Journal of Microbiology and Biotechnology
Vol. 5. No. 4, 234-237, 1995

Production of Gericudranins by Hairy Root Culture of *Cudrania tricuspidata*

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Production of new flavanol derivatives with cytotoxic activity, gericudranin A and B, was studied by using hairy root cultures of *Cudrania tricuspidata*. Schenk and Hildebrandt (SH) medium was chosen for root growth and gericudranin production. After 35 days culture in a half-strength liquid SH medium containing 30 $g^{glucose}/l$, hairy root growth reached 138 g^{FW}/l and gericudranin A and B were produced at concentrations of 27 mg/l and 21 mg/l, respectively. It was also observed that the contents of gericudranin A and B in hairy root were eight and six times higher than those of cudraniae radix, respectively.

Cudrania tricuspidata, which belongs to the family Moraceae, has long been used in Korea as a medicinal herb for its anti-inflammatory, anti-hepatotoxic, anti-hypertensive, and anti-diabetic activities. It contains many flavonoids and xanthones such as morusin, kuwanone derivatives, cyclomulberrin, cyclocarpin, cudraflavone derivatives, kaemferol derivatives, 5-O-methyl-genistein, cyclocarpesin, and cudraxanthone derivatives etc. (2, 3, 5, 9, 13). These flavonoids have been considered as the major components for biological activities.

We have isolated new flavanol derivatives, gericudranin A and B from the plant, which have cytotoxic activities against various cancer cell lines (7). To improve their productivity, a hairy root culture system of *Cudrania tricuspidata* was developed and the result is herein reported.

MATERIALS AND METHODS

Induction of Hairy Root

Seeds of *C. tricuspidata* were aseptically germinated in a growth chamber at 25°C and a photoperiod of 12 h at 2400 lux. Aseptically grown 4 week-old plantlets were used for hairy root induction. Hairy roots were induced from the cut end of the stem segments infected with *Agrobaterium rhizogenes* A4, and by subsequent treatment with carbenicillin to eliminate *Agrobacterium* contamination.

Culture Media and Hairy Root Culture

Three-fold diluted basal salt media of Murashige and Skoog (MS) (8), Schenk and Hildebrandt (SH) (11), Gamborg's B5 (B5) (4), and White's (12) were tested for hairy root growth. All media contained 5 mg/l of vitamin B_1, 5 mg/l of niacin, 0.5 mg/l of vitamin B_6, 1 g/l of myo-inositol, and 30 g/l of sucrose. The pH was adjusted to 5.8 with 0.1N-NaOH before autoclaving. Hairy roots were cultivated in a gyratory shaking incubator at 25°C and 80 rpm in the dark. Root clones were subcultured every 4 weeks with an inocolum of 0.2 g cell fresh weight of root tips (2-3 cm long) in 250 ml Erlenmayer flasks containing 50 ml culture medium.

Analytical Methods

After separating the hairy roots from the culture broth, cell fresh weight (FW) was first measured, and then cell dry weight (DW) was determined by drying the samples in an oven at 80°C to constant weight. Conductivity of the culture broth was measured with a conductance meter (YSI-35).

Methanol extract of fresh root was hydrolysed with 2N-HCl at 100°C for 30 min and then an aliquot was subjected to HPLC (Toso UV 8010 with an UV detector) equipped with a stainless steel column (ODS, 4.6×150 mm). The mobile phase was 28% of acetonitrile. The flow rate was 1 ml/min, and the detection wavelength was 254 nm. The peak area was measured using a computing integrator (YOUNG-IN D520A). Calibration curves for gericudranin A and B

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Key words: *Cudrania tricuspidata*, hairy root culture, production of gericudranins

were established from the peak areas, respectively. Standard gericudranin A and B were isolated from cudraniae radix by the reported method (7).

Glucose concentration was determined by dinitrosalicylic acid method (1). Opine analysis was performed by the method of paper electrophoresis as described by Petit *et al.* (10). All experiments were duplicated and averaged.

RESULTS AND DISCUSSION

Establishment of Hairy Root Clone

Hairy roots of *C. tricuspidata* were successfully induced by infection with *Agrobacter rhizogenes* A4. Incubation for 15 days under the experimental condition in Materials and Methods resulted in a formation of tumorous tissues on the cut end of the stem explant, and adventitious roots were formed from the tumorous tissue (Fig. 1 A). The adventitious roots about one centimeter long were cut and placed on SH agar medium containing 500 mg/l of carbenicillin (SHC) to avoid possible bacterial contamination. After 4 to 5 transfers on fresh SHC medium, fifty root clones without bacterial contamination were obtained. Among these, root clone No. 21 (HR 21), was selected because it showed fairly good growth and highly branching morphology (Fig. 1 B and C).

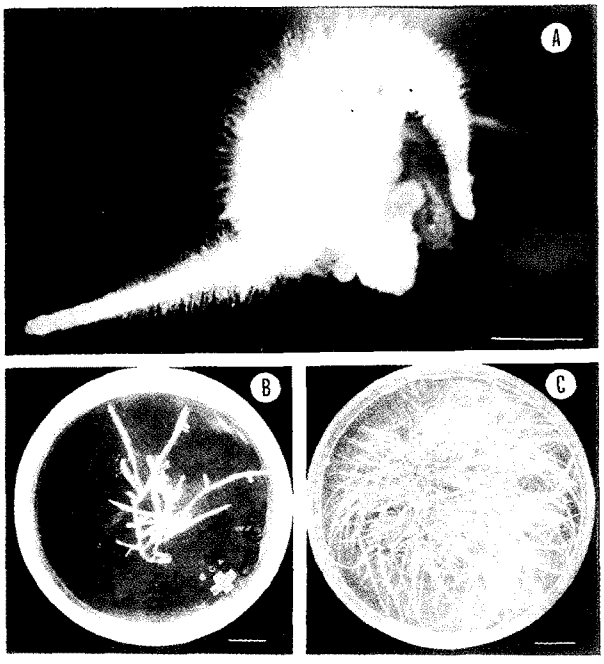

Fig. 1. Photography of hairy root induction (A) and root growth (B and C) in hormone-free Schenk and Hildebrandt medium (bar size: 1 cm).

Table 1. Effects of culture medium on hairy root growth and gericudranin derivatives production.

Medium	Root growth (g^{FW}/l)	Gericudranin (mg/l)	
		A	B
SH	91	18	12
MS	153	10	0
B5	98	5.4	5
White's	5	1	2

Hairy Root Growth and Gericudranin Production in Various Culture Media

To find a suitable culture medium for root growth and gericudranin production, the hairy root clone, HR 21, was cultured in three-fold diluted liquid SH, MS, B5, and White's medium. The media which showed good growth were SH, MS, and B5. In White's medium growth of the hairy root was not observed (Table 1). The result indicated that ammonia-type nitrogen may be necessary for the initiation of root growth. It was also observed that SH medium resulted in the highest titre of gericudranin. Therefore, SH medium was chosen for the further studies for gericudranin production.

Gericudranin A and B were well separated by reverse phase HPLC at retension times of 7.1 min and 11.4 min, respectively (Fig. 2).

Effects of salt strength of the media on hairy root growth and gericudranin production are shown in Table 2. Hairy root growth was highest in non-diluted SH medium, but gericudranin production was most favorable in half-strength SH medium.

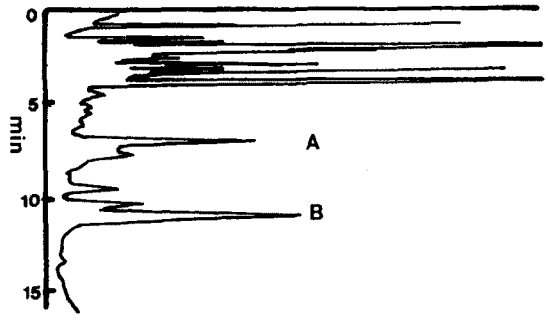

Fig. 2. HPLC pattern of hairy root extract of *C. tricuspidata*. A: gericudranin A, B: gericudranin B.

Table 2. Effect of medium salt strength on hairy root growth and gericudranin derivatives production.

Medium strength	Root growth (g^{FW}/l)	Gericudranin (mg/l)	
		A	B
1/1	185	13.7	12.8
1/2	100	20	18
1/3	85	17	19

Table 3. Effects of carbon sources on hairy root growth and gericudranin derivatives production.

Carbon source	Root growth (g^{FW}/l)	Gericudranin (mg/l)	
		A	B
Sucrose	103	18	15
Glucose	100	22	18
Fructose	10	1.8	1.6
Maltose	15	3	4

Table 4. Effect of glucose concentrations on hairy root growth and gericudranin derivatives production.

Glucose (g/l)	Root growth (g^{FW}/l)	Gericudranin (mg/l)	
		A	B
10	71	14.3	10.8
20	87	20	12.4
30	99	21	13.2
40	101	15.8	11
60	59	4.8	6.8
80	12	1	2.3

Effect of Carbon Sources

Sucrose, glucose, fructose, and maltose were examined for hairy root growth and gericudranin production (Table 3). Sucrose and glucose were found to be most appropriate for hairy root growth, while fructose and maltose apparently caused cell necrosis. Glucose was therefore selected as the most appropriate carbon source for the hairy root growth and gericudranin production.

Effects of glucose concentration on hairy root growth and gericudranin production in flask culture are summarized in Table 4. Hairy root growth increased in glucose concentrations up to 40 g/l. Production of gericudranin was highest at glucose concentration of 30 g/l. Higher sugar concentrations than 40 g/l of glucose were thought to give osmotic shock to the cells, yielding reduced production of gericudranins.

Time courses of Hairy Root Growth and Gericudranin Production

A typical time course of hairy root growth and gericudranin production in half-strength SH medium containing 30 g/l of glucose was shown in Fig. 3. Root fresh weight yielded 138 g/l (15 g^{DW}/l) after 35 days, which is approximately a 34 times increase in cell growth compared to the inoculated amount. Yields of gericudranin A and B were 27 mg/l (0.175% dry wt) and 21 mg/l (0.136% dry wt), respectively. The growth of hairy roots could be well estimated indirectly by measuring the conductivity of the culture broth during the whole culture period. There was a very good linearity between root growth and conductivity decrease in a range of 1.84 mmho to 0.30 mmho. With the half-strength SH medium, the conductivity decrease of 1mmho corresponded to the increase in hairy root biomass of 90 g^{FW}/l.

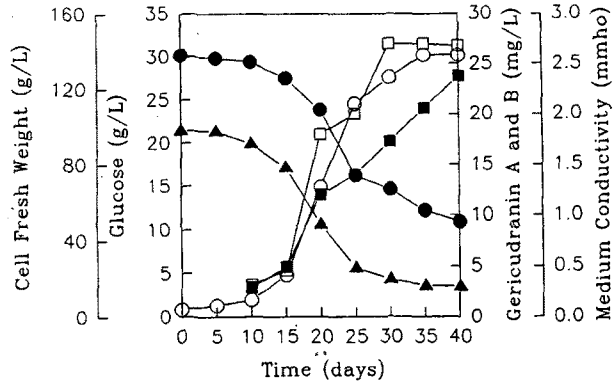

Fig. 3. Typical time courses of hairy root growth and gericudranin derivatives production.
○ : cell fresh weight, ● : glucose, ▲ : conductivity, □ : gericudranin A, ■ : gericudranin B.

In conclusion, it was possible to achieve higher titres of gericudranin A and B, typically eight and six times higher, respectively, using hairy root culture (as compared to those from the donor plant). The cudraniae radix contains only about 0.02% of gericudranins on dry weight basis (7). Further improvement is currently being sought by optimizing the culture conditions and developing hairy root lines with a higher yield.

REFERENCES

1. Chaplin, M. F. and J. F. Kennedy. 1986. *Carbohydrates analysis, a practical approach.* p. 3. IRL Press, Oxford.
2. Fujimoto, T. Y. Hano., T. Nomura, and J. Uzawa. 1984. Components of root bark of *Cudrania tricuspidata*; structure of two new isoprenylated flavones, cudraflavones A and B. *Planta Med.* **50**: 161-163.
3. Fujioto, T. and T. Nomura. 1985. Components of root bark of *Cudrania tricuspidata*; isolation and structure studies on the flavonoids. *Planta Med.* **51**: 190-193.
4. Gamborg, O. L., R. A. Miller, and K. Ojima. 1968. Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res.* **50**: 151-158.
5. Hano, Y., Y. Matsumoto, J. Y. Sun, and T. Nomura. 1990. Structures of three new isoprenylated xanthones, cudraxanthones E, F, and G. *Planta Med.* **56**: 399-402.
6. Harborne, J. B. 1988. *The flavonoids, advances in research since 1980.* p. 275. Champman and Hall Press. London.
7. Lee, I. K., C. J. Kim, J. P. Kim, I. D. Yoo, H. Koshino, and M. Uramoto. 1994. Isolation and structure of new flavonoids, gericudranins, which have a cytotoxic activity. *Nippon Nogeikagaku Kaishi* **68**: 389.

8. Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**: 473-479.
9. Park, J. C., H. S. Young, and J. S. Choi. 1992. Constituents of *Cudrania tricuspidata* in Korea. *Yakhak Hoeji* **36**: 40-45.
10. Petit, A., C. David, A. A. Dahl, J. G. Elis, P. Guyon, F. Casse-Delbart, and J. Temp. 1983. Further extension of opine concepts: plasmid in *A. rhizogenes* cooperate for opine degradation. *Mol. Gen. Genet.* **190**: 214-240.
11. Schenk, R. U. and A. C. Hildebrandt. 1972. Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell culture. *Can. J. Bot.* **50**: 199-204.
12. White, P. R. 1954. *The cultivation of animal and plant cells.* Ronald Press, New York.
13. Young, H. S, J. H. Park, H. J. Park, and J. S. Choe. 1989. Chemical study on the stem of *Cudrania tricuspidata. Arch, Pharm. Res.* **12**: 39-41.

(Received April 8, 1995)