compound by cultured cell is apparently higher than that of intact plants. Although some promising data have already been obtained, more efforts are needed to improve the productivity of ginsenosides, by biochemical and genetic regulation of secondary metabolism.

LITERATURE CITED

1. Ando, T., Tanaka, O. and Shibata, S. : Syoyakugaku Zasshi 25(1), 28(1971).
2. Butenko, R.G., Grushvitsky, R.V. and Slepyan, L.I : Bot. Zh. 53(7), 906(1968).
3. Chang W.C. and Hsing, Y.I : Natl. Sci. Counc. M. 6(1), 76(1978).
4. Chang, W.C. and Hsing, Y.I. : Natl. Sci Counc. M 6(8), 77(1978).
5. Chang, W.C. and Hsing, Y.I. : Theor. Appl. Genet. 57, 133 (1980).
6. Choi, K.T., Kim, M.W. and Shin, H.S. : Proc. 5th Intl. Cong. Plant Tissue & Cell Culture, Tokyo, Japan, 171(1982).
7. Choi, K.T., Kim, M.W. and Shin, H.S. : Korean J. Ginseng Sci. 6(2), 162(1982).
8. Choi, K.T., Kim, M.W., Bae, H.W. and Kang, Y.H. : Korean J. Plant Tissue Cult. 9(1), 7(1982).
9. Choi, K.T., Yang, D.C., Kim, N.W. and Ahn, I.O. : Proc. 4th Intl. Ginseng Symp., Korea Ginseng & Tobacco Research Insitute, Taejon, Korea, 1(1984).
10. Davies, M.E. : Planta 104, 50(1972).
11. Furuya, Y., Kojima, H., Syono, K. and Ishii, T. : Chem. Pharm. Bull. 18, 2371(1970).
12. Furuya, T., Kojima, H., Syono, K., Ishii, T., Uotani, K. and Nishio, M. : Chem. Pharm. Bull. 21, 98(1973).

13. Furuya, T., Yoshikawa, T., Ishii, T. and Kajii, K. : Planta Med. 47, 183(1983).
14. Furuya, T., Yoshikawa, T., Ishii, T. and Kajii, K. : Planta Med. 47, 200(1983).
15. Furuya, T., Yoshikawa, T., Orihara, Y. and Oda, H. : Planta Med. 48, 83(1983).
16. Ikeda, T., Matsumoto, T. and Noguchi, M. : Agric. Biol. Chem. 40, 1765(1976).
17. Jhang, J.J., Staba, E. J. and Kim, J. Y. : *In Vitro* 9, 253(1974).
18. Kim, M.W., Choi, K.T., Bae, H.W. and Kang, Y.H. : Korean J. Botany 23(3, 4), 91(1980).
19. Koul, S., Ahuja, A. and Grewal, S. : Planta Medica 47, 11(1983).
20. Nakagawa, K., Fukui, H. and Tabata, M. : Plant Cell Reports 5, 69(1986).
21. Zenk, M.H., El - Shagi, H., Arens, H., Stockigt, J., Weller, E.W., and Deus, B. : in Plant Tissue Culture and its Biotechnological Application, Springer - Verlag, 27(1977).