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High production of ginsenosides by transformed root cultures of *Panax ginseng*: Effect of basal medium and *Agrobacterium rhizogenes* strains

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Successful transformation of *Panax ginseng* was achieved when petiole segments were infected with *Agrobacterium rhizogenes* ATCC 15834 and MAFF 03-01724. Transformed roots were obtained after galls developed at infected sites. The root morphology, growth and ginsenoside productivity of roots transformed with different bacterial strains differed, and the roots from *A. rhizogenes* ATCC 15834 grew better and produced much more ginsenosides. Using the ATCC transformed root clone, various liquid culture media were tested to determine the optimum culture medium for ginsenoside production. The root growth was optimum in phytohormone-free Gamborg B5 liquid medium, however highest content of ginsenosides (a total of five ginsenosides 1.88 % dry weight) was obtained when the roots were cultured in half-macro-salt strength Gamborg B5 liquid medium. Growth of the roots over a period of 8 weeks showed that their fresh and dry weight continued to increase. The ginsenoside Rb1 content was optimum after 5 weeks of culture. Ginsenoside Rc content began to decrease slightly after the third week of culture. Ginsenosides Rd and Rg1 contents fluctuated, while ginsenoside Re content continued to rise throughout the 8 weeks of culture. Ginsenoside production, however, did not peak within the 8 weeks of culture.

Keywords: *Panax ginseng*, transformed roots, *Agrobacterium rhizogenes*, ginsenoside content

Introduction

The root extracts of ginseng, *Panax ginseng* C.A. Meyer, is traditionally used as a health tonic for preserving life and longevity[1]. Its roots contain saponins called ginsenosides. Among its many therapeutic properties, *P. ginseng* is said to have the ability to counter stress, cancer, hypothermia and hyperglycemia. It not only stimulates the central nervous system but also acts as a sedative[1]. The demand for ginseng roots and extracts has increased over the years. In addition, the long and difficult cultivation method of this plant has resulted in the high costs of ginseng roots.

Plant tissue culture may be an alternative to conventional cultivation methods for producing valuable plant chemicals. Biosynthesis of secondary metabolites using cell cultures was first suggested in the 1970s. One of the problems encountered when using cell cultures is the low rate of secondary metabolite production after prolonged culture. Screening for cells with high productivity must be regularly conducted. In *P. ginseng*, ginsenoside production from callus cultures has been reported by Furuya et al.[2-4], however, the use of phytohormones was crucial in maintaining growth of cells and its saponin productivity.

The use of plant organ culture has its advantages over cell cultures in that it has the ability to accumulate secondary metabolites more easily. This is especially true in bioactive compounds which accumulate in organized tissues such as roots. Transformed root cultures have a further advantage over cultured adventitious roots because transformed roots grow more rapidly in culture medium without any phytohormones. Researchers have demonstrated that transformed roots of *P. ginseng* accumulated the same type of ginsenosides as field-cultivated roots[5-7]. Ginsenoside contents of their transformed roots were equal or higher than field-grown roots on a dry weight basis. In this study, the transformation of *P. ginseng* petiole segments was successfully carried out using *Agrobacterium rhizogenes* ATCC 15834 and MAFF 03-01724. Growth and ginsenoside production of *P. ginseng* transformed root clones in various basal media are reported.

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Materials and Methods

Establishment and maintenance of hairy root cultures Direct infection of petiole segments of a 4-year old *Panax ginseng* plant with *Agrobacterium rhizogenes* ATCC 15834 and MAFF 03-01724 was carried out according to methods described by Yoshimatsu et al.[8]. Transformed roots developed from the galls after six weeks culture on Gamborg B5 (B5)[9] medium supplemented with 1.0 mg/l indole-3-butyric acid (IBA). Nine ATCC 15834 and three MAFF 03-01724 transformed root clones were obtained. One transformed root clone of each bacterium strain (ATCC 15834 or MAFF 03-01724) which showed good growth was selected for further experiments. Transformation was confirmed by opine detection using paper electrophoresis[10] and PCR analysis for T-DNAs[8]. The transformed *P. ginseng* root clones were maintained in a half macro salt strength Murashige and Skoog (1/2 MS)[11] liquid medium supplemented with 3% sucrose without phytohormone on a gyratory shaker (100 rpm) in the dark at 25±2℃. Approximately 0.1g (fresh weight) of transformed roots was inoculated in a 100 ml Erlenmeyer flask containing 50 ml of liquid medium. Cultures were transferred to fresh media every 4 weeks.

Culture media for optimum growth and ginsenoside production

Transformed roots of *P. ginseng* were inoculated into different phytohormone-free liquid media. Culture media containing a half and full macro salt strength MS (1/2 MS and MS), Gamborg B5 (1/2 B5 and B5), Woody Plant (1/2 WP and WP)[12], White (1/2 W and W)[13] and Root Culture (1/2 RC and RC)[14] media were used. All media tested were supplemented with 3 % sucrose. The roots (0.1g fresh weight) were inoculated into a 100 ml Erlenmeyer flask containing 50 ml of liquid medium and placed on a gyratory shaker at 100 rpm in the dark at 25±2℃. After 4 weeks of culture, the roots were harvested. Growth (fresh and dry weight) and ginsenoside contents were determined. All subsequent experiments were conducted using the similar culture conditions unless otherwise stated.

Time course and ginsenoside production in 1/2 B5 liquid medium

Transformed roots were cultured in 1/2 B5 liquid medium and flasks were incubated on a gyratory shaker in the dark. Growth and ginsenoside contents were determined weekly.

HPLC analysis for ginsenosides

Ginsenoside fraction was extracted as reported previously[8]. Freeze dried transformed root tissues were ground prior to extraction. The powdered sample (50mg each) was extracted with methanol (7ml) at 70℃ for 1 h. This procedure was repeated three times. The combined extract was centrifuged and evaporated to dryness. The residue was dissolved in 2 ml water and adsorbed on a Sep-Pak C18 cartridge (MILLIPORE®). The cartridge was then washed with water (5ml) and 30 % methanol (5ml) and eluted with 5ml methanol. After evaporation to dryness, the residue was redissolved in 1 ml methanol and filtered. Ginsenosides Rb1, Rc and Rd in the resulting supernatant were analyzed by HPLC using either column: TSKgel ODS 80Ts, 4.6 mm i.d.x150 mm (TOSOH); flow rate: 1.1 ml min^{-1}; temperature: 40 ℃; solvent: acetonitrile / water (3/7); detection : UV 203 nm; retention time for ginsenosides: Rb1=13.01 min, Rc=16.73 min, Rd=35.01 min or column Wakosil II ODS 3C18 HG, 4.0 mm i.d. x 100 mm (Wako Chemicals, Japan); flow rate: 0.45 ml min^{-1}; temperature: 40 ℃; solvent: acetonitrile / water (3/7); detection : UV 203 nm; retention time for ginsenosides: Rb1=13.36 min, Rc=17.06 min, Rd=38.60 min. Ginsenosides Rg1 and Re were analyzed separately under these HPLC conditions using either column: Hibar Mightysil RP-18, 4.6 mm i.d. x 150 mm (MERCK); flow rate: 0.75 ml min^{-1}; temperature: 40 ℃; solvent: acetonitrile / water (2/8); detection : UV 203 nm; retention time for ginsenosides: Rg1=29.03 min, Re=30.40 min; or column Wakosil II ODS 3C18 HG, 4.0 mm i.d. x 100 mm (Wako Chemicals, Japan); flow rate: 0.45 ml min^{-1}; temperature: 40 ℃; solvent: acetonitrile / water (2/8); detection : UV 203 nm; retention time for ginsenosides: Rg1=33.34 min, Re=35.10 min or column TSKgel ODS 80Ts, 4.6 mm i.d. x 150 mm (TOSOH); flow rate: 1.1 ml min^{-1}; temperature: 40 ℃; solvent: acetonitrile / water (2/8); detection : UV 203 nm; retention time for ginsenosides: Rg1=25.13 min, Re=26.47 min.

Results and Discussion

The transformation of *P. ginseng* using *Agrobacterium rhizogenes* has been shown to be more difficult than transformation of other plant species. Yoshikawa and Furuya[5] tried various methods including the use of excised leaf discs, root discs and *in vitro* plantlets for infection with *Agrobacterium rhizogenes* A4, agropine strain. Transformed roots were successfully obtained when *A. rhizogenes* A4 was co-cultivated with ginseng callus which had been pretreated with cellulase and macerozyme. Ko et al.[6] infected 3-year old plants cultivated in soil with *A. rhizogenes* ATCC 15834 and sterilized root discs. Inomata et al.[7] used 1-year old field root sections for *A. rhizogenes* ATCC 15834 inoculation. In our experiments, petiole segments from a 4-year old plant were used. Direct infection

of *A. rhizogenes* resulted in the formation of galls at the infected sites after 2 weeks on 1/2 MS solid medium. Transformed roots could only develop after the excised galls were transferred to medium containing 1.0mg/l IBA. The supplement of auxin has been beneficial in some plant species which were recalcitrant for *Agrobacterium*-mediated transformation. Agropine and mannopine (ATCC 15834) or mikimopine (MAFF 03-01724), opines specific to these *Agrobacterium* strains, were detected in the transformed roots using paper electrophoresis (data not shown).

The root morphology of ATCC and MAFF clones was completely different from each other. Seven of the ATCC clones were thick roots and exhibited remarkable lateral root branching (Fig. 1A) and two of them formed callus, whereas all the MAFF clones were thinner than ATCC clones and elongated showing less lateral root branching (Fig.1B).

One of each clone which showed the best growth among the clones was selected and their growth (Fig.2A) and ginsenoside productivity (Fig.2B and C) were compared after 8 weeks of culture in 1/2 MS liquid medium in the dark. The ATCC roots grew slightly better than MAFF roots (1.4 fold as fresh weight basis, Fig. 1, 2A) and superiority of ATCC clone was much more pronounced in its ginsenoside productivity (Fig. 2B and C). When the contents of ginsenosides Rb1, Rc, Rd, Re and Rg1 were compared, the ATCC clone accumulated 5 times (4.1 mg/culture) higher than the MAFF clone (0.8 mg/culture). Different ginsenoside pattern was also observed between the clones (Fig.2C). The main ginsenoside in ATCC was ginsenoside Rb1, while that in MAFF was ginsenoside Rg1 (Fig. 2B). The ATCC clone was chosen for further experiments because of its high ginsenoside productivity.

Effect of culture conditions (basal media, illumination and sucrose concentration) on the growth and ginsenoside productivity of ATCC clone was investigated (Fig.3-5). Growth of the roots measured by fresh and dry weights after 4 weeks of culture was optimum in B5 culture medium (Fig.3A).

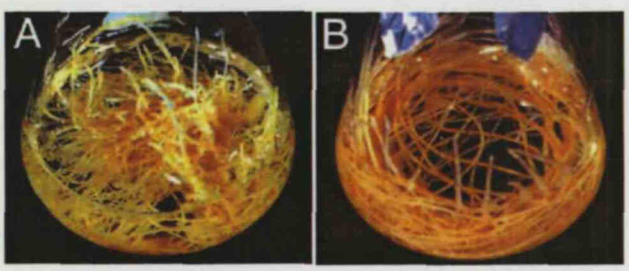

Fig.1.Transformed roots of *Panax ginseng* induced by *Agrobacterium rhizogenes* ATCC 15834 (A) and MAFF 03-01724 (B) cultured in 1/2 MS liquid medium at 25℃ in the dark for 8 weeks

Inferior growth was observed in the roots cultured in low concentration of salts media such as W and RC. Ginsenoside contents in the roots grown in W and RC media were relatively higher than those of the roots grown in other culture media (Fig. 3B). It was interesting to note that ginsenoside contents of transformed roots cultured in a half macro salt strength media were higher than those in full macro salt strength media (Fig. 3B). These results indicate that ginsenoside contents of ginseng transformed roots were higher when culture medium with a low salt concentration was used. *P. ginseng* transformed roots produced an optimum yield of ginsenosides in 1/2 B5 medium (Fig.3C). Our results show that when growth of the transformed roots is low, ginsenoside contents are high and vice versa. Physiological stress on roots due to low growth may have caused an increase in ginsenoside contents. In suspension cultures of *P. ginseng*, the increase in cell growth was not always accompanied by an increase in saponin production[4, 15]. In both cases, higher growth was obtained when the culture conditions were modified, but saponin production did not increase accordingly. On the contrary, Yoshikawa and Furuya[5] demonstrated that the addition of IBA and kinetin could increase both growth and saponin production in both cultured roots and

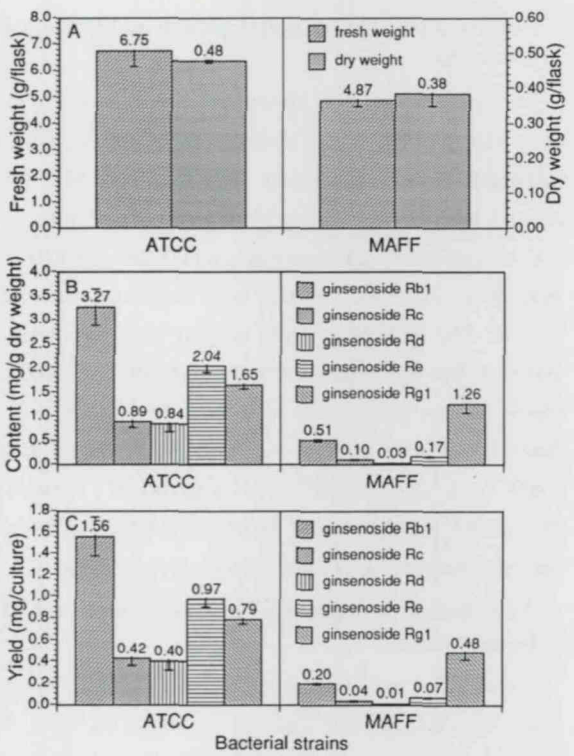

Fig.2.Growth (A), ginsenoside content (B) and yield (C) of *P. ginseng* transformed roots cultured in 1/2 MS liquid medium for 8 weeks in the dark
Bars represent standard errors.

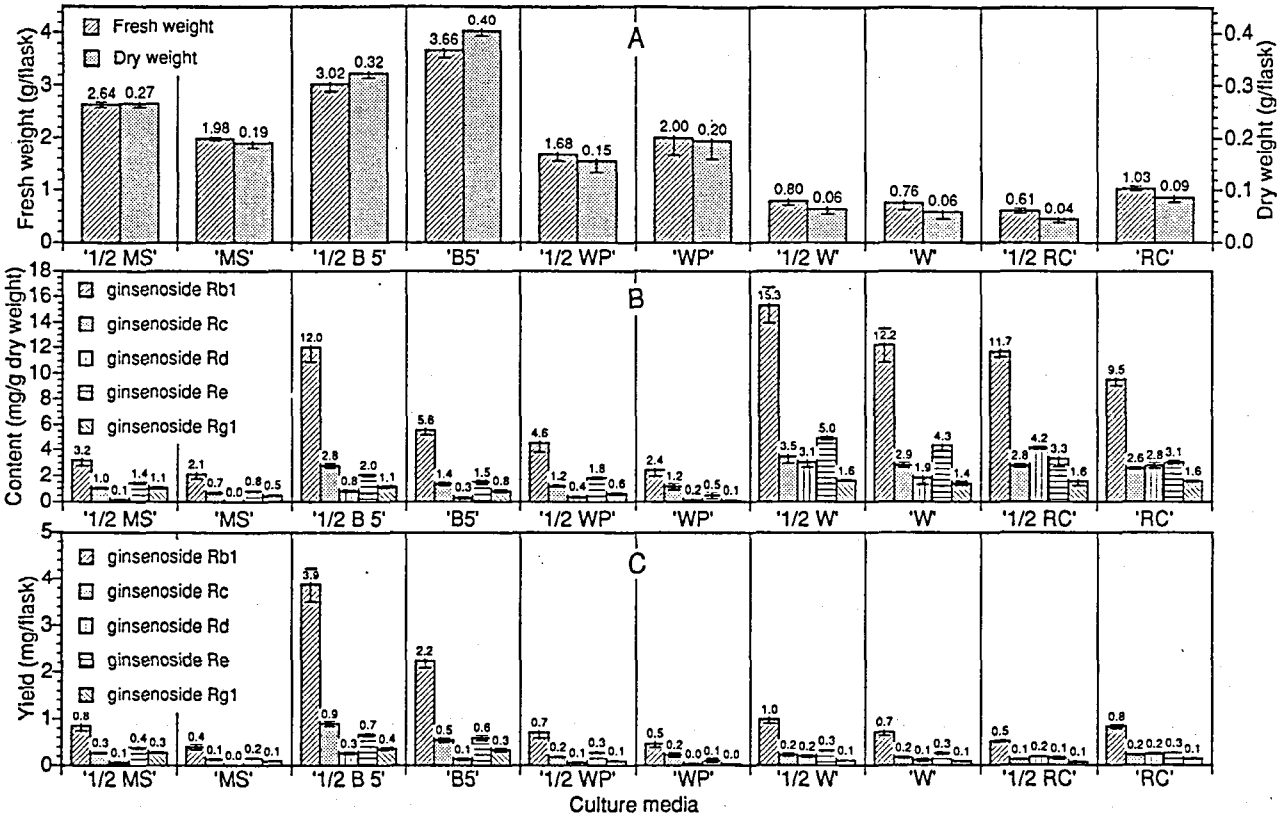

Fig. 3. Growth (A), ginsenoside content (B) and yield (C) of *P. ginseng* transformed roots (ATCC) cultured in various liquid media for 4 weeks in the dark

Bars represent standard errors.

transformed roots of *P. ginseng*.

Growth rates of ginseng callus cultures varied from 1.5 to 8.2 fold after 20 to 30 days of culture[4,5,16]. Contents of ginsenosides Ra, Rb1, Rb2, Rc, Rd, Re, Rf, Rg1, Rg2 and Rh measured 0.65 % dry weight[5]. In root cultures, a growth rate of 4 to 6 fold was achieved after 21 to 30 days[5, 16, 17]. Content of ginsenosides Ra, Rb1, Rb2, Rc, Rd, Re, Rf, Rg1, Rg2 and Rh varied from 0.38 to 0.91 % dry weight[5] and 0.53 % dry weight (ginsenosides Rb1, Rb2, Rc, Rd, Re, Rf and Rg1)[16]. Higher growth exhibited by transformed roots of *P. ginseng* is an important consideration for the production of ginsenosides in culture. Growth of *P. ginseng* roots transformed with *A. rhizogenes* A4 was reported to be much lower than those in many other plant species[5]. *P. ginseng* roots transformed with *A. rhizogenes* ATCC 15834 exhibited higher growth rates: 20-fold growth after 30 days[6]; 31-fold growth in 32 days[7]. In our experiments, the fresh weight after 4 weeks in 1/2 B5 liquid medium was 30 fold (Fig.3A). The ginsenoside content of transformed roots previously reported varied from 0.36 - 0.95 % dry weight[5], 0.47-0.82 % dry weight[6] and 1.19-1.69 % dry weight[7]. By comparison, it is noted that transformed roots could produce a higher yield of ginsenoside than callus or root culture. Furthermore, the total content of five ginsenosides (Rb1, Rc, Rd, Re and Rg1) in this

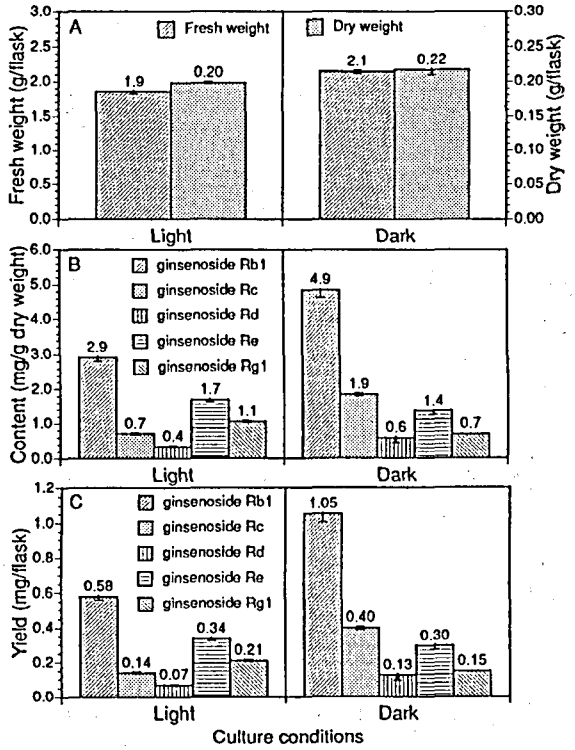

Fig. 4. Growth (A), ginsenoside content (B) and yield (C) of *P. ginseng* transformed roots (ATCC) cultured in 1/2 B5 liquid media for 4 weeks either under 16 h light (68 μ Em^{-2}s^{-1}) or in the dark

Bars represent standard errors.

transformed root clone was 18.8 mg/g dry weight (1.88 % dry weight). The total content of the same five ginsenosides in a 4-year old field-cultivated root was 14.2 mg/g dry weight (1.42 % dry weight). The ginsenoside content of hairy roots can equal or exceed that of a field plant as shown by results obtained here and elsewhere[5-7].

Growth of *P. ginseng* transformed roots was higher in the dark than that under the light (16h photoperiod, 68 $\mu Em^{-2}s^{-1}$) (Fig. 4A). The morphology of light and dark-grown roots was not much different. Ocassionally, purple pigments appeared in the aerial parts of the roots which were exposed to light. Similar results were observed in rhizoids developing from ginseng callus under a 16 h photoperiod[18]. The formation of red pigments in ginseng callus cultures grown under a 12 h photoperiod was also reported by Odnevall and Björk[19]. The ginsenoside contents of transformed roots grown in the dark and under light are shown in Fig. 4B. Ginsenosides Rb1, Rc and Rd contents of the roots grown in the dark were higher than those in the roots grown under the light. On the other hand, ginsenosides Re and Rg1 contents were lower in the dark (Fig.4B). Ginsenoside yield of transformed roots grown in the dark was higher than that under the light (Fig.4C). This indicates that under different light conditions, production of different ginsenosides can be optimized. This may be useful when specific ginsenosides are required. Although the effect of light on *Panax ginseng* transformed roots have not so far been studied, Choi[1] reported that light inhibited growth of ginseng callus though ginsenoside contents were not measured. In another study, growth of ginseng callus in various phytohormone-supplemented media was higher under the light (2500-4000 lux, 16 h photoperiod) than in the dark[4]. The ginsenoside contents did not exhibit any particular trend due to light. Odnevall and Björk[19] showed that light influenced the presence of specialized cells in ginseng callus and that the formation of specialized cells was essential for ginsenoside accumulation. Light therefore had a stimulatory effect on ginsenoside accumulation in callus cultures of ginseng. This stimulatory effect of light was not seen in callus-derived root cultures[19].

Fig.5A shows the effects of sucrose on the growth of ginseng transformed roots. Growth of the roots depended entirely on the concentration of sucrose. This was demonstrated by the poor growth of the roots in culture media without sucrose (Fig.5A). Culture media supplemented with 1 % sucrose produced the roots with high fresh weight but low dry weight. This indicates

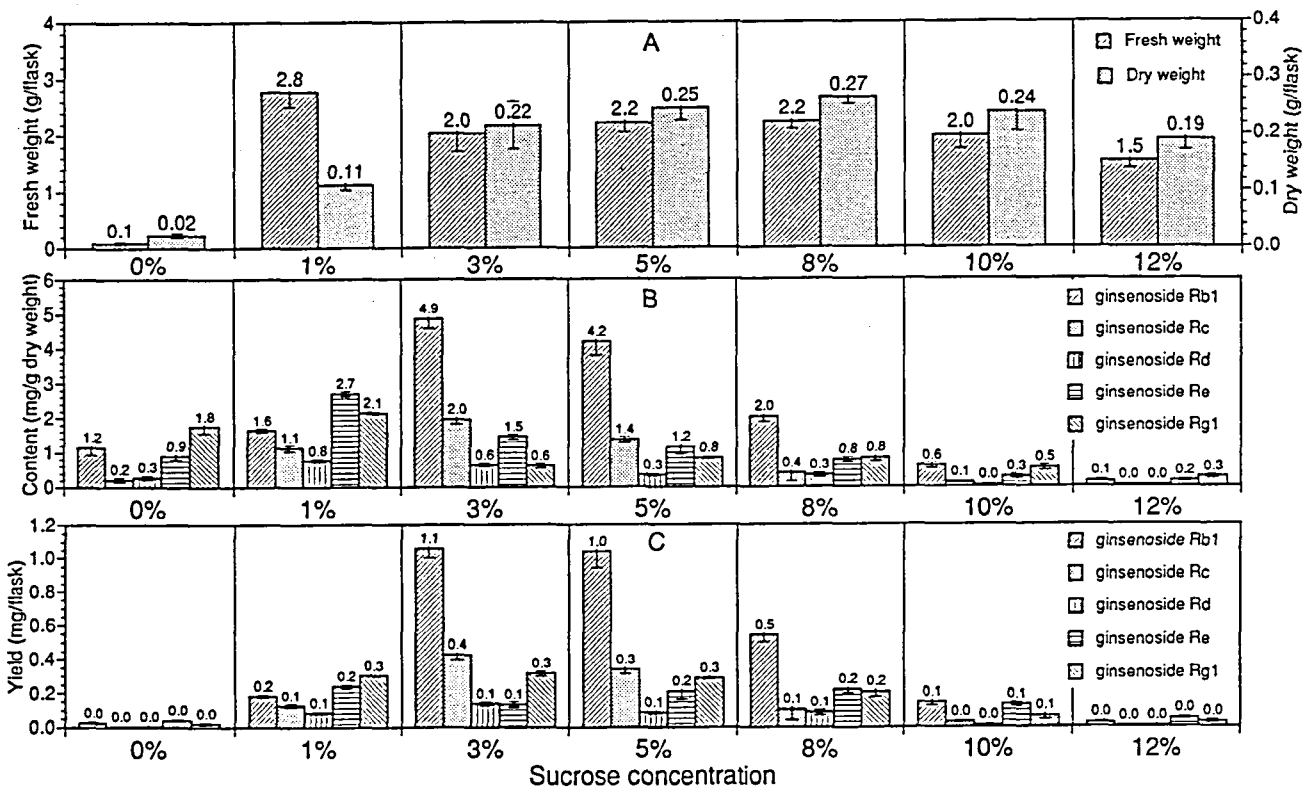

Fig. 5. Growth (A), ginsenoside content (B) and yield (C) of *P. ginseng* transformed roots (ATCC) cultured in 1/2 B5 liquid media containing various sucrose concentration for 4 weeks in the dark
Bars represent standard errors.

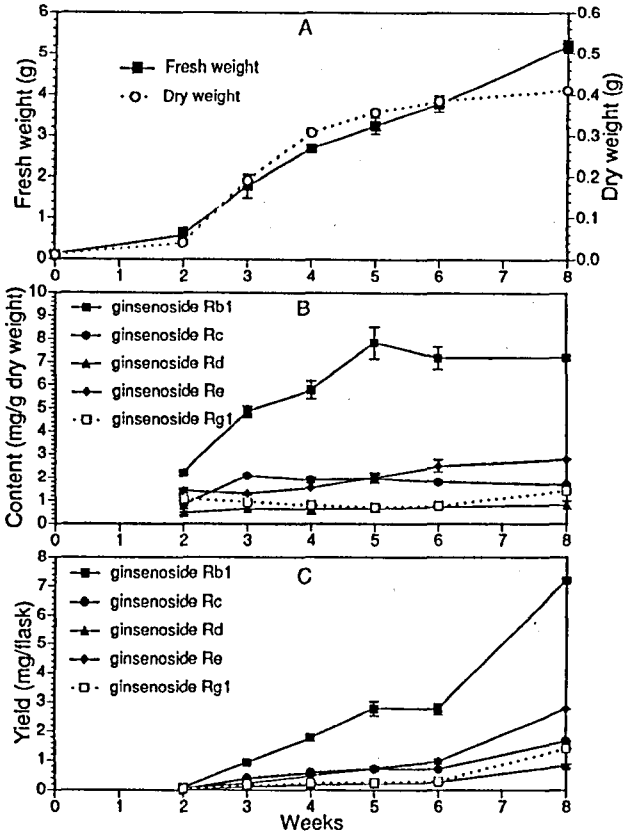

Fig. 6. Growth (A), ginsenoside content (B) and yield (C) of *P. ginseng* transformed roots (ATCC) cultured in 1/2 B5 liquid media containing 3 % sucrose in the dark over a period of 8 weeks
Bars represent standard errors.

that the fresh weight increase was more likely due to the accumulation of water in the root cells cultured in 1 % sucrose. Although growth of the roots were optimum in media with 5 % and 8 % sucrose, the ginsenoside content and yield of ginsenosides per culture flask were highest in media supplemented with 3 % sucrose (Fig.5B and C). The individual ginsenoside content changed with the concentration of sucrose used in the culture media. Ginsenosides Rd, Re and Rg1 levels were highest when 1 % sucrose was used while ginsenosides Rb1 and Rc levels peaked when 3 % sucrose was used (Fig.5B). Ginsenoside contents of transformed roots grown in 10 % and 12% sucrose were very low (Fig.5B). Inomata et al.[7] found that growth of transformed ginseng hairy roots slowed down after the initial 2 weeks of culture in 3 % sucrose. This was due to the conversion of sucrose to glucose and fructose. Periodic changes of culture medium containing 3 % sucrose could maintain high growth rates and increase ginsenoside contents of transformed roots[7]. Higher concentrations of sugars have been shown to retard growth of ginseng callus cultures[1, 16]. This may have been caused by the high osmotic pressure exerted by high sugar concentrations[16].

The growth of ginseng transformed roots was measured over a period of 8 weeks (Fig.6A). Fig. 6A shows that fresh and dry weights continued to increase throughout the culture period. By the 8th week of culture, the original inoculum of 0.1 g of fresh transformed roots had grown to 5.2 g (ca. 52 times). Dry weight increased approximately 41 times in 8 weeks of culture (Fig.6A). The ginsenoside Rb1 content was optimum after 5 weeks of culture (Fig.6B). Ginsenoside Rc content began to decrease after the 3rd week of culture. The ginsenosides Rd and Rg1 contents fluctuated whereas the ginsenoside Re content continued to rise during the 8 weeks of culture. Ginsenoside production, however, did not peak within the 8 weeks of culture. Using this data, a suitable culture period may then be chosen for the production of ginsenosides. This pattern of ginsenoside accumulation was slightly different from that reported by Ko et al.[6] and Inomata et al.[7]. In the transformed root cultures established by Ko et al.[6]: ginsenoside Rb1 contents reached a maximum after 10 days then decreased to half its content by the 30th day, ginsenosides Re and Rg1 contents reached their maximum after 30 days whereas ginsenosides Rc and Rd maintained the same levels. Studies conducted by Inomata et al.[7] showed that ginsenoside contents remained at the same level within the 30 days of culture. When the transformed roots were cultured with culture medium exchange every 7 days, total ginsenoside content reached its highest levels after 2 weeks[7].

Our results have shown that the use of transformed roots of *P. ginseng* may be more feasible for the production of ginsenosides *in vitro* when compared to callus or root cultures if growth and ginsenoside production are the only two criteria used for evaluation. Unlike callus and root cultures, transformed root cultures do not depend on exogenously-supplied phytohormones to maintain growth or the capability for saponin production. Results from this study show that the culture of ginseng transformed roots in 1/2 B5 medium without phytohormones for 4 weeks could proliferate 30 fold its original inoculum. There was no need to supply fresh media every week and the ginsenoside content was higher than field-cultivated roots (1.88 % dry weight).

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