Pergamon

PII: S0031-9422(98)00308-2

Phytochemistry Vol. 49, No. 8, pp. 2331–2335, 1998

Printed in Great Britain
0031-9422/98/$ - see front matter

GINSENOSIDES IN HAIRY ROOTS OF A PANAX HYBRID†

DAISUKE WASHIDA, KOICHIRO SHIMOMURA,‡ YOSHIO NAKAJIMA,§ MICHIO TAKIDO and SUSUMU KITANAKA*

College of Pharmacy, Nihon University, 7-7-1, Narashinodai, Funabashi, Chiba 274, Japan; ‡Tsukuba Medicinal Plant Research Station, National Institute of Health Sciences, 1 Hachimandai, Tsukuba, Ibaraki 305, Japan; §Nagano Agricultural Technique and Training Center, 4857–1, Ooazayamaura, Komoro, Nagano 384, Japan

(*Received in revised form* 14 *March* 1998)

Key Word Index—*Panax ginseng*; *Panax quinquefolium*; Araliaceae; interspecific hybrid; hairy root culture; ginsenoside.

Abstract—Hairy roots of an interspecific hybrid ginseng (*Panax ginseng* × *P. quinquefolium*), named Pgq, were established by the infection of *Agrobacterium rhizogenes* ATCC 15834. Growth and ginsenosides content of hairy roots cultured in various basal liquid media were measured periodically from 2 to 8 weeks. In Gamborg B5 liquid medium, the hairy roots showed best growth (5.87 g fresh weight per flask) at week 8. The highest content of ginsenoside was 2.87% as dry weight at week 8 when cultured in 1/8 Murashige–Skoog liquid medium. The ginsenoside content of Pgq hairy roots was comparable to that of Pgq root cultivated in the field. However the highest yield of ginsenosides was obtained in B5 liquid medium (3.85 mg per flask at week 8).

INTRODUCTION

Hairy root culture can be an effective method of produce useful secondary metabolites in medicinally important plants. The production of secondary metabolites by hairy root cultures have been studied for many plants, including production of shikonin in *Lithospermum erythrorizon* [1], geraniim in *Geranium thunbergii* [2], and hyoscyamine in *Datura innoxia* [3]. Generally, growth and secondary metabolite contents of hairy roots are higher than those of intact plants, callus and adventitious roots. *Panax ginseng* and *P. quinquefolium*, the parental species of Pgq used in this study, are medicinally important plants used for their tonic properties. However, they are very expensive because of the long and laborious cultivation period of 5 to 7 years. We investigated the possibility of developing a new crossbred ginseng and found that Pgq bred between *P. ginseng* and *P. quinquefolium* showed higher ginsenoside production than either parental species [4]. However, seed propagation of Pgq is impossible, since Pgq is sterile. Tissue cultures of Pgq were therefore studied for regeneration of plantlets from callus and production of secondary metabolites. Callus, adventitious roots and hairy roots were successfully induced and cultured [4]. In this report, we described the optimal medium for ginsenoside production in Pgq hairy roots.

*Author to correspondence should be addressed.

†Part 3 in the series 'Ginsenoside Production, Tissue Culture and Induction of Interspecific Hybrid Ginseng (*Panax ginseng* × *P. quinquefolium*)'

RESULTS AND DISCUSSION

In a preliminary experiment Pgq hairy roots were cultured in six hormone-free liquid media (Gamborg B5 (B5) [5], Murashige and Skoog (MS) [6], 1/2 MS, Nitsch and Nitsch [7], White [8] and Woody Plant (WP) [9]) for 3 weeks (Table 1). The hairy roots grew best in B5, 1/2 MS and White liquid media. We studied in detail, growth and ginsenoside production of hairy roots cultured in B5, White, MS, 1/2 MS and more diluted MS liquid media. However, the growth of hairy roots cultured in 1/4, 1/8 and 1/16 MS medium was poor, and a modified dilute MS medium was adopted. In this modified MS medium, 1/4, 1/8 and 1/16 MS media were prepared by diluting only the macro elements (KNO_3, NH_4NO_3, $CaCl_2$, $MgSO_4$ and KH_2PO_4), keeping the minor and organic elements the same as full strength MS medium.

Table 1. Effect of basal media on growth of Pgq hairy roots

Medium	Fresh weight (mg)
B5	192.0 ± 3.8
MS	65.1 ± 4.2
1/2 MS	90.3 ± 9.1
NN	50.6 ± 6.6
White	74.4 ± 1.9
WP	50.5 ± 7.8

Hairy roots (Ca 10 mg fresh weight) were cultured in various liquid basal media for 3 weeks.
Fresh weight shows the average (±S.D.) of 3 roots.

Table 2. Ginsenoside content of Pgq hairy roots, and roots of Pgq, *P. ginseng* and *P. quinquefolium* cultivated in the field

Materials	Ginsenoside content (%) Rb group	Rg group
Hairy roots[a]		
Pgq	2.08	0.80
Roots cultivated in a field[b]		
Pgq	1.71	0.85
P. ginseng	1.11	0.79
P. quinquefolium	2.14	0.90

Rb group: ginsenoside Rb_1, Rb_2, Rc and Rd.
Rg group: ginsenoside Re and Rg_1.
a. Hairy roots were cultured in 1/8 MS liquid medium for 8 weeks.
b. Cultivated roots were 5 years old.
5-year-old roots were quantified by HPLC.

Hairy roots were cultured in B5, White, MS, 1/2 MS, and 1/4, 1/8 and 1/6 modified MS liquid media, and harvested weekly from week 2 to week 8. In the first 3 weeks, hairy roots showed slow growth at the induction phase, then grew rapidly in B5 medium compared to the other media (Fig. 1). Growth of hairy roots cultured in 1/8 modified MS medium reached a maximum at week 7 (1.30 g fresh weight). In other media, growth of hairy roots reached a maximum at week 8. Growth of Pgq hairy roots was optimum when cultured in B5 medium. Similar results were reported in *Platycodon grandiflorum* [10] and *Scutellaris baicalensis* [11].

Six major ginsenosides (ginsenoside Rb_1, Rb_2, Rc, Rd, Re and Rg_1) were quantified by HPLC as shown in Fig. 2. The content of ginsenoside Rb_1 was the highest among the six ginsenosides in all media examined. The Rb_1 content reached 1.0% in White medium and 0.5–0.9% in the other media at week 8. The content of ginsenoside Rb_2 and Rc remained at the same levels (ranging 0.1–0.4%) throughout the culture period in all media. In White medium, 0.3% of ginsenoside Rd was detected by HPLC, but in other media, Rd could not be detected or was fairly low. The contents of ginsenoside Re and Rg_1 remained at the same levels (ranging 0.1–0.4%) throughout the culture period in all media. Time course of ginsenoside accumulation in *P. ginseng* hairy roots was reported by Ko *et al.* [12] and Inomata *et al.* [13]. For *P. ginseng*, the content of ginsenoside Rb_1 decreased over time in contrast to our results with Pgq which accumulated a total content of six ginsenosides over 2.7% in 1/8 MS medium after week 7. The ginsenoside content of *P. ginseng* hairy roots was reported to be 0.34–0.82% by Ko *et al.* [12], 0.6% by Inomata *et al.* [13] and 0.36–0.95% by Yoshikawa *et al.* [14]. The highest total ginsenoside content in this study was 2.88% of dry weight when cultured in 1/8 MS medium for 8 weeks. We compared the ginsenoside content of hairy roots cultured in 1/8 MS medium for 8 weeks with that of Pgq, *P. ginseng* and *P. quinquefolium* roots cultivated for 5 years in the field (Table 2). The total ginsenosides content of Pgq hairy roots was comparable to that of cultivated *P. quinquefolium* and Pgq. Therefore, Pgq hairy root culture is an effective method to obtain tissue with a high content of ginsenosides in a short time.

From the perspective of total ginsenoside yield per flask in the seven media, the results are somewhat different. The total ginsenoside yield of Pgq hairy roots cultured in White medium was low since the poor growth counteracted the relatively high ginsenosides content of the roots. In full

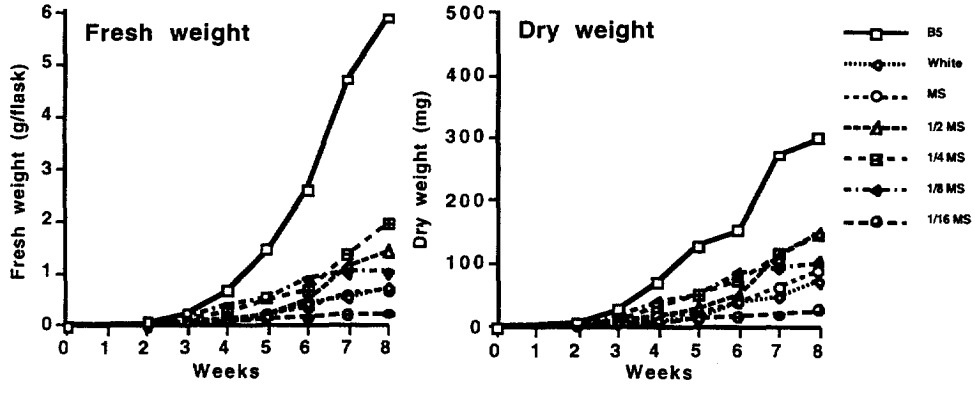

Fig. 1. Growth of hairy roots cultured in several liquid media.

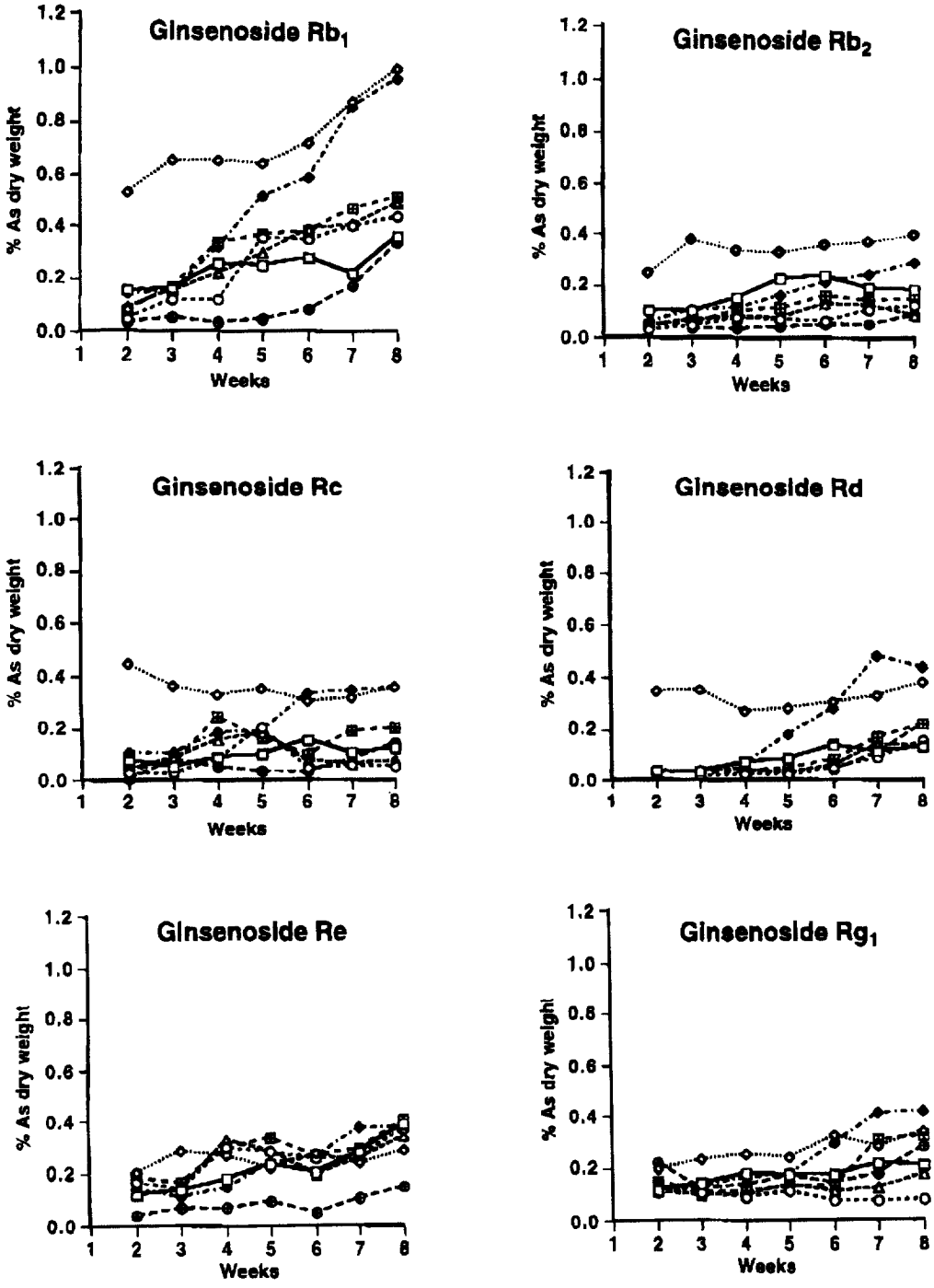

Fig. 2. Contents of six ginsenosides in hairy roots cultured in various liquid media. —□— BS; ···○··· white; ---○--- MS, ---△--- 1/2MS; – – -⊞- – – 1/4MS; – ·-●-·- 1/8MS; – – -⊕- – – 1/16MS.

strength or diluted MS media, the yield of total ginsenosides increased in proportion to the decrease in macro components of MS medium. The yields in B5 and 1/8 MS media increased markedly over time, and at week 8, the highest yield was observed in B5 medium (3.85 mg per 100 ml flask). From those results, we concluded that B5 medium was the best overall for ginsenosides production in Pgq hairy root culture.

EXPERIMENTAL

All culture media containing 30 g l^{-1} sucrose were adjusted to pH 5.7 before autoclaving at 121° for

15 min. All cultures were grown at 25°. Data shown are the mean of three experiments.

Plant material

Immature pollen was removed from the bud of *P. ginseng*, taking care not to injure the stigma, and the stigma was pollinated with mature pollen of *P. quinquefolium* which had been stored at 4°, before the artificial pollinations. The seeds of a hybrid crossbred between *Panax ginseng* × *P. quinquefolium* were obtained from mature fruits and cultivated [4, 15].

Hairy root cultures

Petioles of Pgq were sterilized with 70% ethanol for 30 s, then in 1% NaOCl for 10 min and rinsed three times with sterilized water. The explants were cultured on MS agar medium supplemented with 3% sucrose, 1 mg l^{-1} 2,4-dichlorophenoxy acetic acid (2,4-D) and 0.1 mg l^{-1} kinetin. Callus obtained after one month of culture was propagated on MS agar medium containing 3% sucrose and 1 mg l^{-1} 2,4-D. Embryoids were formed on the callus tissue grown on MS agar medium containing 1 mg l^{-1} 2,4-D as shown by the method of Asaka *et al.* [16]. The embryos formed on Pgq callus were subcultured onto MS agar medium containing 1 mg l^{-1} 2,4-D at 25° in the dark. Multiple shoots were obtained after the callus was cultured on 1/2 MS agar medium containing 1 mg l^{-1} benzy ladenine and 1 mg l^{-1} gibberellic acid under 14 hours/day light (3000 lux) for 6 weeks at 25°. The petioles (2–3 cm) of plantlets were cut off from shoots and *Agrobacterium rhizogenes* ATCC 15834 (subcultured on YEB agar medium [17]) was inoculated onto the cut ends. Four weeks after inoculation, hairy roots appeared at the infected sites. Hairy roots were transferred and cultured in the dark at 25° on hormone-free 1/2 MS agar medium containing antibiotic (0.5 g l^{-1} Claforan) to eliminate the bacteria. After elimination of bacteria, six clones of the axenic hairy roots were transferred to hormone-free 1/2 MS liquid medium (30 ml per 100 ml flask) and cultured on a rotary shaker (100 rpm) in the dark. Opines (agropine and mannopine) of hairy roots were extracted and detected using paper electrophoresis [18] (data not shown). One confirmed transformant clone was selected for used in those experiments. Voucher specimens are deposited at the Department of Pharmacognosy, Nihon University colleage of pharmacy.

Hairy roots cultures in different media

Hairy roots (*ca*, 0.01 g fresh weight, 3 root tips of 1 cm length) were inoculated into seven kinds of hormon-free liquid media (B5, White, MS, 1/2 MS, 1/4 MS, 1/8 MS and 1/16 MS, 30 ml medium per 100 ml flask) and cultured (100 rpm, on a rotary shaker) in the dark at 25°. Half strength, quarter strength, eighth strength and sixteenth strength modified MS media were prepared by diluting the macro elements (KNO_3, NH_4NO_3, $CaCl_2$, $MgSO_4$ and KH_2PO_4), leaving the minor and organic elements the same as full strength MS medium. Growth (fresh and dry weight) and ginsenoside production were determined once a week for 8 weeks.

Determination of ginsenoside content

Lyophilized hairy roots (approximately 10 mg) were extracted with 80% MeOH (0.5 ml) using sonication (60 min) at 40° and the procedure was repeated 3 ×. The extract after filtration was evaporated to dryness and dissolved in 0.1 ml of 80% MeOH. Samples thus obtained were analyzed by HPLC (column LiChrosorb RP-18 (4.6 mm i.d. × 250 mm); Mobile phase: MeCN–0.5%H_3PO_4 (31:69) for ginsenoside Rb group (ginsenoside Rb_1, Rb_2, Rc and Rd) and MeCN–0.5% H_3PO_4 (20:80) for ginsenoside Rg group (Re and Rg_1); flow rate 1.0 ml min^{-1}; column temp. 40°; detection 203 nm (UV). R_t values (min): ginsenoside Rb_1(16.8), Rb_2(26.7), Rc(21.1), Rd(43.4), Re(28.8) and Rg_1(26.5)).

REFERENCES

1. Shimomura, K., Sudo, H., Saga, H. and Kamada, H., *Plant Cell Report*, 1991, **50,** 151.
2. Ishimaru, K. and Shimomura, K., *Phytochemistry*, 1991, **30,** 825.
3. Shimomura, K., Sauerwein, M. and Ishimaru, K., *Phytochemistry*, 1991, **30,** 2275.
4. Kitanaka, S., Kunishi, M., Taira, T., Takahashi, S., Yazawa, S., Miyazawa, Y., Nakajima, Y., Okuno, K., Fukuoka, S. and Takido, M., *Natural Medicines*, 1997, **51,** 1.
5. Gamborg, O. L., Miller, R. A. and Ojima, K., *Exp. Cell Res.*, 1968, **50,** 151.
6. Murashige, T. and Skoog, F., *Physiol. Plant.*, 1962, **15,** 473.
7. Nitsch, J. and Nitsch, C., *Science*, 1969, **85,** 163.
8. White, P. R., In *The Cultivation of Animal and Plant Cells*. Ronald Press, New York, 1954.
9. Lloyd, G. B. and McCown, B. H., *Int. Plant Prop. Soc.*, 1980, **30,** 421.
10. Tada, H., Shimomura, K. and Ishimaru, K., *J. of Plant Physiol.*, 1995, **145,** 7.
11. Zhou, Y., Hirotani. M., Yoshikawa, T. and Furuya, T., *Phytochemistry*, 1997, **44,** 83.
12. Ko, K. S., Noguchi, H., Ebizuka, Y. and Sankawa, U., *Chem. Pham. Bull.*, 1989, **37,** 245.
13. Inomata, S., Yoshikawa, M., Gozu, Y., Shimizu, T. and Yanagi, M., *Plant Cell Rep.*, 1993, **12,** 681.

14. Yoshikawa, T. and Furuya, T., *Plant Cell Rep.*, 1987, **6,** 449.
15. Miyazawa, Y. and Hagiwara, H., *Bull. Nagano Hortic. Exp. Stn.*, 1972, **10,** 32.
16. Asaka, I., Yoshikawa, T., Hirotani, M. and Furuya, T., *Planta Med.*, 1993, **59,** 345.
17. Vervliet, G., Holsters, M., Teuchy, H., van Montague, M. and Schell, J., *J. Gen. Virol.*, 1975, **26,** 33.
18. Petit, A., David, C., Dahl, G. A., Ellis, J. G., Guyon, P., Casse-Delbert, F. and Tempé, J., *Mol. Gen. Genet.*, 1983, **190,** 204.