

## Production of Isoflavonoids in Callus Cultures of *Pueraria candollei* var. *mirifica*

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*Pueraria candollei* Wall. ex Benth. var. *mirifica* (Airy Shaw & Suvat.) Niyomdham was investigated for callus induction using Murashige and Skoog (MS) medium containing different plant growth regulators. After 8 weeks of culture, 66–100% of leaf or stem explants formed calli. Calli from stem explants cultured on MS medium supplemented with 0.5 mg/l thidiazuron (TDZ) gave the maximum of shoot induction (16%) and the highest level of total isoflavonoids [ $(50.39 \pm 7.06)$  mg/g dry wt], which was 7-fold higher than that of the native tuber [ $(7.04 \pm 0.29)$  mg/g dry wt]. These results suggest that addition of TDZ to the culture medium markedly enhances the production of isoflavonoids in calli induced from stem explants of *P. candollei* var. *mirifica*.

**Key words:** *Pueraria candollei* var. *mirifica*, Isoflavonoids, Callus Cultures

### Introduction

*Pueraria candollei* Wall. ex Benth. var. *mirifica* (Airy Shaw & Suvat.) Niyomdham (Leguminosae) has been used in Thai traditional medicine for rejuvenation. The tuberous root of this plant shows estrogenic activity (Chansakaow *et al.*, 2000a, b; Trisomboon *et al.*, 2006; Cherdshewasart *et al.*, 2007a; 2008), preventive action of bone loss (Urasopan *et al.*, 2008), and antioxidant activity (Cherdshewasart and Sutjit, 2008). The major chemical constituents isolated from the plant tuber are isoflavonoids, phytoestrogen compounds including puerarin, daidzin, genistin, daidzein and genistein (Chansakaow *et al.*, 2000a; Cherdshewasart *et al.*, 2007b). The concentration of isoflavonoids in the tuber of *P. candollei* var. *mirifica* is variable due to cultivation and harvest season (Cherdshewasart *et al.*, 2007b). Therefore, *in vitro* cultures are necessary for the selection of cell lines with high yield of bioactive phytoestrogen compounds. The establishment of callus cultures of *P. candollei* var. *mirifica* and the production of isoflavones like daidzein and genistein using 2,4-dichlorophenoxyacetic acid (2,4-D) and

kinetin as plant growth regulators has been reported previously (Thanonkeo and Panichajakul, 2006). However, the production of the major isoflavone glycosides, puerarin, daidzin and genistin, from *P. candollei* var. *mirifica* by means of a tissue culture has not yet been reported. Thidiazuron (TDZ) has been shown to be an efficient plant growth regulator enhancing the production of secondary metabolites in various plants such as asiaticoside in whole plant cultures of *Centella asiatica* (Kim *et al.*, 2005), artemisinin in regenerated shoots of *Artemisia annua* (Lualon *et al.*, 2008), glycyrrhizin in callus cultures of licorice (Wongwicha *et al.*, 2008), and pseudojubilogenin glycosides in plantlets of *Bacopa monnieri* (Kamonwannasit *et al.*, 2008). It would, therefore, be interesting to select calli yielding a high active compound through *in vitro* cultures using TDZ, compared with  $\alpha$ -naphthaleneacetic acid (NAA) and 6-benzyladenine (BA), as plant growth regulator. In the present study, we investigated the efficacy of different plant growth regulators for optimizing the callus induction, a regeneration system, and isoflavonoids production in *P. candollei* var. *mirifica*.

## Material and Methods

### Chemicals

$\alpha$ -Naphthaleneacetic acid (NAA) and 6-benzyladenine (BA) were purchased from Fluka Chemical (Buchs, Switzerland). *N*-Phenyl-*N'*-1,2,3-thiadiazol-5'-yl urea (thidiazuron, TDZ) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Daidzin, genistin, daidzein and genistein were purchased from Nacalai Tesque, Inc. (Tokyo, Japan). Puerarin was obtained from Chromadex Inc. (Irvine, CA, USA). All other chemicals were standard commercial products of analytical grade.

### Plant materials

*P. candollei* var. *mirifica* seeds were obtained from Botanical Garden, Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, Thailand. The seeds were washed with sterile distilled water and 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  $(25 \pm 1)^\circ\text{C}$  under 16 h light/day.

### Callus induction

Leaf and stem segments (0.5 cm) from *in vitro* plantlets fully grown within 4 weeks were cultured on MS medium with growth regulators, *i.e.*, TDZ alone (0.1–1.5 mg/l) and combinations of NAA (0.5–1 mg/l) and BA (0.5–1 mg/l). After 8 weeks, the calli were subcultured on the same medium. 8-Week-old calli were harvested for isoflavonoids analysis. The regenerated shoots were subcultured on MS medium without hormones for elongation and rooting. Shoot regeneration was observed for rooting at the end of the fourth week. The regenerated shoots were maintained on MS medium without hormones at  $(25 \pm 1)^\circ\text{C}$  under 16 h light/day and subcultured every 6 weeks.

### Extraction of callus samples and isoflavonoids analysis

Dried samples (30 mg) of 8-week-old calli 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. Puerarin, daidzin, genistin, daidzein and genistein contents in the extracted solutions were determined by HPLC. The HPLC analysis was performed 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<sup>®</sup>,

Table I. Percentage of callus and shoot induction, and number of shoots per explant of *Pueraria candollei* var. *mirifica* after 8 weeks of culture.

Plant growth regulator [mg/l]			Stem <sup>a</sup> /leaf <sup>a</sup>	Callus induction (%)	Shoot induction (%)	Number of shoots/ explant
NAA	BA	TDZ				
–	–	0.1	Stem	100.00	–	–
			Leaf	84.38	–	–
–	–	0.5	Stem	100.00	15.62	1.40
			Leaf	65.62	–	–
–	–	1.0	Stem	100.00	–	–
			Leaf	75.00	–	–
–	–	1.5	Stem	100.00	3.12	1.00
			Leaf	87.50	–	–
0.5	1.0	–	Stem	87.50	3.12	2.00
			Leaf	71.88	–	–
1.0	0.5	–	Stem	100.00	6.25	3.00
			Leaf	93.75	–	–
1.0	1.0	–	Stem	100	–	–
			Leaf	100	–	–

<sup>a</sup>  $n = 32$ .

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 determination was done in triplicate.

## Results and Discussion

Callus induction from *P. candollei* var. *mirifica* was evaluated on MS medium with TDZ or a combination of NAA and BA. After 8 weeks of culture, we succeeded in inducing callus formation. The initial calli were subcultured for further studies after an 8-week induction. The percentages of callus and shoot induction are shown in Table I. 66–100% of leaf or stem explants had formed calli. In the case of the stem explants, the percentage of callus induction was ranging from 88–100%. This result indicated that stem explants are suitable for callus induction. Among these results, the percentage of callus induction from stem explants was 100% independent of the concentration of TDZ in the culture medium (Table I). This result suggested that TDZ was effective for callus induction from stem explants of *P. candollei* var. *mirifica*.

The maximum of shoot induction was achieved from the stem explant cultured on MS medium supplemented with 0.5 mg/l TDZ (16%) while medium with 1 mg/l NAA and 0.5 mg/l BA gave maximum shoots per explant (3 shoots per explant) (Table I). The initial regenerated shoots were subcultured on MS medium without hormones after 8 weeks of callus induction. After subcultured for 4 weeks, rooting of the regenerated shoots was observed. The regenerated plants were not different in appearance from the normally grown *P. candollei* var. *mirifica* plants. We investigated also total isoflavonoids content in leaves of regenerated plants compared with that in the native plant. We found that leaves of plants regenerated from 0.5 mg/l TDZ [(7.51 ± 0.22) mg/g dry wt] contain a slightly higher level of total isoflavonoids than those of the native plant [(5.72 ± 0.46) mg/g dry wt].

We determined the isoflavonoids contents of 8-week-old calli by HPLC as shown in Table II. The total isoflavonoids content in induced calli from stem explants cultured with TDZ was high (31.79–50.39 mg/g dry wt) while that with NAA and BA was lower (11.21–20.07 mg/g dry wt) (Table II). The highest level of total isoflavo-

Table II. Isoflavonoids content in calli from *Pueraria candollei* var. *mirifica* after 8 weeks of culture.

Plant growth regulator [mg/l]			Stem/leaf					Isoflavonoids content [mg/g dry wt]				
NAA	BA	TDZ			Puerarin	Daidzin	Genistin	Daidzein	Genistein	Total		
-	-	0.1	Stem	Leaf	0.34 ± 0.01	40.37 ± 2.21	5.01 ± 0.32	1.31 ± 0.33	0.38 ± 0.09	47.43 ± 2.65		
					0.34 ± 0.01	22.05 ± 0.85	2.61 ± 0.24	1.17 ± 0.07	0.59 ± 0.02	26.76 ± 1.05		
-	-	0.5	Stem	Leaf	3.05 ± 0.58	39.58 ± 4.71	5.64 ± 1.31	0.68 ± 0.42	0.44 ± 0.09	50.39 ± 7.06		
					0.35 ± 0.01	32.46 ± 6.64	4.54 ± 0.48	1.42 ± 0.35	0.62 ± 0.06	39.37 ± 7.52		
-	-	1.0	Stem	Leaf	2.32 ± 0.02	24.11 ± 0.93	3.54 ± 0.55	1.39 ± 0.16	0.42 ± 0.07	31.79 ± 1.35		
					1.42 ± 0.10	18.99 ± 1.27	2.73 ± 0.61	0.92 ± 0.04	0.39 ± 0.02	24.45 ± 2.00		
-	-	1.5	Stem	Leaf	2.11 ± 0.21	24.34 ± 2.40	3.70 ± 0.20	1.35 ± 0.22	0.34 ± 0.33	31.85 ± 2.25		
					1.75 ± 0.10	19.46 ± 2.18	2.96 ± 0.14	1.08 ± 0.19	0.42 ± 0.14	25.67 ± 2.07		
0.5	1.0	-	Stem	Leaf	1.58 ± 0.07	15.71 ± 0.93	1.78 ± 0.36	0.66 ± 0.08	0.34 ± 0.04	20.07 ± 1.16		
					2.21 ± 0.38	30.90 ± 4.64	5.08 ± 0.85	1.21 ± 0.28	0.49 ± 0.04	39.91 ± 6.14		
1.0	0.5	-	Stem	Leaf	1.32 ± 0.03	11.69 ± 0.73	1.30 ± 0.01	0.66 ± 0.04	0.35 ± 0.04	15.32 ± 0.80		
					1.93 ± 0.18	22.49 ± 1.34	2.49 ± 0.30	0.91 ± 0.16	0.44 ± 0.02	28.26 ± 1.57		
1.0	1.0	-	Stem	Leaf	1.34 ± 0.14	8.03 ± 0.82	1.05 ± 0.19	0.49 ± 0.02	0.30 ± 0.01	11.21 ± 1.15		
					1.34 ± 0.13	12.11 ± 2.26	1.51 ± 0.39	0.86 ± 0.26	0.39 ± 0.07	16.22 ± 3.05		

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

noids was achieved in calli from stem explants cultured on medium with 0.5 mg/l TDZ [ $(50.39 \pm 7.06)$  mg/g dry wt]. HPLC analysis revealed that daidzin displayed the highest concentration of the isoflavonoids in callus cultures, followed by genistin, puerarin, daidzein and genistein, respectively. These results suggest that 0.5 mg/l TDZ showed high efficiency for callus induction, shoot regeneration, and enhanced production of isoflavonoids in *P. candollei* var. *mirifica*. According to previous reports, *in vitro* cultures of various plants on medium with TDZ gave also high levels of secondary metabolites (Kim *et al.*, 2005; Lualon *et al.*, 2008; Wongwicha *et al.*, 2008; Kamonwannasit *et al.*, 2008). Therefore, TDZ may hold a potential to enhance the yield of isoflavonoids in callus cultures of *P. candollei* var. *mirifica*.

We also studied the total isoflavonoids content in the native tuber (2-year-old), which was found to  $(7.04 \pm 0.29)$  mg/g dry wt [puerarin,  $(1.47 \pm 0.09)$  mg/g; daidzin,  $(3.66 \pm 0.15)$  mg/g; genistin,  $(1.10 \pm 0.03)$  mg/g; daidzein,  $(0.48 \pm 0.01)$  mg/g; and genistein,  $(0.32 \pm 0.01)$  mg/g dry wt, respectively]. Daidzin was the isoflavonoid with the highest concentration in the native tuber, *i.e.* the isoflavonoids profile in calli from *in vitro* cultures was the same as that from the native tuber. The total isoflavono-

noids content in callus cultures was 7-fold higher than that of the native tuber. In addition, the total isoflavonoids content in callus cultures from our study showed a higher level than that found in *in vitro* cultures of *P. tuberosa* (Goyal and Ramawat, 2008), *P. lobata* (Matkowsk, 2004; Li and Zhang, 2006), and *P. candollei* var. *mirifica* (Thanonkeo and Panichajakul, 2006). Therefore, calli of *P. candollei* var. *mirifica* from the present study are suitable source for studying the biosynthesis pathway of isoflavonoids in cell suspension culture.

In conclusion, stem explants of *P. candollei* var. *mirifica* cultured on MS medium supplemented with 0.5 mg/l TDZ are optimum for induction of callus, shoot regeneration and increased yield of total isoflavonoids. The present study reports a high production of bioactive phytoestrogens in *P. candollei* var. *mirifica* calli cultures. The callus developed here will be useful for cell suspension cultures to select the cell line with a high yield of isoflavonoids and large scale cultures.

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- Chansakaow S., Ishikawa T., Sekine K., Okeda M., Higuchi Y., Kudo M., and Chaichantipyuth C. (2000a), Isoflavonoids from *Pueraria mirifica* and their estrogenic activity. *Planta Med.* **66**, 572–575.
- Chansakaow S., Ishikawa T., Sekine K., Okeda M., Higuchi Y., and Chaichantipyuth C. (2000b), Identification of deoxymiroestrol as the actual rejuvenating principle of “kwao keur”, *Pueraria mirifica*. The known miroestrol may be an artifact. *J. Nat. Prod.* **63**, 173–175.
- Cherdshewasart W. and Sutjit W. (2008), Correlation of antioxidant activity and major isoflavonoid contents of the phytoestrogen-rich *Pueraria mirifica* and *Pueraria lobata* tubers. *Phytomedicine* **15**, 38–43.
- Cherdshewasart W., Kitsamai Y., and Malaivijitnond S. (2007a), Evaluation of the estrogenic activity of the wild *Pueraria mirifica* by vaginal cornification assay. *J. Reprod. Dev.* **53**, 385–393.
- Cherdshewasart W., Subtang S., and Dahlan W. (2007b), Major isoflavonoid contents of the phytoestrogen rich-herb *Pueraria mirifica* in comparison with *Pueraria lobata*. *J. Pharm. Biomed. Anal.* **43**, 428–434.
- Cherdshewasart W., Traisup V., and Picha P. (2008), Determination of the estrogenic activity of wild phytoestrogen-rich *Pueraria mirifica* by MCF-7 proliferation assay. *J. Reprod. Dev.* **54**, 63–67.
- Goyal S. and Ramawat K. G. (2008), Synergistic effect of morphactin on cytokinin-induced production of isoflavonoids in cell cultures of *Pueraria tuberosa* (Roxb. ex Willd.) DC. *Plant Growth Regul.* **55**, 175–181.
- Kamonwannasit S., Phrompittayarat W., Ingkaninan K., Tanaka H., and Putalun W. (2008), Improvement of pseudojubogenin glycosides production from regenerated *Bacopa monnieri* (L.) Wettst. and enhanced yield by elicitors. *Z. Naturforsch.* **63c**, 879–883.
- Kim O. T., Kim M. Y., Ahn J. C., Li M. Y., and Hwang B. (2005), Effects of precursor and thidiazuron on triterpene saponin biosynthesis in *Centella asiatica* (L.) Urban. *Korean J. Med. Crop Sci.* **13**, 91–94.
- Li L. and Zhang C. R. (2006), Production of puerarin and isoflavonones in cell suspension cultures of *Pueraria lobata* (Willd.): effects of medium supplementation with casein hydrolysate and coconut milk. *J. Environ. Biol.* **27**, 21–26.
- Lualon W., De-Eknamkul W., Tanaka H., Shoyama Y., and Putalun W. (2008), Multiple shoot regeneration and artemisinin production in *Artemisia annua* L. using thidiazuron. *Z. Naturforsch.* **63c**, 96–100.
- Matkowsk A. (2004), *In vitro* isoflavonoid production in callus from different organs of *Pueraria lobata* (Willd.) Ohwi. *J. Plant Physiol.* **161**, 343–346.

- Thanonkeo S. and Panichajakul S. (2006), Production of isoflavones, daidzein and genistein in callus cultures of *Pueraria candollei* Wall. ex Benth. var. *mirifica*. Songklanakarin J. Sci. Tech. **28**, 45–53.
- Trisomboon H., Malaivithnond S., Watanabe G., Cherdshewasart W., and Taya K. (2006), The estrogenic effect of *Pueraria mirifica* on gonadotrophin levels in aged monkeys. Endocrine **29**, 129–134.
- Urasopan N., Hamada Y., Cherdshewasart W., and Malaivithnond S. (2008), Preventive effects of *Pueraria mirifica* on bone loss in ovariectomized rats. Maturitas **59**, 137–148.
- Wongwicha W., Tanaka H., Shoyama Y., Tuvshintokh I., and Putalun W. (2008), Production of glycyrrhizin in callus cultures of licolice. Z. Naturforsch. **63c**, 413–417.