Optimization of organic nutrients for ginseng hairy roots production in large-scale bioreactors

G. Sivakumar\textsuperscript{1,}\textsuperscript{*}, K. W. Yu\textsuperscript{2}, E. J. Hahn\textsuperscript{2} and K. Y. Paek\textsuperscript{2}

\textsuperscript{1}ENEAA, Biotech Genomics, Casaccia, Via Anguillarese 30100060, Rome, Italy
\textsuperscript{2}Research Center for the Development of Advanced Horticultural Technology, Chungbuk National University, Cheongju 361-763, South Korea

Macro and micro-elements, carbon sources and elicitors play a central role during \textit{Panax ginseng} hairy root growth, biomass and ginsenoside production and also influence numerous root developmental stages in large-scale bioreactor culture. Due to the lack of biosynthetic nutrients and signalling-inducing nutrients, the regulatory role of mineral elements is not well understood. We have genetically engineered \textit{Agrobacterium rhizogenes} KCTC 2703 to have the rol C gene in T-DNA of ginseng calluses that produce hairy roots. A normal MS medium nutrient in the bioreactor gave low hairy roots growth, biomass and ginsenoside content, indicating that an optimal mineral element ratio is required to increase hairy root growth and biomass. In addition, elicitors also play an important role in increasing ginsenoside production. These results suggest that mineral elements are an important regulatory factor in hairy root growth and biomass, while elicitors decrease biomass and increase ginsenoside content.

Keywords: \textit{Agrobacterium rhizogenes}, bioreactors, ginseng, ginsenosides, hairy roots.

\textit{Panax ginseng} C.A. Meyer (Araliaceae) is an important industrial medicinal crop in South Korea known for its high ginsenoside content. However, it is difficult to cultivate the crop in the field and disease control remains a major problem in commercial cultivation\textsuperscript{1}. Thus, bioreactor technology is necessary for large-scale cultivation of this plant. Hairy roots are formed by infection with \textit{Agrobacterium rhizogenes}. Transferred DNA (T-DNA) on the root-inducing (Ri) plasmid (~200 kbp) of \textit{A. rhizogenes} is integrated into plant genomic DNA and several genes on the T-DNA are expressed in the transformed plant cells. Hairy roots are then formed in the infected area. Though the adventitious roots in most species require phytohormones, i.e. auxins to grow on a medium, hairy roots grow vigorously on the medium without phytohormones\textsuperscript{2}. Other mineral nutrients such as macro and micro-elements, carbon source and elicitor levels have been deemed essential for ginseng hairy root growth, biomass and ginsenoside production in large-scale bioreac-
tors. An organic nutrient serves as a chemical signal and energy source, and regulates growth and differentiation of hairy roots in a bioreactor\textsuperscript{3}. Root nutrition is entering an exciting phase, where a molecular understanding of key transport processes is beginning to emerge. The ability of roots to respond to a wide range of nutrients is probably a reflection of their large gene families of transporters that differ in affinities for substrates and are differentially expressed in different tissues. So far, optimization of mineral elements on ginsenoside production has not been reported in hairy root cultures in large-scale bioreactors, nor have there been reports about hairy root cultures. Hwang \textit{et al.}\textsuperscript{3} have established a two-step culture process of mass culture and ginsenoside production by ginseng hairy roots. They used 3 l bubble-type bioreactors and found that the light condition was suitable for ginsenoside synthesis and dark condition was favourable for hairy root growth. Ginseng hairy roots have shown biotransformation abilities of 2,3-oxidosqualene\textsuperscript{4} and ginsenoside\textsuperscript{5}. For a more detailed understanding of nutrient range, we need to understand ginseng hairy root well enough to undertake truly predictive engineering of biomass and ginsenoside production. This would allow us to efficiently produce hairy roots that are more productive of cosmetic and medicinal as well as novel industrial ginsenosides. We usually find new nutritional limiting steps that prevent production of ginsenoside or catabolic pathway. At its most basic level, the goal of optimizing nutritional supplementation in a bioreactor is to create a comprehensive multidimensional representation of ginsenoside biosynthetic reactions in large-scale cultivation of \textit{P. ginseng} hairy roots.

Materials and methods

\textit{Plant material}

\textit{P. ginseng} C.A. Meyer roots (6-year-old) were collected from Pung-Gi Province, South Korea. Roots were washed with a detergent solution (AIC Co, Korea) for 5 min and rinsed with running tap water for 5 min. After being soaked in 70\% aqueous EtOH for 1 min, the roots were rinsed in sterilized water, further sterilized with 1\% (v/v) sodium hypochlorite for 15 min, and rinsed repeatedly with sterile

\*For correspondence. (e-mail: sivakumar@libero.it)
distilled water. The sterilized roots were cut into sections of 5 mm and then inoculated into nutrient broth (DIFCO, USA) liquid medium containing actively growing Agrobacterium rhizogenes KCTC 2703 for 16 h at 28°C. After overnight culture, explants were transferred to Murashige and Skoog (MS) solid medium containing 3% sucrose for 2 days, and finally transferred to half MS solid medium containing 300 mg/l Cloran (Cefotaxime sodium, HANBOK, Korea) until roots emerged. Rapidly growing roots with no bacterial contamination were used to establish lines of hairy root cultures. Transformed roots were transferred to 100 ml Erlenmeyer flasks containing 40 ml phytohormone-free MS liquid medium supplemented with 3% sucrose and suspension cultured on an orbital shaker at 100 rpm. All root cultures were initiated from inoculation of 0.15 g FW, maintained in dark at 23 ± 2°C and subcultured to fresh medium every four weeks. Transformation was confirmed by PCR experiments.

Identification of transformation

DNA extraction was carried out according to White and Sinkar. The amplification reaction was carried out in a GeneAmp PCR System DNA Thermal Cycler (PERKIN-ELMER, USA) using 22-mer oligonucleotides as primers. The primers for rol C gene were 5'-ATG-GCT-GAA-GAC-GAC-CTG-TGT-T-3' and 5'-TTA-GCC-GAT-TGC-AAA-CTT-GCA-C-3'. The reaction mixture consisted of 1 μl of 1 Unit Taq polymerase, 2.5 μl of 100 mM dNTP, 1 μl of 20 mM primer, 1 μl of 20 ng/μl template DNA and 2.5 μl of 10X reaction buffer; it was replenished up to 25 μl by sterilized distilled H2O. The following process was set up for the PCR reaction: predenature for 4 min, 94°C; denature for 1 min, 94°C; annealing of primer for 1 min, 60°C; amplification for 2 min, 72°C. The last amplification was for 5 min at 72°C. After amplification for 30 cycles, the PCR–DNAs were stabilized at last amplification for 5 min. PCR results were checked using agarose gel electrophoresis with HindIII-digested λDNA maker.

Proliferation of hairy roots

Hairy roots were selected and subcultured every 4 weeks in 400 ml conical flasks with 100 ml of phytohormone-free MS medium containing 3% sucrose on a gyratory shaker (100 rpm) at 23 ± 2°C in a dark room. Different types of bioreactors (5–20 l; Figure 1) were employed to proliferate hairy roots. These proliferated roots were used as explants for further experiments. pH of the medium was adjusted to 6.0 before autoclaving at 121°C and 1.2 kg cm −2 pressure for 40–50 min. The volume of input air was adjusted to 0.1vvm (air volume/min). Bioreactors were maintained at 23 ± 2°C in a dark room for 4 weeks before harvest.

Determination of medium inorganic compounds

The filtered samples were used for analysing inorganic compounds after determination of soluble sugars. Inorganic compounds, including cations such as Na+, NH4+, K+, Mg2+ and Ca2+, as well as anions such as Cl−, NO3−, HPO4 − and SO42− were analysed using HPLC system (Waters 2690 separation module; Waters 996 photodiode array detector; Waters millennium 2010 chromatography manager). The cations were analysed by a 432 conductivity detector and IC-Pak™ cation M/D column using 0.1 mM EDTA/2 mM HNO3 at a flow rate of 1.0 ml min −1, while anions were analysed by a suppressed 432 conductivity detector using 1.6 mM NaHCO3/1.2 mM Na2CO3 at a flow rate of 1.0 ml/min.

Salt strength on hairy root growth and ginsenoside production

The following media containing 3% sucrose were tested: 1/2 MS, 3/4 MS, 1.0 MS and 1.5 salt strength MS. Root growth index and ginsenoside production were checked after 4 weeks of culture.

Ammonia/nitrate ratio

Ratios of ammonia/nitrate were tested by ammonium chloride/potassium nitrate (NH4Cl/KNO3). Concentrations of total nitrogen sources tested were 19.7, 29.55, 39.4, 59.1, and 78.8 mM. The following ammonia/nitrate (NH4/NO3 mM) ratios were tested: 0/18.5, 7.19/18.5, 14.38/18.5, 21.57/18.5, 28.75/18.5, 14.38/0, 14.38/9.4, 14.38/18.8, 14.38/28.2, 14.38/37.6.

Macro-elements

Concentrations of calcium chloride, magnesium sulphate, and potassium phosphate were tested as 0, 0.5, 1.0, 1.5 and 2.0 times the normal concentration in MS medium.
Microelements

Concentrations of metal elements such as CoCl₂, CuSO₄, KI, MnSO₄, and ZnSO₄ were tested as 1, 5, 10 times the normal concentration in MS medium.

Carbon source and osmotic agents

Sucrose (1–9%) were tested in MS medium. In addition, fructose (3%), glucose (3%), maltose (3%), as well as combinations of glucose and fructose (1.5:1.5%), glucose and sucrose (1.5:1.5%), and fructose and sucrose (1.5:1.5%) were tested in 3/4 salt strength MS medium. For experiments on osmotic pressure, 0.1, 0.3, 0.5 M sorbitol and mannitol were also tested.

Elicitors

Cultured roots were transferred into new medium with elicitors to be cultured for 7 days before harvest. The elicitors used were: (i) Phenolic compounds such as 300 mg/l phenylalanine, 300 mg/l caffeic acid, 100 and 300 mg/l catechin, and 300 mg/l catechol. (ii) Glycosides such as 0.1% chitin, 0.1% Gum karaya, 200 mg/l Fucoidan, and 300 mg/l pepton (SIGMA, USA); (iii) 1, 2, 5, and 10 mg/l jasmonate.

Level of pH

The media pH was adjusted to 3, 4, 5, 6 and 7 before culturing and pH changes in the media during root cultures in bioreactors were checked every week.

Determination of root biomass

Roots were separated from the medium by passing through a stainless-steel sieve. Fresh weight was found after rinsing once with tap water and blotting away surface water, and dry weight was recorded after roots were dried to constant weight at 50°C for several days.

The hairy root growth yield was calculated as: Growth rate = Harvest dry weight (g)/inoculated dry weight (g).

Determination of ginsenosides and productivity

Analysis of ginseng ginsenosides was modified according to Fuzzati et al. Briefly, dried pulverized roots (1 g) were Soxhlet extracted with 80% methanol (50 ml) at 60°C for 1 h, repeated 2 times, and filtered through filter paper (Whatman No. 1). The extract was evaporated to dryness and dissolved in 50 ml HPLC-grade water. The water-soluble fraction was again washed with the same volume of ether twice. The water insoluble fraction was extracted with 50 ml of n-butanol saturated with water three times and washed with 20 ml water. The organic phase was evaporated to dryness under vacuum at 35°C. The residue was dissolved in LC–MS CHROMASOLV-grade methanol (5 ml) into vials and filtered through a 0.45 μm millipore PTEF membrane filter (ADVANTEC MFS, Inc, USA). The ginsenoside fraction was analysed using HPLC system (Waters 2690 separation module; Waters 996 photodiode array detector; Waters millennium 2010 chromatography manager) on an Altex Platinum C18 column (diameter 1.5 μm, 33 × 7 mm), with water and acetonitrile. The flow rate of water and acetonitrile for the first 10 min and the next 15 min was 75 : 25 and 63 : 37 respectively. Flow rate of the mobile phase was 1.0 ml/min and monitoring of ginsenoside was 203 nm. Authentic ginsenosides were purchased from Whan In Phar. Co, Ansung, Korea. The amount of panaxadiol (Rb group) as calculated as the sum of Rb1, Re, Rh2, and Rd and the amount of panaxatriol (Rg group) as the sum of Rg1, Rg2, and Rg6. Total content of ginsenoside was calculated as the sum of Rg and Rb groups analysed by HPLC.

Results and discussion

Hairy root growth and ginsenoside production

Ginseng (Figure 2) hairy roots induced by A. rhizogenes KCTC 2703 were confirmed to have rol C gene in T-DNA (Figure 2). These hairy roots had a typical growth curve. The first week was the lag period of hairy root growth; then it began to increase gradually during the second week. The exponential growth stage during the second and the third weeks was followed by the stationary phase during the fourth and fifth weeks (Figure 3). Ginsenoside accumulation gradually decreased from 5.01 to 3.39 mg/DW during the first two weeks, and later increased gradually to 7.9 mg/DW. The exponential growth phase occurred during the fourth and...

**Figure 2.** PCR analysis of each transformed and non-transformed root with rol C primer, showing 500 bp rol C fragment: arrow (lanes 1–3). Lane M, HindIII-digested λDNA as molecular weight marker; lanes 1–3, Transformed hairy roots; lane 4, Adventitious root.
fifth weeks. During the first two weeks, many new multiple roots were generated from the inoculated hairy roots, and these roots began to grow. As the ginseng hairy roots began to accumulate ginsenosides after two weeks, formation of new multiple roots was necessary for ginsenoside accumulation in ginseng hairy root culture.

Medium component during hairy root culture in bioreactors

Figure 4 shows that the regular of sucrose content changes was similar with that of electric conductivity (EC) changes. During the first three weeks, the values decreased gradually; after addition of the same medium, the values increased and then decreased gradually. However, fructose and glucose contents increased gradually, though they decreased after the addition of the new medium. Changes in the contents of anions such as Cl\textsuperscript{-}, NO\textsubscript{3}\textsuperscript{-}, H\textsubscript{2}PO\textsubscript{4}\textsuperscript{-}, SO\textsubscript{4}\textsuperscript{2-} were similar with those of sucrose and EC; the values decreased gradually during the whole culture process, and increased after the addition of fresh medium (Figure 5a). As shown in Figure 5b, changes in cation contents were different from each other. The contents of Na\textsuperscript{+} and K\textsuperscript{+} were maintained stable during the whole process, indicating that they were taken up by the hairy roots, while NH\textsubscript{4}\textsuperscript{+} was consumed quickly before and after adding the new medium. Mg\textsuperscript{2+} and Ca\textsuperscript{2+} were absorbed gradually, like anions. This indicates that sucrose, NH\textsubscript{4}\textsuperscript{+} anions, as well as Mg\textsuperscript{2+} and Ca\textsuperscript{2+} were important elements for both hairy root growth and ginsenoside accumulation. In roots, K\textsuperscript{+} channel activity is the major determination of cell membrane electrical properties\textsuperscript{9}. Depolarization-activated Ca\textsuperscript{2+} permeable channels are present in the root plasma membrane\textsuperscript{10}. It could involve Ca\textsuperscript{2+} signalling, Na\textsuperscript{+} uptake and/or K\textsuperscript{+} releases. In addition to growth and biomass maintenance, Johnson\textsuperscript{11} proposed two supplementary specific components for root respiration, one being the energy cost of ion uptake and other cost of ion re-uptake.

Nitrogen is the essential mineral element required in the greatest amount in plants, comprising 1.5 to 2% of plant dry matter and approximately 16% of total plant protein\textsuperscript{12}. Roots can utilize mineral nitrogen (NO\textsubscript{3} and NH\textsubscript{3})\textsubscript{2}; however, nitrate (NO\textsubscript{3}) is the most important source of nitrogen for root development. Nitrate efflux and influx are independent processes under distinct regulations. Once in the root cell cytoplasm, nitrate may be stored in the vacuole for later use, transported into xylem and translocated to other parts for assimilation and storage, reduced to nitrite and then ammonia via nitrate reductase and nitrite reductases. The reduction of nitrate to nitrite and then ammonia generates nitrogen in a form that can be assimilated into amino acids\textsuperscript{13}. Nitrate serves to repress the expression of a key enzyme in the starch biosynthesis pathway, presumably to divert carbon skeletons toward the nitrogen assimilatory pathway.

Effect of salt strength on hairy root growth and ginsenoside production

As shown in Table 1, 3/4 salt strength MS medium was optimal for ginseng hairy root growth compared with other media, though hairy roots also grew well in other media. Maximum growth rate (6.43) was obtained in MS medium. Lower salt strengths of MS media such as 1/2 MS and 3/4 MS were favourable for ginsenoside accumulation. Maximum ginsenoside production (161.04 mg/l) was obtained in 1/2 strength MS medium. In addition, 3/4 strength MS medium was also suitable for ginsenoside production, the ginsenoside yield being 133.99 mg/l in this medium. Hence, 3/4 strength MS medium was favourable for both hairy root growth and ginsenoside production. Hairy roots are also building blocks of organic matter or cofactors in addition to their potential role as signalling molecules. Cloned Arabidopsis transporters have begun to define the molecular basis for regulation of uptake for many of the macronutri-

![Figure 3. Growth curve and changes in ginsenoside content in hairy root cultures of ginseng using 400 ml flask containing 100 ml MS medium with 3% sucrose.](image1)

![Figure 4. Changes in sugar content and electric conductivity during ginseng hairy root culture in a 201 bubble bioreactor containing MS medium. Initial inoculating volume was 20 g/4l and then 41 of the same fresh medium was added (→ Time of feeding the medium.).](image2)
Table 1. Optimization of strength of MS medium on hairy root growth and ginsenoside productivity. Data collected after 4 weeks of culture using 400 ml conical flask with 100 ml MS medium

<table>
<thead>
<tr>
<th>Salt strength</th>
<th>FW (g)</th>
<th>Biomass DW (g)</th>
<th>Percentage DW</th>
<th>Growth rate*</th>
<th>Ginesoside (mg/g)</th>
<th>Ginesoside yield (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS 0.50</td>
<td>15.6 ± 0.43**</td>
<td>1.22 ± 0.02</td>
<td>7.82</td>
<td>5.73</td>
<td>13.20 ± 0.25</td>
<td>161.04</td>
</tr>
<tr>
<td>0.75</td>
<td>18.7 ± 0.30</td>
<td>1.37 ± 0.01</td>
<td>7.33</td>
<td>6.43</td>
<td>9.78 ± 0.30</td>
<td>133.99</td>
</tr>
<tr>
<td>1.00</td>
<td>16.9 ± 0.12</td>
<td>1.20 ± 0.01</td>
<td>7.10</td>
<td>5.63</td>
<td>6.82 ± 0.81</td>
<td>81.84</td>
</tr>
<tr>
<td>1.50</td>
<td>14.2 ± 0.50</td>
<td>1.04 ± 0.12</td>
<td>7.32</td>
<td>4.88</td>
<td>5.65 ± 1.12</td>
<td>58.76</td>
</tr>
</tbody>
</table>

*Growth rate was calculated from increase in DW. Values are quotients of DW after cultivation and DW of the inoculum. Experiments were repeated three times.
**Mean ± standard error of three replicates.

Figure 5. Changes in anion content (a) and cation content (b) during ginseng hairy root culture in a 20 l balloon-type bubble bioreactor containing MS medium. Initial inoculating volume was 20 g/l and then 4 l of the same fresh medium was added (→, Time of feeding the medium).

Granulents (NH4+, NO3-, K+, Cu2+, H2PO4-, SO42- and Mg2+) and micronutrients (Cl-, Zn, Mn2+, Fe3+ and Cu2+) can also affect transcription of the transporter gene14.

Carbon source on hairy root growth and ginsenoside production

Sucrose, glucose, fructose, glucose + fructose, and sucrose + glucose were tested as carbon sources for ginseng hairy root growth. Results showed that sucrose increased ginseng hairy root growth rate higher than any other carbon source, followed by sucrose + glucose (data not shown). Thus sucrose was the optimal carbon source for ginseng hairy root growth.

Different concentrations of sucrose were tested. Table 2 shows that dry weight and growth rate of ginseng hairy root increased with increase in sucrose concentration in the range of 1–9%, while 2–7% sucrose was suitable for fresh weight increase. Low sucrose concentrations such as 2–5% were suitable for ginsenoside production, while 1 or 7–9% sucrose was not suitable for ginsenoside production. The maximum ginsenoside content (8.01 mg/g DW) as well as the maximum ginsenoside productivity were obtained with 2% sucrose. Sucrose at 1–3% was favourable for both ginsenoside Rg group and Rb group synthesis. Among the different kinds of ginsenosides, the contents of Rg, Rb1, Rb2 and Rd decreased compared to other ginsenosides (Table 3). The above results indicated that a two-stage culture system is suitable for ginseng hairy root culture, during the growth stage, higher sucrose concentration is needed, while during the ginsenoside production stage, a relatively low concentration of sucrose (2%) should be maintained.

In addition, the reducing sugar, total sugar and starch contents in hairy roots cultured at different concentrations of sucrose were analysed (Figure 6). Results indicated that reducing sugar, total sugar and starch contents in hairy roots increased with increasing sucrose concentration. This implies that the amount of sucrose absorbed and assimilated by hairy roots increased with increase in sucrose in the medium. The importance of sugars in controlling root processes is also provided by reports that sugars help in regulating the expression of a significant number of plant root genes15. For example, a calcium-dependent protein kinase acts in sugar-regulated gene expression in plants16. Although sucrose and glucose may act directly as signalling molecules in some sugar-response pathways, other pathways may sense the level of a different sugar or sugar metabolite. In addition, some sugar-response pathways may sense the rate of flux through a particular metabolic pathway, rather than the absolute levels of sugars or sugar metabolites17.
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### Table 2. Optimization of sucrose concentration on hairy root growth and ginsenoside yield of ginseng. Data collected after 5 weeks of culture using 400 ml conical flask with 100 ml MS medium.

<table>
<thead>
<tr>
<th>Sucrose (%)</th>
<th>FW (g)</th>
<th>Biomass DW (g)</th>
<th>Percentage DW</th>
<th>Ginsenosides (mg/g DW)</th>
<th>Growth rate*</th>
<th>Rg group</th>
<th>Rb group</th>
<th>Total</th>
<th>Ginsenoside yield (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15.40 ± 0.04**</td>
<td>0.69 ± 0.01</td>
<td>4.51</td>
<td>4.14</td>
<td>3.00</td>
<td>4.13</td>
<td>7.13 ± 0.05</td>
<td>49.20</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>21.50 ± 0.50</td>
<td>1.30 ± 0.22</td>
<td>5.59</td>
<td>7.74</td>
<td>2.80</td>
<td>5.22</td>
<td>8.01 ± 0.49</td>
<td>104.13</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>23.29 ± 0.40</td>
<td>1.31 ± 0.01</td>
<td>5.63</td>
<td>7.80</td>
<td>3.20</td>
<td>5.30</td>
<td>6.53 ± 0.24</td>
<td>85.54</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>22.50 ± 0.03</td>
<td>1.83 ± 0.01</td>
<td>8.15</td>
<td>10.92</td>
<td>1.70</td>
<td>3.28</td>
<td>4.98 ± 0.29</td>
<td>91.13</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>21.10 ± 0.80</td>
<td>1.90 ± 0.05</td>
<td>8.60</td>
<td>11.31</td>
<td>1.12</td>
<td>1.56</td>
<td>2.67 ± 0.26</td>
<td>50.73</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>19.80 ± 0.05</td>
<td>2.09 ± 0.06</td>
<td>10.56</td>
<td>12.44</td>
<td>1.02</td>
<td>0.74</td>
<td>1.77 ± 0.24</td>
<td>36.99</td>
<td></td>
</tr>
</tbody>
</table>

*Growth rate was calculated from increase in DW. Values are quotients of DW after cultivation and DW of the inoculum.

**Mean ± standard error of three replicates.

### Table 3. Optimization of sucrose concentration on ginsenosides of ginseng. Data collected after 5 weeks of culture using 400 ml conical flasks with 100 ml MS medium.

<table>
<thead>
<tr>
<th>Sucrose (%)</th>
<th>Rg group</th>
<th>Rb group</th>
<th>Rb group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rg1</td>
<td>Re</td>
<td>Rf</td>
</tr>
<tr>
<td>1</td>
<td>2.54</td>
<td>0.32</td>
<td>0.12</td>
</tr>
<tr>
<td>2</td>
<td>2.50</td>
<td>0.21</td>
<td>0.08</td>
</tr>
<tr>
<td>3</td>
<td>2.29</td>
<td>0.19</td>
<td>0.05</td>
</tr>
<tr>
<td>5</td>
<td>1.53</td>
<td>0.13</td>
<td>0.04</td>
</tr>
<tr>
<td>7</td>
<td>0.99</td>
<td>0.09</td>
<td>0.04</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>0.08</td>
<td>0.95</td>
</tr>
</tbody>
</table>

**Figure 6.** Changes in ginsenoside, starch, reducing sugar and total sugar contents in MS medium with various sucrose concentrations in Panax ginseng hairy root culture.

**Osmotic agents on hairy root growth and ginsenoside production**

As shown in Table 4, ginsenoside content and ginsenoside productivity decreased after treatment with sorbitol and mannitol. Ginsenoside content and ginsenoside productivity decreased with increase in concentration of sorbitol and mannitol in the range of 0–0.5 M. The hairy root dry weight and growth rate increased with 0.1 M mannitol, while 0.1 M sorbitol almost had no influence on ginseng hairy root growth. To respond to a high external osmotic environment, the cells accumulate glycerol, seemingly to compensate for differences between the extracellular and intracellular water potential. Sorbitol and mannitol showed a much higher percentage of retention than glycerol, indicating that membrane permeability to sorbitol and mannitol was lower compared to glycerol. Less leakage of sorbitol during salt stress could be an advantage for the replacement of glycerol by sorbitol. The H+ pump might be acting as a detector or effector in turgor maintenance, the physiological consequences of its up-regulation may be increased uptake of nutrients. In peach, boron is translocated in the phloem as sorbitol–boron–sorbitol and mannitol–boron–mannitol respectively. Under osmotic condition, ion fluxes across the plasma membrane, activation of mitogen-activated protein (MAP) kinase pathways took place and induced production of reactive oxygen species.

**Elicitors on hairy root growth and ginsenoside production**

Tables 5 and 6 show that jasmonic acid strongly increased the accumulation of ginsenoside in hairy roots. With higher
Table 4. Optimization of sorbitol and mannitol on growth and ginsenoside productivity of ginseng hairy root for 5 weeks in 400 ml conical flask supplemented with 100 ml MS medium

<table>
<thead>
<tr>
<th>Treatment (mol)</th>
<th>Biomass</th>
<th>Growth rate*</th>
<th>Ginsenosides (mg/g DW)</th>
<th>Ginsenoside yield (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.61 ± 0.20**</td>
<td>5.37</td>
<td>20.31 ± 0.03</td>
<td>326.99</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>1.60 ± 0.01</td>
<td>5.33</td>
<td>11.43 ± 1.36</td>
<td>182.88</td>
</tr>
<tr>
<td></td>
<td>1.35 ± 0.03</td>
<td>4.50</td>
<td>5.64 ± 0.74</td>
<td>76.14</td>
</tr>
<tr>
<td></td>
<td>0.52 ± 0.08</td>
<td>1.73</td>
<td>3.51 ± 0.47</td>
<td>18.25</td>
</tr>
<tr>
<td>Mannitol</td>
<td>1.77 ± 0.01</td>
<td>5.90</td>
<td>12.11 ± 0.48</td>
<td>214.35</td>
</tr>
<tr>
<td></td>
<td>1.53 ± 0.08</td>
<td>5.10</td>
<td>6.01 ± 0.09</td>
<td>91.95</td>
</tr>
<tr>
<td></td>
<td>0.57 ± 0.11</td>
<td>1.90</td>
<td>3.27 ± 0.67</td>
<td>18.64</td>
</tr>
</tbody>
</table>

*Growth rate was calculated from increase in DW. Values are quotients of DW cultivation and DW of the inoculum. Similar experiments were done three times.

**Mean ± standard error of three replicates.

Table 5. Optimization of jasmonic acid on hairy root growth and ginsenoside productivity of ginseng. Data collected after 5 weeks of culture using 400 ml conical flask with 100 ml MS medium

<table>
<thead>
<tr>
<th>Jasmonic acid (mg/l)</th>
<th>FW (g)</th>
<th>Biomass DW (g)</th>
<th>Percentage DW</th>
<th>Growth rate*</th>
<th>Ginsenosides (mg/g DW)</th>
<th>Ginoside yield (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>30.2 ± 2.30*</td>
<td>1.52 ± 0.02</td>
<td>5.05</td>
<td>7.12</td>
<td>10.31 ± 0.41</td>
<td>5.51 ± 0.48</td>
</tr>
<tr>
<td>1.0</td>
<td>24.5 ± 1.00</td>
<td>1.31 ± 0.02</td>
<td>5.35</td>
<td>6.12</td>
<td>30.08 ± 0.08</td>
<td>5.87 ± 1.21</td>
</tr>
<tr>
<td>2.0</td>
<td>20.0 ± 0.90</td>
<td>1.08 ± 0.03</td>
<td>5.50</td>
<td>5.04</td>
<td>41.59 ± 5.43</td>
<td>6.05 ± 1.02</td>
</tr>
<tr>
<td>5.0</td>
<td>14.1 ± 0.40</td>
<td>0.86 ± 0.01</td>
<td>6.13</td>
<td>4.04</td>
<td>52.98 ± 6.02</td>
<td>5.60 ± 0.20</td>
</tr>
</tbody>
</table>

*Growth rate calculated from increase in DW. Values are quotients of DW after cultivation and the DW of the inoculum. Similar experiments were done three times.

**Mean ± standard error of three replicates.

Table 6. Optimization of jasmonic acid on hairy root ginsenoside productivity of ginseng. Data collected after 5 weeks of culture using 400 ml conical flask with 100 ml MS medium

<table>
<thead>
<tr>
<th>Jasmonic acid (mg/l)</th>
<th>Ginoside (mg/g DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rg1</td>
</tr>
<tr>
<td>0.0</td>
<td>1.63 ± 0.17*</td>
</tr>
<tr>
<td>1.0</td>
<td>1.85 ± 0.28</td>
</tr>
<tr>
<td>2.0</td>
<td>1.73 ± 0.40</td>
</tr>
<tr>
<td>5.0</td>
<td>1.34 ± 0.11</td>
</tr>
</tbody>
</table>

*Mean ± standard error of three replicates.

jasmonic acid concentration, higher ginsenoside content and ginsenoside productivity were obtained. After treatment with 5.0 mg/l jasmonic acid for 7 days, the total ginsenoside content increased about 4 times and ginsenoside productivity improved about twofold (Table 5). However, the ginseng hairy root growth rate decreased with increase in jasmonic acid concentration in the range of 0–5.0 mg/l; this shows that jasmonic acid inhibits hairy root growth. Among ginsenosides, the Rb group showed an increase, while the Rg group was stable. Increase in ginsenosides Rb1, Rb2, Re and Rd was more compared to other kinds of ginsenosides (Figure 6).

In addition, phenolic compounds such as phenylalanine, caffeic acid, catechin, and catechol and glycosides such as chitin, gum karaya, fucoidan, and pepton were also added in the initial medium as elicitors in ginseng hairy root cultures in flasks. After 4 weeks of culture, 300 mg/l peptone showed effective results for the increase in ginsenoside accumulation in hairy root, and the hairy root growth rate was similar to that of control (Table 7). Other elicitors did not show clear effects on ginsenoside increase. Maximum ginsenoside productivity (244.0 mg/l) was obtained after treatment with 300 mg/l peptone for 4 weeks. Addition of 100 mg/l catechin resulted in highest hairy root growth rate (8.06) and ginsenoside productivity was similar to that of control. Caffeic acid (300 mg/l) and fucoidan (200 mg/l) could not be used as elicitors since they inhibited both the hairy root growth and ginsenoside production. The results indicate that 300 mg/l peptone can be used for improving ginsenoside accumulation, whereas 100 mg/l catechin can be employed to increase the hairy root growth. Among the elicitors we tested, jasmonic acid showed the best effect on ginsenoside production. Jasmonates act as stress hormones and play a role in plant...
growth and development. After 48 h of treatment with jasmonate, a general decrease in protein synthesis is caused by the ribosome-inactivating protein JIP 60. Generally, in plant tissues treated with jasmonates, distinct effects are exerted on gene expression. For instance, the amount of small and large subunits of Rubisco proteins decreased with time of incubation in the presence of jasmonic acid. This could be a reason for decrease in the biomass of ginseng hairy roots after treatment with jasmonic acid.

**Medium pH on hairy root growth and ginsenoside production**

Table 8 shows the effects of initial medium pH on ginseng hairy root growth and ginsenoside production in MS medium. The initial pH range of 6.0–6.5 before autoclaving was optimal for hairy root growth and ginsenoside production, the maximum growth rate (7.44) and maximum ginsenoside productivity (239.68 mg/l) were obtained at pH 6.0 and 6.5 respectively. Hairy root growth and ginsenoside production were strongly inhibited when the initial pH was below 4.0 or above 7.0. When the pH is too low, levels of aluminum and hydrogen in the medium are toxic to the root systems of ginseng. If the pH is too high, micronutrients may be bound in forms unavailable for hairy root uptake.

In bioreactor culture, pH is an important factor influencing cell growth and secondary metabolite accumulation. pH 6.5 is the acidic optimal pH of the H+-ATPase, assuming that the NO3-dependent H+ pumping rate accounted for the rate of the electroneutralizing NO3 unipor in plasma membrane. A transport assay, independent of the H+-ATPase, was required to characterize further the intrinsic properties of the NO3 unipor, especially its pH dependence. The NO3 unipor in root plasma membrane vesicles exhibits strong voltage dependence with an optimum E 
v at pH 6.5. Nevertheless, as it is thought to be involved in passive NO3 efflux, the affinity of the unipor may appear high compared with concentrations used to measure the transport activity of root anion efflux channels. From our results on ginseng hairy root culture, the initial pH of the medium should be controlled in the range of 6.5 before autoclaving. Physiological experiments have shown that the kinetic control by H+ ions is exerted at the cytoplasmic domains of the H+ ATPase.

In conclusion, optimization of mineral elements competence in bioreactor cultivation technology may be an increasing nutrient transport mechanism on plasma membrane that is responsible for the growth and biomass production of ginseng hairy roots. It could be due to changes in the structure or expression of ginseng growth regulatory signal transduc-
Figure 7. Large-scale bioreactors (10,000 l) system.

cation phytomolecules. Further studies on differences in structure and expression pattern of ginseng growth from mineral elements ratio may contribute to understanding the enhanced growth and ginsenosides production of ginseng hairy roots in large-scale bioreactors. Finally, increasing ginsenosides by elicitors could allow some uncertainty as to the precise order of the early pathway. Cyclization steps diverging from confirmed intermediate ginsenosides might be new powerful method for improving the yield of useful secondary metabolites production in large-scale bioreactors (Figure 7).


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