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Somatic Embryogenesis and Ginsenoside Production of *Panax ginseng* in Phytohormone-free Medium

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Embryogenic cultures of *Panax ginseng* were established without using phytohormones. Somatic embryos developed from the roots of an *in vitro* seedling and from excised leaf and petiole segments cultured in half-macro-salt strength Murashige and Skoog medium. Excised leaf and petiole segments were obtained from *in vitro* germinated seedlings. Plantlets were subsequently obtained from developing somatic embryos in phytohormone-free media. Shoot formation from somatic embryos was influenced by light intensity. The rate of growth and frequency of embryogenesis were improved when cut-up embryogenic tissues were inoculated into liquid media in the dark. The ginsenoside contents of a 4 year-old field-cultivated root, seedlings from zygotic embryos, somatic embryos and embryogenic tissues were determined and compared. Somatic embryos contained 1.7 times the amount of ginsenoside Rb1 and 2.3 times the amount of ginsenoside Re compared to seedlings from zygotic embryos. Ginsenoside Rd, which was absent in the seedlings derived from zygotic embryos, was detected in somatic embryos. Higher ginsenosides Rd and Rg1 levels were found in embryogenic tissues grown on solid media than in tissues grown in liquid media. The total ginsenoside yields, including the ginsenosides Rb1 and Rg1 levels of cut-up embryogenic tissues, were higher than those of clump tissues.

Keywords: *Panax ginseng*, somatic embryogenesis, phytohormone-free, ginsenoside contents

Introduction

Panax ginseng C.A. Meyer is a valuable medicinal plant, of which roots are used as a traditional tonic to preserve life and longevity[1)] . Its pharmacological activity is attributed to the triterpene saponins, namely ginsenosides, produced in the root [2)]. Propagation of ginseng by conventional methods is a difficult and long process, making the ginseng roots an expensive commodity. In the field, seeds are used for plant propagation and this often results in the variable quality of the roots. *In vitro* propagation via somatic embryogenesis represents a promising method in obtaining clonal plants. There have been numerous reports on somatic embryogenesis in *P. ginseng*, however in most of papers published, the use of phytohormones in the media was essential in achieving embryogenesis[1-11)] . Recently, Choi and Soh[12)] reported somatic embryogenesis in the cultures of *P. ginseng* immature zygotic embryos without phytohormone and they subsequently reported regenerative ability of somatic embryos from cotyledons[13)] . However in their system, the somatic embryos induced from cotyledonous explants without phytohormone ceased to grow and required either gibberellic acid or chilling treatment for germination[13, 14)] . In this paper, somatic embryogenesis, embryo propagation via secondary embryogenesis and plantlet formation of *P. ginseng* occur readily in phytohormone-free medium. In addition, ginsenoside content of these somatic embryos was compared to seedlings derived from zygotic embryos and a 4-year old field-cultivated root.

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Materials and Methods

Establishment of embryogenic cultures

Immature seeds of *Panax ginseng* C.A. Meyer were surface sterilized in 75 % ethanol for 30 seconds and then in 2 % (v/v) sodium hypochlorite solution with Tween 20 (1 drop / 40 ml) for 10 min. Seeds were rinsed three times with sterile deionized water and cultured on 0.5 % agar medium containing 0.5 % sucrose and incubated in the dark at 25 ± 2 ℃. After 9 to 10 months of culture, germination occurred. Seedlings removed of leaves, excised leaf and petiole segments from seedlings were transferred to phytohormone-free (HF) a half macro salt strength Murashige and Skoog (1/2 MS)[15)] solid medium supplemented with 3 % sucrose and 0.2 % Gelrite and cultured under dim light (16 h photoperiod, 9 $\mu Em^{-2}s^{-1}$) at 25 ± 2 ℃.

After 1 month, embryogenic tissues formed on the surface of the root of the seedling (Pg, Fig. 1A). Leaf and petiole segments excised from seedlings gave rise to embryogenic tissues (Pga and Pgb, respectively) on HF 1/2 MS solid medium.

Maintenance of embryogenic cultures

Embryogenic cultures of *P. ginseng* derived from root of the seedling (Pg), leaf (Pga) and petiole (Pgb) could be maintained via secondary embryogenesis on HF 1/2 MS solid medium at 25 ± 2 ℃ under dim light (16 h photoperiod, 9 μ Em^{-2}s^{-1}). Approximately 0.4 g of embryogenic tissues was inoculated into culture tubes (40 i.d. x 130 mm, 30 ml solid medium) and transferred to fresh HF 1/2 MS medium every 8 weeks.

Effect of light on frequency of somatic embryogenesis and shoot formation

Embryogenic tissues (Pg) were inoculated into culture tubes containing HF 1/2 MS solid medium as described above. Cultures were subjected to full light at 57 μ Em^{-2}s^{-1}, dim light at 9 μ Em^{-2}s^{-1} or complete darkness. After 6 weeks, frequency of somatic embryogenesis and shoot formation were determined.

Effect of cutting up embryogenic tissues prior to culture on solid or in liquid media

Embryogenic tissues (Pg) grown on HF 1/2 MS solid medium were prepared into clumps (10 - 20 mm) or into small pieces (1 - 2 mm, cut-up using a scalpel). On solid media (0.2 % Gelrite), 0.4 g of tissues was inoculated into a culture tube (40 i.d. x 130 mm, 30 ml medium). In liquid media, 0.5 g of tissues was inoculated into a 100 ml Erlenmeyer flask containing 50 ml liquid media. Culture tubes were incubated as described above and flasks were incubated at 25 ± 2 ℃ in the dark on a gyratory shaker at 100 rpm. After 6 weeks, growth (fresh weight) and somatic embryogenesis frequency were determined.

Effect of light on cut-up embryogenic tissues in liquid media

Cut-up embryogenic tissues were cultured in 1/2 MS liquid medium with light (16 h photoperiod at 68 μ Em^{-2}s^{-1}) or in the dark. Small pieces of tissues (0.5 g) were inoculated into a 100 ml Erlenmeyer flask containing 50 ml liquid media and flasks were incubated at 25 ± 2 ℃ in the dark on a gyratory shaker at 100 rpm. After 6 weeks, growth (fresh weight) and somatic embryogenesis frequency were determined.

HPLC analysis for ginsenosides

Ginsenoside fraction was extracted as reported previously[16]. Freeze dried tissues (50 mg) of a 4-year old field-cultivated root, seedlings from zygotic embryos (2 months after germination), somatic embryos (2 months of culture in 1/2 MS liquid media) and embryogenic tissues (6 weeks of culture in 1/2 MS media) were extracted with methanol (7 ml) at 70 ℃ for 1 h. This procedure was repeated three times. The combined extract was centrifuged and the supernatant was evaporated to dryness. The residue was dissolved in 2 ml water and adsorbed on a Sep-Pak C18 cartridge (MILLIPORE®). The cartridge was washed with water (5 ml) and 30 % methanol (5 ml) and eluted with 5 ml 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 [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 (3/7); detection : UV 203 nm; retention time for ginsenosides: Rb1=13.01 min, Rc=16.73 min, Rd=35.01 min]. Ginsenosides Rg1 and Re were analyzed by HPLC conditions using either 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: 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.

Results

Establishment of embryogenic cultures

Immature seeds just after harvest from the field-grown plants germinated after a long culture period (9 - 10 months) on agar-sucrose medium at 25 ℃ in the dark. Embryogenic tissues (Pg) developed on the surface of the seedling root when the seedlings were transferred and cultured on 1/2 MS solid medium without any phytohormones for 1 month (Fig. 1A). Leaf and petiole segments excised from the seedlings gave rise to embryogenic tissues (Pga and Pgb, respectively) on 1/2 MS solid medium without any phytohormones. The frequency of embryogenesis was highest in the tissues derived from the seedlings (Pg), followed by the tissues derived from the petiole (Pgb) and leaf (Pga) (Fig. 2A). A total of 82 somatic embryos could develop via secondary embryogenesis from a piece of embryogenic tissue (Pg) of 0.4 g cultured for 6 weeks under dim light (Fig. 1B). Subsequently plantlet formation via

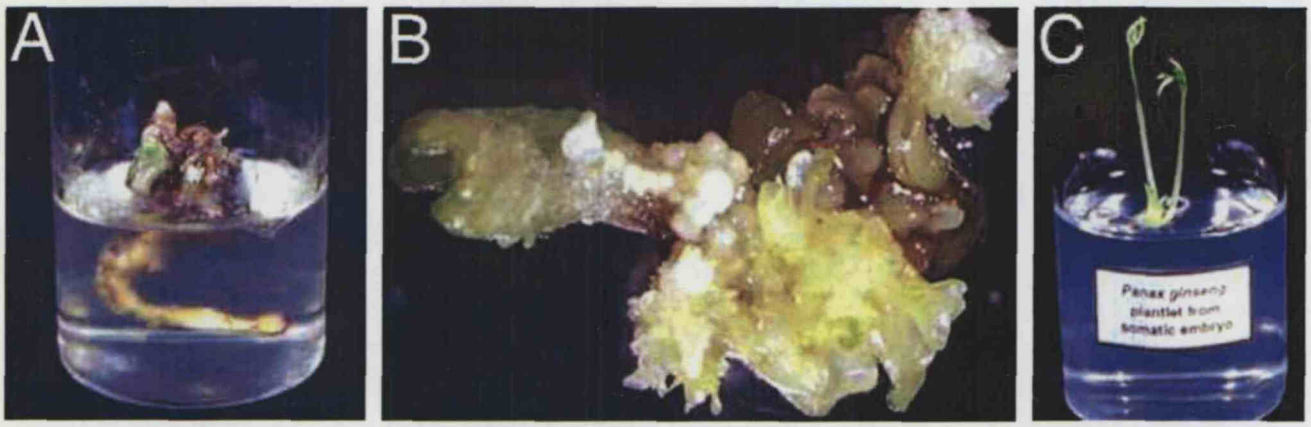

Fig. 1. **Somatic embryogenesis of *Panax ginseng* in phytohormone-free medium A, embryogenic tissues formed on the surface of the root part of the plantlet; B, further somatic embryos could be obtained via secondary embryogenesis; C, somatic embryos developed into plantlets without the use of phytohormones**

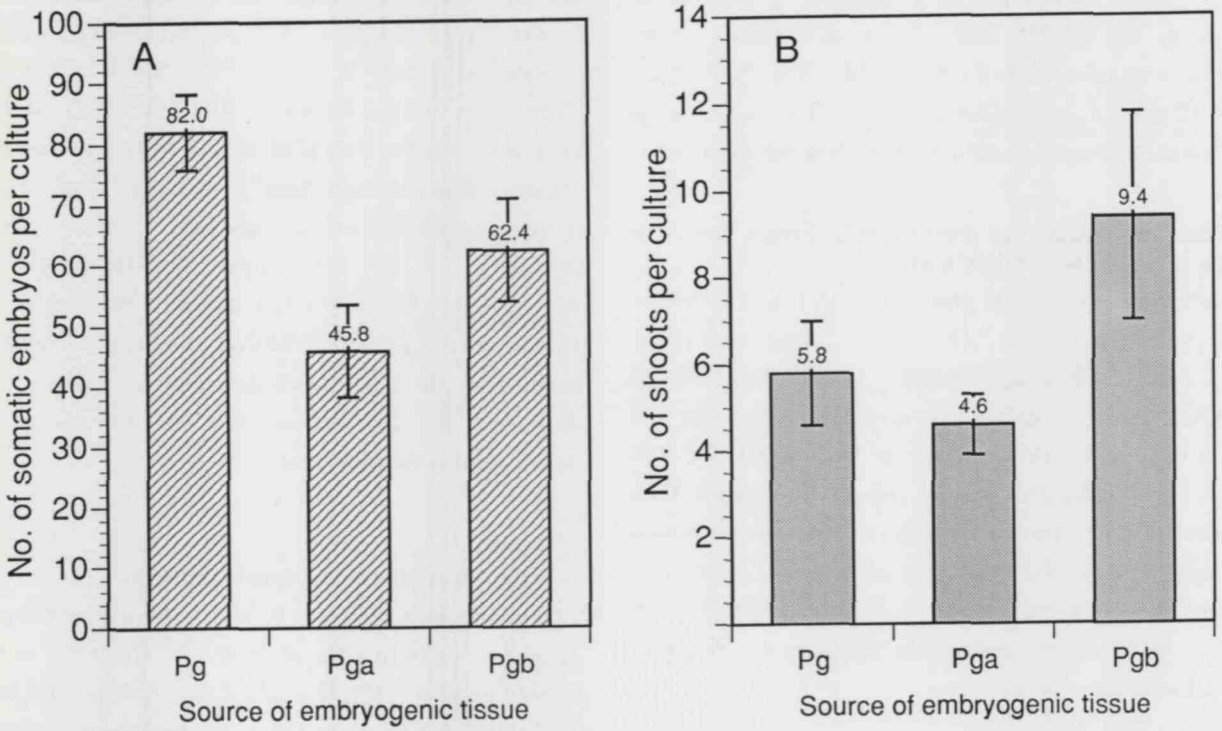

Fig. 2. **Influence of plant sources on the frequency of somatic embryogenesis (A) and shoot formation (B) of *P. ginseng* after 6 weeks of culture on 1/2 MS solid medium under dim light condition**
Bars represent standard errors.

shoot formation in germinating somatic embryos occurred on phytohormone-free medium (Fig. 1C). Highest number of shoots per culture was obtained from somatic embryos of petiole tissues (Pgb) (Fig. 2B). *P. ginseng* plantlets could be transplanted into pots and acclimatized in a phytotron (25 ± 1 ℃, 35 $\mu Em^{-2}s^{-1}$). All further experiments were carried out using Pg tissues as this source of embryogenic tissue produced the highest number of somatic embryos.

Effect of light on frequency of somatic embryogenesis and shoot formation

Number of somatic embryos formed per culture was optimum when embryogenic tissues were cultured on 1/2 MS solid medium in the dark (Fig. 3A). Full light intensity at 57

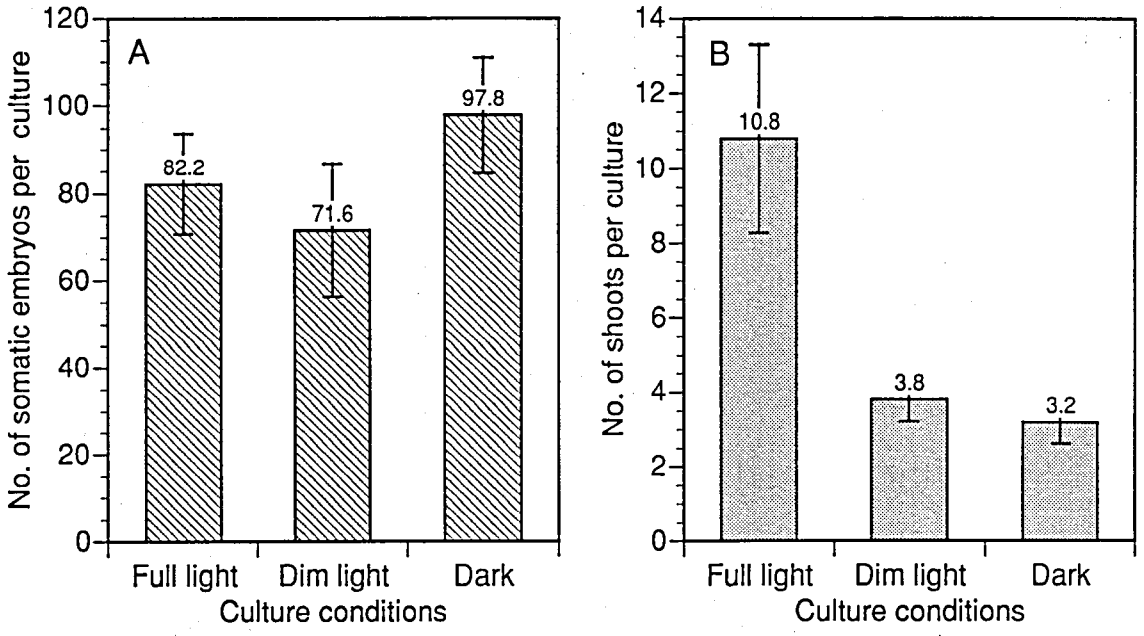

Fig. 3. Effect of light on the frequency of somatic embryogenesis (A) and shoot formation (B) of *P. ginseng* after 8 weeks of culture on 1/2 MS solid medium
Bars represent standard errors.

$\mu Em^{-2}s^{-1}$ was found to be beneficial for the shoot formation in somatic embryos (Fig. 3B).

Effect of cutting up embryogenic tissues prior to culture on solid or in liquid media

Growth of embryogenic tissues cultured in 1/2 MS liquid medium was approximately 2.2 times higher than that on solid medium (Fig. 4A). An increase in fresh weight was also observed in the tissues which had been cut-up compared to the tissues in clumps (Fig. 4A). In Fig. 4B, the number of somatic

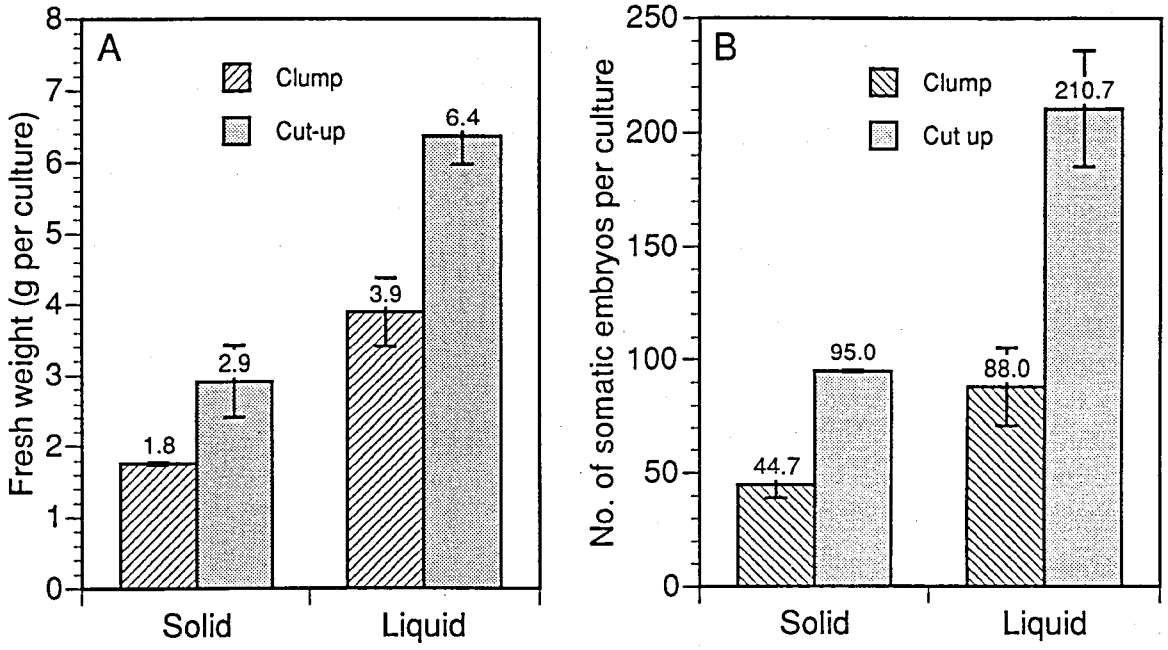

Fig. 4. Effect of cutting up embryogenic tissues on growth (A) and somatic embryogenesis (B) of *P. ginseng*
Embryogenic tissues were cultured either on 1/2 MS solid media or in liquid media in the dark for 6 weeks. Bars represent standard errors.

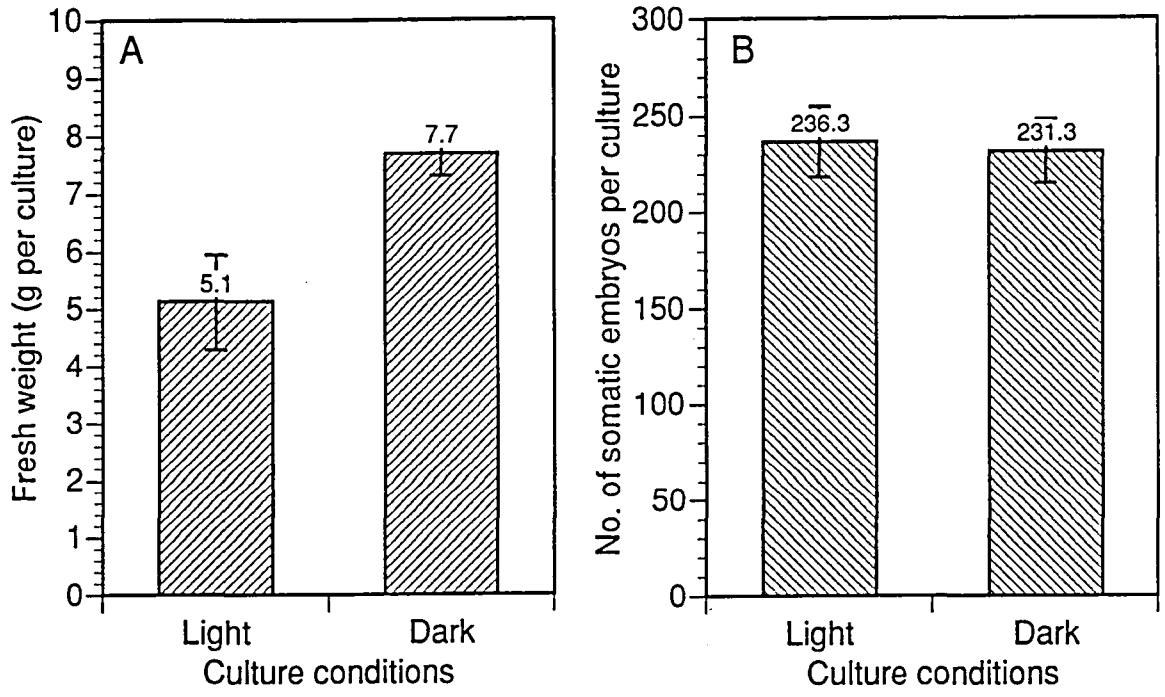

Fig. 5. Effect of light on growth (A) and somatic embryogenesis (B) of *P. ginseng* tissues in 1/2 MS liquid media after 6 weeks of culture

Embryogenic tissues were cut up prior to culture. Bars represent standard errors.

embryos formed in liquid medium was twice the number formed on solid medium. The number of somatic embryos formed per culture in liquid medium increased from 88 in clump tissues to 211 when tissues were cut-up. These results show that a liquid culture system was superior. Growth and frequency of somatic embryogenesis were also enhanced when embryogenic tissues were cut into 1 - 2 mm in size.

Effect of light on cut-up embryogenic tissues in liquid media

Growth of cut-up embryogenic tissues cultured in 1/2 MS liquid medium in the dark was 1.5 times higher than the tissues grown under the light (Fig. 5A). In a period of 6 weeks, tissues in the dark grew approximately 15 times (7.7 g) the original inoculum of 0.5 g per flask. However, the number of somatic embryos formed per culture was the same, 236 in light-grown tissues and 231 in dark-grown tissues (Fig. 5B).

Ginsenoside contents in field-cultivated root, plantlets from zygotic embryo and somatic embryo

The ginsenoside contents of a 4-year old field-cultivated root were compared to ginsenoside contents in seedlings from zygotic and somatic embryos (Fig. 6). Two-month old seedlings and somatic embryos cultured in 1/2 MS liquid medium for 2 months contained less ginsenosides than the 4-year old field-cultivated root. However, somatic embryos contained 1.7 times the amount of ginsenoside Rb1 and 2.3 times the amount of ginsenoside Re when compared to seedlings. It is of interest that ginsenoside Rd which was absent in seedlings, was detected in somatic embryos. It was found that the ginsenoside Re content in somatic embryos was comparable to that of a 4-year old field-cultivated root.

Embryogenic tissues grown on solid medium contained higher ginsenosides Rd and Rg1 levels than those in liquid medium (Fig. 7A). Levels of ginsenosides Rb1, Rc and Re were not much different between the liquid and solid culture system. The ginsenosides Rb1 and Rg1 levels in cut-up tissues were higher than levels in clump tissues. This was observed in both liquid and solid culture systems. The ginsenoside Rg1 content in cut-up embryogenic tissues grown on solid media (Fig. 7A) was as high as that in a 4-year old field-cultivated root (Fig. 6). Fig. 7B shows the ginsenoside yield in embryogenic tissues cultured in liquid and on solid media. Ginsenoside yields were clearly higher in cut-up tissues than in clump tissues.

Discussion

In all previously published reports on somatic embryogenesis

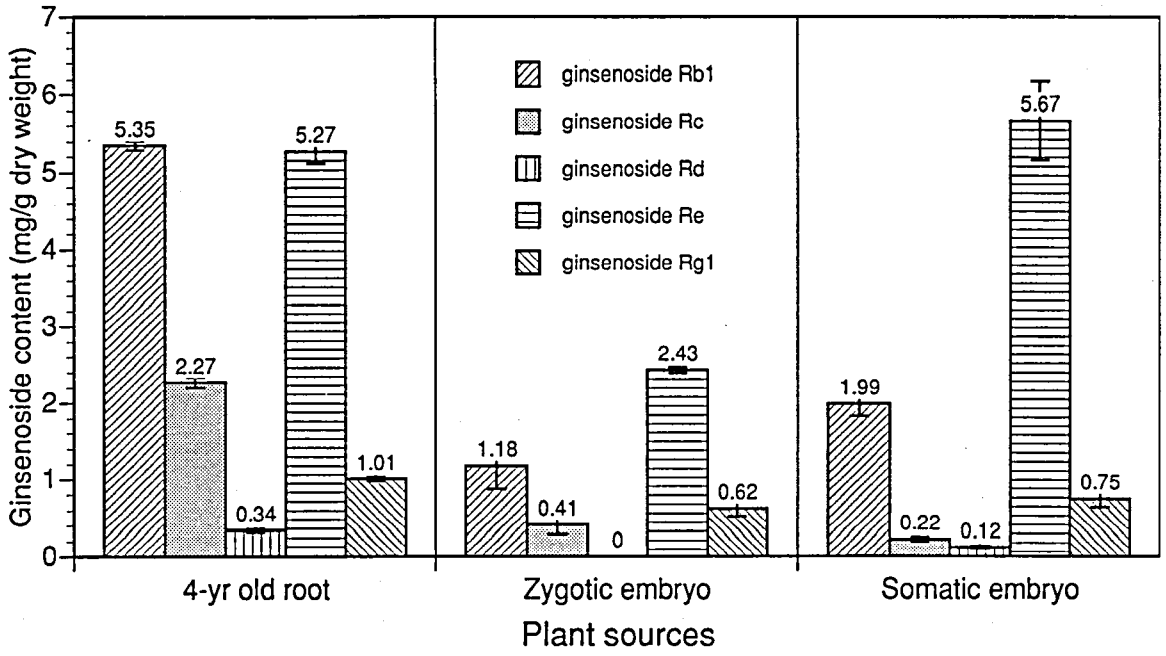

Fig. 6. Ginsenoside content in a 4-year old field-cultivated root, zygotic embryos and somatic embryos of *P. ginseng*
Bars represent standard errors.

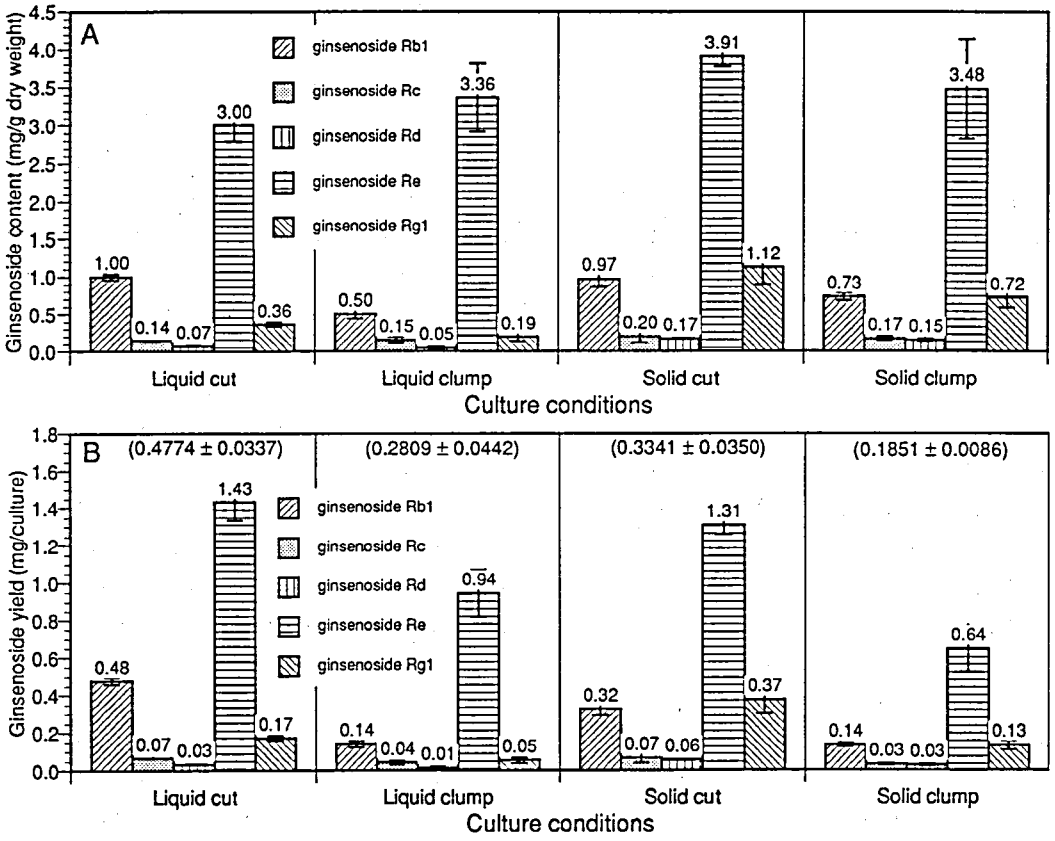

Fig. 7. Ginsenoside content (A) and yield (B) of *P. ginseng* embryogenic tissues cultured in 1/2 MS medium for 6 weeks
Embryogenic tissues were prepared into cutting up tissue (1 - 2 mm) or into clump (10 - 20 mm) prior to culture on solid or in liquid media. Values in the parentheses represent dry weight of tissues per culture ± standard error. Bars represent standard errors.

and plant propagation via somatic embryogenesis of *P. ginseng*, no system has been established without the use of phytohormones. Auxins, cytokinins and/or gibberellins have played important roles in somatic embryogenesis and plantlet formation of *P. ginseng*. In our present study, stress caused by a long culture period in an agar-sucrose medium could have triggered somatic embryogenesis. Similarly, high temperature treatment (30 - 40 ℃ for 12 - 24 h)[17] and high concentration of sugar (100 g/l sucrose)[18] were able to induce somatic embryogenesis in multiple shoots of *P. ginseng* cultured in MS medium supplemented with 1 mg/l kinetin[19]. The physiological state of the germinating zygotic embryo may be an important factor to consider before somatic embryogenesis occurs[12]. This is also supported by the results which show that leaf and petiole segments produced tissues with varying embryogenic capacity in media without phytohormones (Fig. 2) though Choi and Soh reported that somatic embryogenesis was not observed on the germinating zygotic embryos. The supply of somatic embryos in this culture system is maintained via secondary embryogenesis in phytohormone-free medium. This is important because a continuous supply of somatic embryos and clonal plantlets can be maintained using this system. No phytohormones are required for regeneration in this system. This is unlike results obtained by Choi et al[19,20] where gibberellic acid or a cold treatment was required for germination of somatic embryos. This system is preferred as it is a simpler, more manageable and the cost of propagation can be reduced without use of phytohormones. Unlike callus, the embryogenic tissues are organized tissues. This also lowers the chances for genetic instability. Therefore, this culture system offers an *in vitro* propagation system which will produce a supply of genetically-stable plants. To optimize this propagation system, some factors affecting somatic embryogenesis were studied. Light was shown to be an important factor in controlling the germination of *P. ginseng* somatic embryos in phytohormone-free medium. Other reports showed that cytokinin, gibberellin and a half strength media with reduced sucrose concentration (1.5 %) was effective in promoting shoot development in somatic embryos of *P. ginseng*[6,8]. Cut-up embryogenic tissues cultured in liquid media exhibited an increase in growth rate and number of somatic embryos. This could be because there is better absorption of nutrients and higher dissolved oxygen levels in cut-up tissues grown in a liquid culture system. Choi[1] also reported that the growth rate of cultured ginseng roots was higher in liquid medium. Embryogenic tissues in liquid media also grew well in the dark and this is an important consideration for the commercial scale-up of this system.

In comparing the ginsenoside contents of somatic embryos, embryogenic tissues, seedlings from zygotic embryos and field-cultivated root, qualitative and quantitative differences were found. Specific ginsenosides Rb1, Rc, Rd, Re and Rg1 which were measured in the 4-year old field-cultivated root were also produced in somatic embryos and embryogenic tissues. These results show that somatic embryos and embryogenic tissues in culture produced similar types of ginsenosides as a 4-year old field-cultivated root. These data may be useful for the production of specific types of ginsenosides from ginseng cultures. For example, the production of ginsenoside Re in 2-month old somatic embryos (5.67 mg/g dry weight) was equivalent to level produced in a 4-year old field-cultivated root (5.27 mg/g dry weight). Somatic embryos could be used as a source of the ginsenoside Re and studies can be conducted to determine its pharmaceutical role. The ginsenoside levels (ginsenosides Rb1, Rc and Rg1) in somatic embryos and embryogenic tissues are generally lower than that of a 4-year old field-cultivated root. Results of this study also show that contents of ginsenosides Rb1 and Rg1 in somatic embryos were higher than those found in seedlings from zygotic embryos. Other results show that the biosynthetic pathway of ginsenosides in embryos requires further study as it is not well-understood or reported. Evidence of this is seen as only four of the ginsenosides (ginsenosides Rb1, Rc, Re and Rg1) measured were found in seedlings of zygotic origin and the ginsenoside Rd was absent. The *in vitro* production of ginsenosides using somatic embryos and embryogenic tissues seems promising due to the high growth rate, shorter time frame and the use of culture medium without phytohormones. Instead of waiting 4 years to harvest a field-cultivated root, 2 month old somatic embryos can be used for extraction of specific ginsenosides.

Ginsenoside production has been reported in ginseng callus cultures, however, the use of phytohormones was crucial not only in maintaining growth but also in maintaining its capability for saponin production[1, 20-22]. In our study, we examined the ginsenoside production capability of embryogenic tissues in phytohormone-free medium. It was found that the development of embryogenic tissues into somatic embryos increased the ginsenoside production capability. This is observed when comparisons are made between ginsenoside production in embryogenic tissues and somatic embryos. Efforts to increase growth and yield of ginsenosides in embryogenic tissues were made by liquid culture and by cutting up tissues. Ginsenoside yield was comparatively higher in cut up tissues than clump tissues, but culturing on solid or in liquid media did not have much varying effect. The production of

ginsenoside Rg1 (1.12 mg/g dry weight) in cut up embryogenic tissues cultured on solid media was equivalent to level produced in a 4-year old field-cultivated root (1.01 mg/g dry weight). Ginsenoside Rg1 is one of the more important ginsenosides.

This unique culture system in this study not only offers possibilities in producing clonal plants which are genetically stable but also the *in vitro* production of ginsenosides from somatic embryos and embryogenic tissues without the use of phytohormones.

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References

1) Choi, K.T.: Biotechnology in Agriculture and Forestry, Vol. 4, Medicinal and Aromatic Plants I, ed. by Bajaj, Y.P.S., Springer-Verlag, Berlin, Heidelberg, pp. 484-500 (1988)
2) Shibata, S., Tanaka, O., Shoji, J. and Saito, H.: Journal of Economics and Medicinal Plant Research I, eds. by Wagner, H.H. and Farnsworth, N. R., Academic Press, London, pp. 217-284 (1985)
3) Butenko, R., Brushwitzky, I. V. and Slepyan, L. I.: *Bot. Zh.*, 7, 906-911 (1968)
4) Jhang, J.J., Staba, E.J. and Kim, J.Y.: *In Vitro*, **9**, 253-259 (1974)
5) Chang, W.C. and Hsing, W.I.: *Nature*, **284**, 341-342 (1980)
6) Chang, W.C. and Hsing, W.I.: *Theor. Appl. Genet.*, **57**, 133-135 (1980)
7) Lee, H.S., Liu, J. R., Yang, S.G. and Lee, Y.H.: *Hort. Science*, **25**, 1652-1654 (1990)
8) Shoyama, Y., Kamura, K. and Nishioka, I.: *Planta Med.*, **54**, 155-156 (1988)
9) Arya, S., Liu, J. R. and Eriksson, T.: *Plant Cell Rep.*, **10**, 277-281 (1991)
10) Arya, S., Arya, I.D. and Eriksson, T.: *Plant Cell Tiss. Org. Cult.*, **34**, 157-162 (1993)
11) Shoyama, Y., Matsushita, H., Zhu, X. X. and Kishira, H.: Biotechnology in Agriculture and Forestry, Vol. 31, Somatic Embryogenesis and Synthetic Seed II, ed. by Bajaj, Y.P.S., Springer-Verlag, Berlin, Heidelberg, pp. 343-356 (1995)
12) Choi, Y. E. and Soh, W. Y.: *Plant Cell Tiss. Org. Cult.*, **45**, 137-143 (1996)
13) Choi, Y. E., Yang, D. C., Park, J. C., Soh, W. Y. and Choi, K. T.: *Plant Cell Rep.*, **17**, 544-551 (1998)
14) Choi, Y. E., Yang, D. C., Yoon, E. S. and Choi, K. T.: *Plant Cell Rep.*, **18**, 493-499 (1999)
15) Murashige, T. and Skoog F.: *Physiol. Plant.*, **15**, 473-497 (1962)
16) Yoshimatsu, K., Yamaguchi, H. and Shimomura, K: *Plant Cell Rep.*, **15**, 555-560 (1996)
17) Asaka, I., Ii, I., Yoshikawa, T., Hirotani, M. and Furuya, T.: *Planta Med.*, **59**, 345-346 (1993)
18) Asaka, I.,Ii, I., Hirotani, M., Asada, Y., Yoshikawa, T. and Furuya, T.: *Planta Med.*, **60**, 146-148 (1994).
19) Furuya, T., Yoshikawa, T., Ushiyama, K., Oda, H.: *Experientia*, **42**, 193-194 (1986)
20) Furuya, T., Yoshikawa, T., Ishii, T. and Kajii, K.: *Planta Med.*, **47**, 183-187 (1983)
21) Furuya, T., Yoshikawa, T., Ishii, T. and Kajii, K.: *Planta Med.*, **47**, 200-204 (1983)
22) Furuya, T., Yoshikawa, T., Orihara, Y. and Oda, H.: *Planta Med.*, **48**, 83-87 (1983)