Journal of Plant Biology, December 2001, 44(4) : 179-184

Effects of Macro Elements and Nitrogen Source on Adventitious Root Growth and Ginsenoside Production in Ginseng (*Panax ginseng* C. A. Meyer)

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We investigated the effects on ginseng adventitious root growth and ginsenoside production when macro-element concentrations and nitrogen source were manipulated in the culture media. Biomass growth was greatest in the medium supplemented with 0.5-strength NH_4PO_3, whereas ginsenoside accumulation was highest (9.90 mg g^{-1} DW) in the absence of NH_4PO_3. At levels of 1.0-strength KNO_3, root growth was maximum, but a 2.0 strength of KNO_3 led to the greatest ginsenoside content (9.85 mg g^{-1}). High concentrations of $MgSO_4$ were most favorable for both root growth and ginsenoside accumulation (up to 8.89 mg g^{-1} DW). Root growth and ginsenoside content also increased in proportion to the concentration of $CaCl_2$ in the medium, with the greatest accumulation of ginsenoside (8.91 mg g^{-1} DW) occurring at a 2.0 strength. The NH_4^+/NO_3^- ratio also influenced adventitious root growth and ginsenoside production; both parameters were greater when the NO_3^- concentration was higher than that of NH_4^+. Maximum root growth was achieved at an NH_4^+/NO_3^- ratio of 7.19/18.50, while ginsenoside production was greatest (83.37 mg L^{-1}) when NO_3^- was used as the sole N source.

Keywords: bioreactor, macro element, NH_4^+/NO_3^- ratio, nitrogen source

Ginseng (*Panax ginseng* C.A. Meyer), a member of the *Araliaceae* family, is traditionally considered one of the most potent medicinal plants, having been used for centuries as a health tonic. The most important active components in ginseng roots are the ginsenosides, which show anabolic, adaptogenic, antibiotic, minor hyperglycemic, and anti-cancer activities (Lee et al., 1995).

The demand for ginseng roots and extracts has increased over the years. Currently, plants are raised on farms throughout Korea and China. Because field production is a long (4 to 6 years) and laborious process, the cultivated roots are an expensive commodity (Persons, 1995). In addition, disease control practices have led to serious problems with pesticide residues (Yu and Ohh, 1995). Likewise, ginseng cannot be replanted in the same land because of various "replant diseases", which is a dilemma because Korea has a limited amount of arable land (Li, 1995; Yu and Ohh, 1995).

In recent years, plant cell culture technology has been successfully applied to the production of many useful secondary metabolites, including pharmaceuticals, pigments, and other fine chemicals (Zhong, 1995; Tamet and Mavituna, 1997; Gao et al., 2000). Ginsenosides also have been derived through cell culture (Furuya et al., 1983; Liu and Zhong, 1997, 1998; Zhong, 1998; Akalezi et al., 1999), although the high fluctuation in ginsenoside content achieved via culturing is a large obstacle to commercialization. In addition, when ginsenosides have been produced through transformed hairy root cultures (Hwang et al., 1996; Yoshimatsu et al., 1996), the total extracts have contained an opiate-like compound that is potentially harmful to mammalian cells (Yoshikawa and Furuya, 1987).

Fortunately, the adventitious roots of ginseng have proven to be an excellent alternative for the production of ginsenosides (Seon et al., 1999; Son et al., 1999; Yu et al., 2000). Nevertheless, further research is required for identifying the key factors that can increase root biomass and ginsenoside productivity, such as nutrient status in the culture medium (Yeoman and Yeoman, 1996). Regulation of media nutrients significantly improves the production of ginsenosides and polysaccharides in ginseng cell cultures (Furuya et al., 1983; Zhang et al., 1996; Zhong et al., 1996).

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However, no results have been reported regarding the influence of particular nutrient concentrations in the medium when culturing adventitious roots.

Therefore, we have investigated the effects of different macro elements and nitrogen source on ginseng adventitious root growth and ginsenoside production. Our objective in this study was to develop more efficient adventitious root culture techniques for ginseng, thereby resulting in greater production of biomass and ginsenosides compared with tissues grown in conventional culture media.

MATERIALS AND METHODS

Induction and Proliferation of Callus

Calli were induced and proliferated via the method of Seon et al. (1999). Fresh, four-year-old roots of ginseng (*P. ginseng* C.A. Meyer) were collected from the main production area, Pung-Gi province in Korea, then washed with a detergent solution and rinsed with running tap water for 10 min. The roots were sterilized with 70% ethanol for 1 min, further sterilized with a 1% (v/v) sodium hypochlorite solution for 30 min, and rinsed three times with sterile distilled water. They were then cut into small pieces (0.4 to 0.7 cm long) and placed in Petri dishes (9.0 × 1.5 cm) that contained a solid SH medium (Schenk and Hidebrandt, 1972) supplemented with 1 mg L^{-1} 2,4-D, 0.1 mg L^{-1} kinetin, and 3% sucrose. After the pH was adjusted to 5.5, 0.5 g L^{-1} plant agar (DIFCO, USA) was added, and the dishes were autoclaved at 121°C. Cultures were maintained at 25 ± 2°C in the dark. After four weeks, calli were induced from the surfaces of the inoculated root pieces and proliferated on an SH solid medium supplemented with 2.0 mg L^{-1} NAA, 0.1 mg L^{-1} kinetin, and 3% sucrose. The calli were subcultured every four weeks and were maintained at 25 ± 2°C in the dark.

Induction and Proliferation of Adventitious Roots

Adventitious roots were induced and proliferated according to the methods of Seon et al. (1999) and Yu et al. (2000). Calli were cultured for four weeks on an SH medium containing 2.0 mg L^{-1} IBA, 0.1 mg L^{-1} kinetin, and 3% sucrose. Afterward, the induced roots were proliferated in 400-mL conical flasks that contained 100 mL SH liquid medium supplemented with 2.0 mg L^{-1}, 0.1 mg L^{-1} kinetin, and 3% sucrose. Cultures were maintained on an orbital shaker (100 rpm) at 25 ± 2°C in the dark. The finest root line (G10) was selected from 15 established lines, then proliferated in 5-L balloon-type bubble bioreactors containing 3 L of SH liquid medium supplemented with 2.0 mg L^{-1} IBA, 0.1 mg L^{-1} kinetin, and 3% sucrose.

Varying the Concentrations of Macro Elements in the Culture Medium

Four grams of adventitious roots were cultured in a 400-mL conical flask containing 100 mL of an SH medium supplemented with 2.0 mg L^{-1} IBA, 0.1 mg L^{-1} kinetin, and 3% sucrose. The levels of NH_4PO_3, KNO_3, $MgSO_4$, and $CaCl_2$ in the SH medium were each varied at 0, 0.5, 1.0, 1.5, and 2.0 strengths of the original, normal concentration. Air volume in the flask was adjusted to 0.1 vvm. Cultures were maintained at 25 ± 2°C in the dark and each treatment was repeated three times. After five weeks of culture, we determined the root growth yield and ginsenoside productivity. Growth yield was defined as the quotient of root DW after four weeks of culture/root DW of the inoculum.

Effects of Nitrogen Source on Adventitious Root Growth and Ginsenoside Production

Adventitious ginseng roots (25 g FW) were inoculated into a 5-L cone-type bubble bioreactor containing 3 L of an SH medium supplemented with 2.0 mg L^{-1} IBA, 0.1 mg L^{-1} kinetin, and 3% sucrose. The following ratios of NH_4^+/NO_3^- were used: 0.0:18.5, 7.19:18.50, 14.38:18.50, 21.57:18.50, 28.75:18.50, 14.38:0.00, 14.38:9.40, 14.38:18.80, 14.38:28.20, 14.38:37.60 (mM/mM), in an experiment with three replicates. Cultures were maintained in the dark at 25 ± 2°C. The air volume in the bioreactor was adjusted to a constant flow rate of 0.02 to 0.03 vvm using an air flow meter (RMA 14, Dwyer C., USA). Root growth and ginsenoside productivity were measured after five weeks of culture.

Determination of Root Growth Rate

Fresh weight (FW) was measured after the water was absorbed from the root surfaces. To measure dry weight (DW), roots were oven-dried at 70°C until they reached a constant mass. The adventitious root growth rate was then calculated as:

Growth rate = harvested DW (g)/inoculated DW (g).

Determination of Ginsenoside Content and Productivity

Ginsenosides were analyzed according to the method of Son et al. (1999), with the following modification: dried powder from the cultured roots (1 g) was extracted with 60% methanol (40 mL) at 100°C for 3 h and filtered under vacuum. The extract was evaporated to dryness and dissolved in 10 mL HPLC-grade water. This water-soluble extract was then passed slowly through a SEP PAK C_{18} cartridge (Waters, USA) and eluted with 10 mL methanol. The ginsenoside fraction was analyzed, with water and acetonitrile, using an HPLC system (Waters 2690 separation module; Waters 996 photodiode array detector; Waters millennium 2010 chromatography manager, USA) on an Altec Platinum C18 column (ϕ1.5 μm, 33 × 7 mm). The ratios of water to acetonitrile for the first 10 min and the last 15 min were 75:25 and 63:37, respectively. Flow rate for the mobile phase was 1.2 mL/min, and absorbance of the ginsenoside was read at 203 nm. The authentic ginsenosides were purchased from Wako, Japan. Ginsenoside content in the roots was calculated as:

Ginsenoside content (mg g^{-1}) = sample ginsenoside concentration (from HPLC) (mg L^{-1}) × sample volume (L)/adventitious root DW (g). Ginsenoside productivity was calculated as:

Ginsenoside productivity (mg L^{-1}) = total ginsenoside content (mg g^{-1}) × harvested adventitious root DW (g)/volume of culture medium (L).

RESULTS AND DISCUSSION

Effects of Macro Elements on Adventitious Root Growth and Ginsenoside Production

Table 1 and Figure 1 show how growth and yield of ginseng adventitious roots were affected by the concentrations of macro elements in the SH media. Biomass production was greater when 0.5 and 1.0 strengths of NH_4PO_3 were used, with the highest yield (4.58) resulting from the 0.5-strength level. Ginsenoside accumulation also was influenced by macro-element supplements (Fig. 2), increasing at the lower concentration. In fact, the greatest ginsenoside production (9.90 mg g^{-1} DW) was obtained when NH_4PO_3 was absent from the culture medium.

A 1.0 strength of KNO_3 resulted in the maximum FW (16.33 g), DW (1.54 g), and growth yield (4.39), while the 2.0 strength led to the greatest ginsenoside content (9.85 mg g^{-1}). Higher strengths (1.0, 1.5, and 2.0) of $MgSO_4$ were more favorable for both root growth and ginsenoside accumulation, as seen by the highest root DW (1.60 g) and ginsenoside content (8.89 mg g^{-1} DW). Root growth and ginsenoside accumulation also increased with higher $CaCl_2$ concentrations; the greatest ginsenoside content (8.91 mg g^{-1}

Table 1. Biomass growth of ginseng adventitious roots as affected by concentrations of macro elements in the SH medium. Cultures were maintained in 400-mL conical flasks for 4 weeks.

Concentration of macro element		Biomass growth		
		Fresh wt. (g)	Dry wt. (g)	% Dry wt.
NH_4PO_3	0.0	11.93 b[a]	1.17 c	9.79
	0.5	14.88 a	1.60 a	10.77
	1.0	13.73 a	1.53 a	11.15
	1.5	11.43 b	1.36 b	11.90
	2.0	11.38 b	1.26 b	11.05
KNO_3	0.0	5.33 d	0.53 d	9.95
	0.5	13.18 b	1.44 bc	10.89
	1.0	16.33 a	1.54 a	9.42
	1.5	12.73 b	1.48 ab	11.61
	2.0	10.12 c	1.34 c	13.25
$MgSO_4$	0.0	11.40 c	1.14 b	10.04
	0.5	15.60 ab	1.53 a	9.82
	1.0	15.75 ab	1.59 a	10.17
	1.5	16.55 a	1.60 a	9.68
	2.0	15.40 b	1.60 a	10.34
$CaCl_2$	0.0	12.30 c	1.25 b	10.19
	0.5	15.85 b	1.54 a	9.78
	1.0	17.25 a	1.60 a	9.30
	1.5	18.15 a	1.59 a	8.97
	2.0	18.25 a	1.54 a	8.44

[a]Separation within columns by Duncan's multiple range test, 5% level.

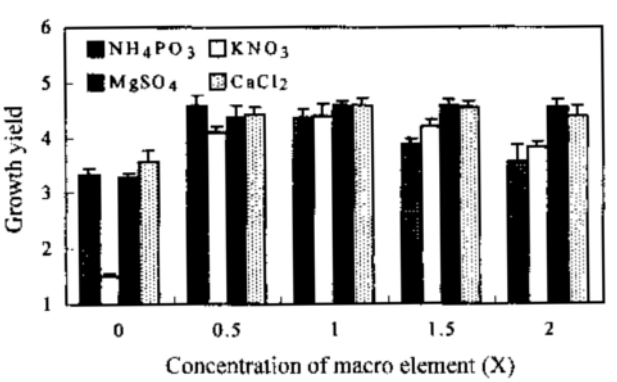

Figure 1. Growth yield of ginseng adventitious roots as affected by concentrations of macro elements. Values are the quotient of the root dry weight after 4 weeks of culture and the root dry weight of the inoculum.

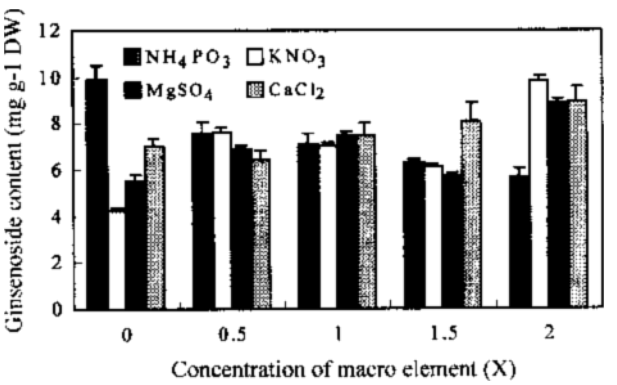

Figure 2. Ginsenoside content in ginseng adventitious roots after 4 weeks of culture as affected by concentrations of macro elements.

DW) was achieved at a 2.0 strength in the medium. Overall, ginseng adventitious root growth and ginsenoside production required higher concentrations of KNO_3, $MgSO_4$, and $CaCl_2$ than those normally used in culture media. In contrast, however, the absence of NH_4PO_3 enhanced ginsenoside accumulation.

Depletion of nitrogen or phosphate is associated with limited root growth and a concomitant increase in the level of secondary metabolism (Yeoman and Yeoman, 1996). Knobloch and Berlin (1981) demonstrated the effect of phosphate limitations on the accumulation of cinnamoyl putrescines in tobacco cultures, while Mantell and Smith (1983) concluded that the *lack* of phosphate stimulated secondary metabolite biosynthesis. In cell suspension cultures of *P. ginseng* and *P. notoginseng*, a low initial concentration of phosphate in the medium sufficiently promoted both cell growth and ginsenoside accumulation (Zhang et al., 1996; Liu and Zhong, 1998), a result that is similar to our own. Likewise, Zhong and Wang (1998) reported that NH_4^+ in the culture medium inhibited ginsenoside accumulation in *P. notoginseng* cell suspension cultures and that maximum ginsenoside production was obtained when NH_4^+ was absent. Therefore, optimizing macro-element concentrations, especially for nitrogen and phosphate, in the culture media is a key step toward higher production of secondary metabolites in plant cell, tissue, or organ culture.

Effects of Nitrogen Source on Adventitious Root Growth and Ginsenoside Production

The particular NH_4^+/NO_3^- ratio in the culture medium affected ginseng adventitious root growth and ginsenoside production (Table 2), with both parameters increasing when the NO_3^- concentration was higher. Maximum root growth (35.95 g DW) was achieved at an NH_4^+/NO_3^- ratio of 7.19/18.5 (1:2). Likewise, the greatest ginsenoside production (83.37 mg L^{-1}) was found with an NH_4^+/NO_3^- ratio of 0/18.5 (0:1). In contrast, root growth rates and the accumulation of ginsenoside were severely inhibited when the NH_4^+ concentration was increased. In particular, root growth and ginsenoside productivity reached only one-third and one-tenth of their respective maximum values when NH_4^+ was used as the sole nitrogen source.

Liu and Zhong (1997) also reported increased root growth of *P. ginseng* at low NH_4^+/NO_3^- ratios, as well as the greatest ginsenoside productivity in the absence of NH_4^+. They presented similar results with their *P. quinquefolium* cell cultures. Maximum levels of saponin and polysaccharide also were obtained when NO_3^- was the sole nitrogen source (Zhong and

Table 2. Effects of NH_4^+/NO_3^- ratio in SH medium on growth of ginseng adventitious roots, growth yield, and ginsenoside productivity. Cultures were maintained in 5-L cone-type bubble bioreactors for 20 d.

Concentration of NH_4^+/NO_3^- (mM)[a]	Biomass growth (g DW)	Growth yield[b]	Residual NO_3^- (mM)	Ginsenoside productivity (mg L^{-1})
0.00/18.50	31.67 b[c]	7.89	1.60	83.37
7.19/18.50	35.59 a	5.59	0.39	66.30
14.38/18.50	30.30 b	5.12	7.02	51.71
21.57/18.50	28.29 bc	4.32	7.69	40.74
28.75/18.50	26.90 c	3.65	9.09	32.73
14.38/ 0.00	10.21 c	2.44	0.50	8.30
14.38/ 9.40	31.79 ab	5.63	4.50	59.66
14.38/18.80	34.20 a	5.05	12.49	57.57
14.38/28.20	33.59 a	4.15	29.01	46.47
14.38/37.60	30.10 b	3.85	35.05	38.63

[a]NH_4^+/NO_3^- = NH_4Cl/KNO_3 (mM/mM)
[b]Values are the quotient of the root dry weight after 4 weeks of culture and the root dry weight of the inoculum.
[c]Separation within columns by Duncans multiple range test, 5% level.

Wang, 1998). These results suggest that the nitrate and ammonium ions have different effects on primary and secondary metabolism in plant cell and tissue cultures.

In general, nitrate enhances the accumulation of secondary metabolites, while ammonium inhibits this. Many published reports have confirmed that callus and root growth, as well as secondary metabolite accumulation, are promoted under the influence of NO_3^- in cell cultures of *Pinus strobus* (Kaul and Hoffman, 1993), *Lithospermum erythrorhizon* (Tabata and Fujita, 1985), and *Aralia cordata* (Sakamoto et al., 1994). Further experiments are required for identifying the optimal nitrogen source and ammonium to nitrate ratio that will maximize ginsenoside productivity according to culture stage.

ACKNOWLEDGMENTS

This work was supported in part by a grant from the Research Center for the Development of Advanced Horticultural Technology, Chungbuk National University, Korea.

Received September 5, 2001; accepted October 11, 2001.

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