Isoflavonoid Production in a Hairy Roots Culture of *Pueraria candollei*

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Z. Naturforsch. **64c**, 687–691 (2009); received April 6/May 8, 2009

A hairy roots culture of *Pueraria candollei* was established using *Agrobacterium rhizogenes* ATCC15834 and grown in half-strength Murashige and Skoog (MS) medium. The highest production of total isoflavonoids was found to be (36.48 ± 4.09) mg/g dry wt [(3.39 ± 0.20) mg/g dry wt puerarin, (29.91 ± 3.74) mg/g dry wt daidzin, (1.65 ± 0.09) mg/g dry wt genistin, (0.76 ± 0.03) mg/g dry wt daidzein, and (0.76 ± 0.03) mg/g dry wt genistein, respectively]. The total isoflavonoid content in hairy roots of *P. candollei* was 5.18-fold higher than that of the native tuber. Effects of sucrose content and medium type on growth and isoflavonoid production were investigated. 5% (w/v) Sucrose was an optimum content for the growth and isoflavonoid accumulation in *P. candollei* hairy roots. Half-strength MS medium had the highest effect for biomass production whereas woody plant medium had mostly stimulated isoflavonoid content in hairy roots.

Key words: Isoflavonoid, Hairy Roots Culture, *Pueraria candollei*

Introduction

Pueraria candollei Wall. ex Benth. (Leguminosae), a Thai indigenous herb with the local name White Kwao Krua, has long been used in traditional medicine for rejuvenation. A *P. candollei* tuberous extract exhibited dose-dependent estrogenic effects on the reproductive system (Cherdshewasart *et al*., 2004, 2007). It also showed the ability to prevent bone loss (Urasopan *et al*., 2008) and antioxidant activity (Cherdshewasart and Sutjit, 2008). The major chemical constituents isolated from the plant tuber are isoflavonoids, including puerarin, daidzin, genistin, daidzein, and genistein (Chansakaow *et al*., 2000). These compounds are mainly accumulated in tuberous roots (Ingham *et al*., 1986). The individual and total isoflavonoid contents among tubers collected from different places exhibited different levels caused by significant differences in active compounds. This variability in isoflavonoid levels in mature tubers could seriously affect the quality of any tuber-derived materials or even research results derived from the plants (Cherdshewasart and Sriwatcharakul, 2007).

Plant tissue cultures have been suggested as a potential tool for the production of useful secondary metabolites. Thus, using this technology, secondary metabolites can be produced under controlled and reproducible conditions, independent of geographical and climatic factors. Hairy roots cultures are another alternative method to produce isoflavonoids, because of their rapid growth, genetic stability, and capacity to synthesize secondary metabolites (Shanks and Morgan, 1999). It is known that many factors could affect cell growth and secondary metabolites production in plant tissue cultures including media components and carbon sources (Dicosmo and Misawa, 1995). Therefore, optimum conditions for hairy roots cultures are necessary for biomass and secondary metabolites production. There are many reports which have successfully presented the secondary metabolites production by optimizing the culture conditions such as sucrose content and medium type (Putalun *et al*., 2004, 2006; Kittipongpatana *et al*., 1998). In the present study, we established a hairy roots culture of *P. candollei* and investigated the effect of medium type and sucrose content in

0939–5075/2009/0900–0687 $ 06.00

the hairy roots culture on cell growth and isoflavonoid production.

Material and Methods

Chemicals

Daidzin, genistin, daidzein, and genistein were purchased from Nacalai Tesque, Inc. (Tokyo, Japan). Puerarin was obtained from Chromadex Inc. (Irvine, CA, USA). Cefotaxime (CF) sodium salt was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals were standard commercial products of analytical grade.

Plant material

P. candollei seeds were obtained from the Botanical Garden, Faculty of Pharmaceutical Sciences, Khon Kaen University, Thailand. The seeds were washed with sterile distilled water and were surface-sterilized in 10% sodium hypochlorite for 15–20 min. After being washed three times with sterilized water, the seeds were immersed in 70% ethanol for 1 min and then germinated on hormone-free Murashige and Skoog (MS) medium containing 3% sucrose (w/v), pH 5.5. Germination started within 7 d and was carried out at (25 ± 1) °C under 16 h light/day. Plantlets were used for hairy roots induction.

Hairy roots induction

The young stems were infected with *Agrobacterium rhizogenes* ATCC15834, cultured on MS medium at 25 °C for 48 h, and then transferred to ½ MS medium with 500 mg/l CF. At 2-week-intervals, infected segments were transferred to ½ MS medium with 300 mg/l and 100 mg/l CF, respectively. Transformed roots of *P. candollei* were grown in 125-ml flasks containing 30 ml of ½ MS liquid medium. The medium was agitated on a rotary shaker (100 rpm at 25 °C, under continuous light for 16 h/d). The hairy roots were subculture every 2 weeks into fresh medium.

Growth rate, effects of sucrose and medium type on isoflavonoid production in hairy roots

Fully grown hairy roots were subcultured into 125-ml flasks containing 30 ml of ½ MS liquid medium. Hairy roots were harvest every 5 d to determine the fresh weight (wt), dry wt and isoflavonoid content. Various contents of sucrose (1–9% w/v) and medium types [MS, B5 and woody plant medium (WP)] were used to test their effect on the growth of hairy roots and isoflavonoid production under light (16 h photoperiod) conditions at 25 °C with agitation (100 rpm). After 30 d of culture, the dry wt and isoflavonoid contents were determined. Each experiment was done in triplicate.

Extraction of samples and isoflavonoid analysis

Hairy roots were measured, as dry wt, gravimetrically by harvesting the biomass and drying at 50 °C in a hot air oven to a constant weight. Dried samples (30 mg) of hairy roots were powdered and extracted five times with 0.5 ml methanol with sonication. The extracts were combined, evaporated and then redissolved with 1 ml methanol. The extracted solutions were analyzed for their puerarin, daidzin, genistin, daidzein and genistein contents by HPLC using a PerkinElmer Series 200 LC pump connected with a PerkinElmer 785A UV/VIS detector (254 nm) and a PE Nelson computer. An RP-18 column (LiChroCART®, 125 mm × 4 mm, 5 μm particle size, Merck, Germany) was used. The mobile phase consisted of 25% acetonitrile containing 1.5% acetic acid. The flow rate was 1.0 ml/min. Each sample was examined in triplicate.

Results and Discussion

Stem parts of *P. candollei* were infected with *A. rhizogenes* ATCC15834. After 2 weeks of culture, the hairy roots were emerged from infection sites. The induced hairy roots were transferred to half-strength (½) MS medium with CF to eliminate *A. rhizogenes*. They grew vigorously in the medium and had characteristics of transformed roots, such as fast growth and high lateral branching. Transformed roots of *P. candollei* were grown in 30 ml of ½ MS liquid medium. During incubation, the hairy roots changed gradually from white to brown, and some brown substance was secreted from them into the medium after 3–4 weeks of culture.

The growth pattern of *P. candollei* hairy roots revealed that the hairy roots grew slowly in the first 5 days of culture (Fig. 1). However, the hairy roots grew faster subsequently, and the fastest growth happened during days 10–20. After 20 d of culture, the growth rate began to slow down, but the biomass of the hairy roots still increased

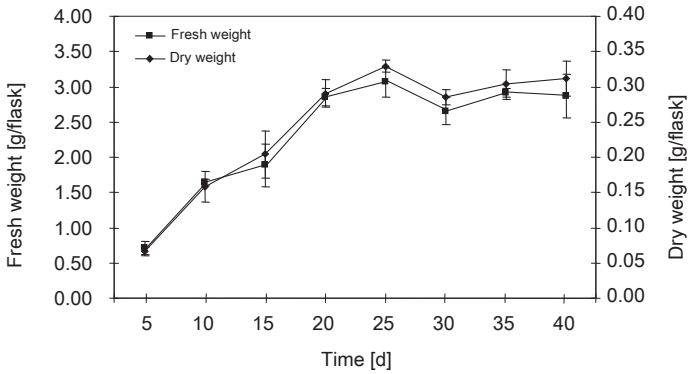

Fig. 1. Time course of the growth of a *P. candollei* hairy roots culture on liquid ½ MS medium for 40 days.

until day 25 of culture (0.33 g dry wt/flask). After 20 days of culture, the colour of the hairy roots changed from white to brown, and the biomass decreased after 25 days of culture. These results were similar to those of previous studies on hairy roots cultures of *P. phaseoloides* (Shi and Kintzios, 2003). The production of the isoflavonoids puerarin, daidzin, genistin, daidzein, and genistein was different from the growth rate. The hairy roots produced isoflavonoids in the first 5 days of culture fast and after that the production rate only slightly increased. The highest production of isoflavonoids was found to be (36.48 ± 4.09) mg/g dry wt [(3.39 ± 0.20) mg/g dry wt puerarin, (29.91 ± 3.74) mg/g dry wt daidzin, (1.64 ± 0.09) mg/g dry wt genistin, (0.76 ± 0.03) mg/g dry wt daidzein, and (0.76 ± 0.03) mg/g dry wt genistein, respectively] at day 35 (Table I). Furthermore, the total isoflavonoid content in native tuberous roots was investigated and found to be (7.04 ± 0.29) mg/g dry wt [(1.47 ± 0.09) mg/g dry wt puerarin, (3.66 ± 0.15) mg/g dry wt daidzin, (1.10 ± 0.03) mg/g dry wt genistin, (0.48 ± 0.01) mg/g dry wt daidzein, and (0.32 ± 0.01) mg/g dry wt genistein, respectively]. The results showed that the isoflavonoid content in hairy roots was about 5.18-fold higher than that in native roots. The hairy roots culturing presented herein may be a promising tool for the large-scale production of isoflavonoids.

Among the nutritional factors, sucrose is a special significant factor for cell growth and secondary metabolites production. ½ MS media containing various sucrose contents were examined for their effects on biomass and isoflavonoid production. The biomass of hairy roots from ½ MS medium containing 3% sucrose was found to be (0.15 ± 0.05) g/flask. The maximum level of biomass was observed for the medium containing 5% sucrose [(0.31 ± 0.04) g/flask]. The biomass of hairy roots decreased at higher sucrose contents (7% and 9%). The suppression of hairy roots

Table I. Time course of the isoflavonoid content in *P. candollei* hairy roots.

Time [d]	Isoflavonoid content [mg/g dry wt]					
	Puerarin	Daidzin	Genistin	Daidzein	Genistein	Total
5	1.80 ± 0.09	23.73 ± 0.95	1.17 ± 0.04	0.90 ± 0.07	0.43 ± 0.02	28.02 ± 1.18
10	1.65 ± 0.04	22.60 ± 0.31	1.14 ± 0.00	0.51 ± 0.02	0.41 ± 0.01	26.31 ± 0.38
15	1.93 ± 0.61	26.08 ± 1.04	1.15 ± 0.29	0.71 ± 0.12	0.55 ± 0.09	30.43 ± 2.15
20	2.59 ± 0.05	28.09 ± 2.12	1.40 ± 0.08	0.66 ± 0.02	0.68 ± 0.02	33.42 ± 2.29
25	2.25 ± 0.29	24.62 ± 3.42	1.20 ± 0.16	0.44 ± 0.00	0.63 ± 0.04	29.14 ± 3.91
30	2.83 ± 0.25	23.47 ± 3.10	1.32 ± 0.11	0.77 ± 0.05	0.71 ± 0.02	29.11 ± 3.52
35	3.39 ± 0.20	29.91 ± 3.74	1.65 ± 0.09	0.76 ± 0.03	0.76 ± 0.03	36.48 ± 4.09
40	2.83 ± 0.14	25.88 ± 0.76	1.50 ± 0.01	0.62 ± 0.02	0.85 ± 0.03	31.69 ± 1.04

Values represent the mean ± S.D. (n = 3).

growth in the media containing 7% and 9% sucrose could be due to the high osmotic pressure at high sucrose contents. The isoflavonoid accumulation in hairy roots appeared to be closely related to biomass accumulation; 5% sucrose produced the highest level of isoflavonoids which was found to be (30.87 ± 3.10) mg/g dry wt (Table II). These results suggested that 5% sucrose was an optimum content for a hairy roots culture of *P. candollei*.

Besides, the medium type is one of the factors that can affect the biomass and isoflavonoid production. The effect of different medium types was investigated. Fig. 2 shows that ½ MS medium produced the highest dry wt of hairy roots [(0.13 ± 0.03) g/flask]. The lowest dry wt of biomass of hairy roots was observed with ½ B5 medium. The isoflavonoid contents in hairy roots were determined as shown in Table III. The maximum isoflavonoid content in hairy roots was found in woody plant medium [(26.39 ± 3.80) mg/g dry wt]. These results indicated that woody plant medium had the highest effect on isoflavonoid accumulation in hairy roots.

In conclusion, hairy roots of *P. candollei* can upregulate the level of isoflavonoid content to about 5.18-fold of that in intact roots. The optimization of growth and isoflavonoid accumulation of *P. candollei* hairy roots depend on the sucrose content – 5% sucrose (w/v) was optimum for biomass and isoflavonoid accumulation. ½ MS medium had the highest effect on biomass production whereas woody plant medium was suitable for isoflavonoid accumulation in hairy roots.

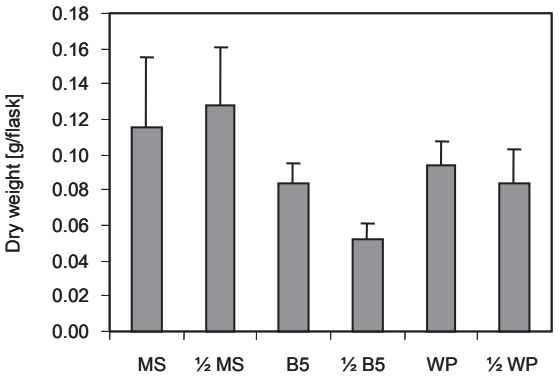

Fig. 2. Effect of medium type on dry weight of *P. candollei* hairy roots.

Table II. Effect of sucrose in medium on isoflavonoid content in *P. candollei* hairy roots.

Sucrose content (% w/v)	Isoflavonoid content [mg/g dry wt]					
	Puerarin	Daidzin	Genistin	Daidzein	Genistein	Total
1	2.51 ± 0.35	16.78 ± 3.57	1.04 ± 0.12	0.94 ± 0.08	0.48 ± 0.02	21.76 ± 4.16
3	2.22 ± 0.04	17.71 ± 3.15	1.09 ± 0.05	0.72 ± 0.01	0.58 ± 0.06	22.32 ± 3.30
5	2.71 ± 0.25	25.67 ± 2.70	1.40 ± 0.11	0.51 ± 0.01	0.59 ± 0.03	30.87 ± 3.10
7	2.18 ± 0.09	21.32 ± 3.32	1.21 ± 0.09	0.44 ± 0.01	0.57 ± 0.01	25.73 ± 3.52
9	2.25 ± 0.29	24.62 ± 3.42	1.20 ± 0.16	0.44 ± 0.00	0.63 ± 0.04	29.14 ± 3.91

Values represent the mean ± S.D. (n = 3).

Table III. Effect of medium type on isoflavonoid content in *P. candollei* hairy roots.

Medium type	Isoflavonoid content [mg/g dry wt]					
	Puerarin	Daidzin	Genistin	Daidzein	Genistein	Total
MS	2.37 ± 0.15	17.55 ± 3.86	1.02 ± 0.13	0.67 ± 0.06	0.61 ± 0.03	22.20 ± 4.24
½ MS	2.22 ± 0.04	17.71 ± 3.15	1.09 ± 0.05	0.72 ± 0.01	0.58 ± 0.06	22.32 ± 3.30
B5	2.13 ± 0.48	11.62 ± 2.23	0.95 ± 0.16	0.85 ± 0.12	0.58 ± 0.04	16.12 ± 3.02
½ B5	2.29 ± 0.22	14.44 ± 1.05	1.09 ± 0.05	0.73 ± 0.03	0.66 ± 0.02	19.21 ± 1.37
WP	1.90 ± 0.21	22.33 ± 3.40	1.14 ± 0.13	0.51 ± 0.01	0.51 ± 0.04	26.39 ± 3.80
½ WP	2.16 ± 0.21	7.24 ± 0.90	0.70 ± 0.04	1.63 ± 0.05	0.47 ± 0.01	12.20 ± 1.20

Values represent the mean ± S.D. (n = 3).

Acknowledgements

This work was supported by a grant from the Thailand Research Fund (RSA5280012) and the Graduate School, Khon Kaen University, Khon Kaen, Thailand. The research in this paper was also supported in part by a Grant in Aid from the JSPS Asian CORE Program, the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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