ORIGINAL ARTICLE

Production of isoflavones, daidzein and genistein in callus cultures of *Pueraria candollei* Wall. ex Benth. var. *mirifica*

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Abstract

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Callus cultures of *Pueraria candollei* var. *mirifica* were first established from various parts of explants with the objective of isoflavones, daidzein and genistein production. The cultures were studied on their growth and isoflavone production by various combinations of growth regulators, auxins and cytokinins at 25±2ºC. Daidzein and genistein accumulated in the cells were determined. The results revealed that callus of *P. candollei* var. *mirifica* was capable of producing high level of both isoflavones consistently. The culture temperature played an important role in the growth and isoflavone production. Over twofold of growth and threefold of isoflavone production were demonstrated at 32±2ºC. The callus established from the stems in MS medium supplemented with 4.5 µM 2,4-D and 0.46 µM kinetin produced the highest yield of daidzein (5.12 mg/g, DW) and genistein (2.77 mg/g, DW), which was remarkably higher than the intact plants.

Key words : callus cuture, *Pueraria mirifica*, daidzein, genistein, isoflavones

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Daidzein and genistein, which have a structure and functions similar to human estrogenic hormones, are recognized as phytoestrogen (Dixon *et al*., 1999). Based on these properties, both compounds have been associated with a reduction of breast and prostate cancers, cardiovascular and osteoporesis incidences (Hsieh *et al*., 1998; Potter *et al*., 1998; Kumar and Dahan, 1999; Ren *et al*., 2001). Recent studies on the effects of these compounds on heart and bone health including post-menopausal symptoms, indicated positive effects that are very attractive for use in health and functional foods (Brouns, 2002). This group of compounds is predominantly found in leguminous plants including, *Pueraria candollei* Wall. ex Benth. var. *mirifica* (Airy Shaw & Suvatabandhu) Niyomdham, the indigenous herb of Thailand. The active components, namely "phytoestrogen" especially isoflavones, daidzein and genistein, are mainly accumulated in tuberous roots (Kashemsanta *et al*., 1952; Ingham *et al*., 1986; Chansakaow *et al*., 2000). To-date, *P. candollei* var. *mirifica* is an endangered medicinal plant due to the limited resources of wild plants and soil erosion (Sahavacharin, 1999). The propagation of these plants, especially planting in the field, has been successful, which is encouraging for a commercial scale production. However, the amount of active components in the tuberous root is highly dependent on various physical and chemical factors such as geographical location, climate and disease (Burbidge, 1993). An alternative method for the production of these compounds is from plant cell cultures, which can be sustained at a desired level (Luczkiewicz and Glod, 2005). Isoflavones have been produced in callus cultures derived from various leguminous plants, such as *Pueraria lobata* (Takeya and Itokawa, 1982; Matkowski, 2004), *Psoralea* sp. (Bouque *et al*., 1998), *Maackia amurensis* (Fedoreyev *et al*., 2000) and *Genista* sp. (Luczkiewicz and Glod, 2003). The previous study showed that the accumulation of isoflavones in callus cultures was much higher than that from the intact plants.

The objective of this research was to study the alternative means of daidzein and genistein production by the establishment of *P. candollei* var. *mirifica* callus cultures. The culture conditions, including growth regulators and temperature, were optimized to increase the biomass yield and the isoflavone production, which would form a basis for a large-scale culture of *P. candollei* var. *mirifica* cells. The ability of isoflavone production in tuberous roots of intact plant and callus cultures were compared.

Materials and Methods

Plant materials

Seeds of *P. candollei* var. *mirifica* were kindly provided by Dr.Prasarn Chaladkid, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Rachasima, Thailand. Voucher herbarium specimens (No.11348) of the plant were identified and deposited at the herbarium of Khon Kaen University (KKU), Department of Biology, Faculty of Science, Khon Kaen University, Khon Kaen, Thailand. The seeds were washed thoroughly with tap water. They were subsequently surface-sterilized by shaking in 20% (v/v) of commercial Clorox containing three drops of Tween 20 for 20 min and thoroughly rinsed 3 times with sterile distilled water. The seeds were then clipped on seed coat and soaked in sterile distilled water for 24 h prior to germination on hormone-free MS medium (Murashige and Skoog, 1962) containing 3% (w/v) sucrose and 1% (w/v) agar in sterile vessels at 25±2ºC with a 16/8 h light/dark cycle (light intensity 20 μmole/m^2/s, cool white fluorescent Philips TLD 18W/33) for two weeks. Seedlings obtained were used for either plant cultivation or callus induction. For plant cultivation, the 1-month old seedlings were transplanted into individual pots (11x11x12 cm) and maintained in a greenhouse. After 1 month, they were cultivated in an open field. The tuberous roots (16-month old) were harvested from the 5 plants for isoflavone analysis by HPLC.

Callus induction

Micro roots from seedlings of *P. candollei* var. *mirifica* were excised and initiated on MS

medium supplemented with 3% (w/v) sucrose, 0.8% (w/v) agar and various combinations of growth regulators, auxin (4.5 and 9 µM 2,4-dichlorophenoxyacetic acid (2,4-D)) and cytokinins (0.46 µM kinetin and 4.4 µM 6-benzylaminopurine (BAP)). Cultures were incubated using the same conditions as the seedlings. Subcultures are routinely carried out every two weeks. Selection for a fast-growing callus lines were performed within 4 cycles. The selected calli were subsequently grown under the initial conditions for 2 months. Before the start of the experimental treatments, the time course of callus growth and isoflavone production were determined to establish the length of time required for callus cultures.

Effect of growth regulators and temperature on callus growth and isoflavone production

Calli were cultured on MS medium supplemented with various combinations of growth regulators, auxin (4.5 and 9 µM 2,4-D) and cytokinins (0.46 µM kinetin and 4.4 µM BAP) for optimization of growth and isoflavone production. The cultures were incubated at 32±2ºC in comparison to the control temperature (25±2ºC). All the experiments were conducted using 500 mg callus as inoculum in 20 mL medium in glass bottles (4.5x4.5x8.5 cm) for a growth period of 4 weeks. Each treatment had at least three replicates and the treatment on each growth regulator combination was repeated three times.

Effect of different parts of explants on callus growth and isoflavone production

Calli originated from various explants of *P. candollei* var. *mirifica* (root, cotyledon, stem, leaf, shoot) were established and screened on the best culture medium and conditions obtained from the previous experiments for the highest growth and isoflavone production. Growth and isoflavone accumulation were monitored for 28 days. All experiments were repeated three times. The data were submitted to the statistical analysis of variance by one-way ANOVA and the means were compared using Duncan's Multiple Range Test at a 5% probability level.

Measurement of callus growth

Callus growth was measured as dry weight (DW) by harvesting the biomass and drying at 60ºC in an hot air oven to a constant weight.

Extraction and HPLC analysis of daidzein and genistein

Three separate extractions and analysis were made from each pooled sample. The method for extraction of daidzein and genistein was modified from Federici *et al*. (2003). One g of mortar-ground dried cell was mixed with 1 g of celite and packed in a 16-mL glass column (BIO-RAD, USA). The elution was done using 50 mL of 80% (v/v) methanol solution. One mL of extracted solution was filtered through 0.45 µM nylon membrane before analysis with HPLC.

The method for HPLC analysis was modified from Hutabarat *et al*. (1998). The extracted samples (20 µl) were analyzed using a reversed-phase column (Nova-Pak C_{18}, 150x3.9 mm I.D.; 4 µm). The mobile phase used were 33% v/v of acetonitrile and 67% v/v of 1% v/v acetic acid with a flow rate of 0.8 mL/min. UV detection was carried out at 254 nm with attenuation of 0.1 AUF full-scale. Calibration curves were constructed by plotting the peak area (y) against concentration in µg/mL of standard solutions (x). The regression equation for daidzein and genistein were: $y = 1.16 \times 10^{-5}x + 0.01501$ and $y = 1.09 \times 10^{-5}x - 0.23630$ with r^2 values of 1 and 0.9998, respectively. The identification was performed by comparing retention times and UV absorption spectra to those of daidzein and genistein standards (Sigma, USA) (Figure 3). Both daidzein and genistein contents were reported as mg/g, DW.

Results

Effect of growth regulators and temperature on callus growth and isoflavone accumulation

In this experiments, the effects of different combination of auxin (2,4-D) and cytokinins (kinetin, BAP) on callus cultures of *P. candollei* var. *mirifica* were investigated at 25±2ºC and 32±ºC. The results in Table 1 and Figure 1A

Table 1. Effect of different concentrations of growth regulators on callus biomass and daidzein and genistein accumulation of *P. candollei* var. *mirifica* in MS medium at 25±2ºC and 32±2ºC

Growth Regulators (μM)			Cell Dry Weight (g)		Daidzein (mg.g^{-1}DW)		Genistein (mg.g^{-1}DW)	
2,4-D	Kinetin	BAP	25±2ºC	32±2ºC	25±2ºC	32±2ºC	25±2ºC	32±2ºC
4.5	0.46	-	0.1±0.01a	0.30±0.01ab	0.29±0.05a	3.04±0.09a	0.07±0.01ab	0.68±0.11a
9.0	0.46	-	0.15±0.01ab	0.29±0.01ab	0.15±0.02b	2.79±0.20a	0.04±0.01bc	0.51±0.15ab
4.5	-	4.4	0.13±0.01b	0.27±0.02b	0.07±0.01c	0.64±0.12b	0.03±0.01c	0.38±0.06b
9.0	-	4.4	0.15±0.01ab	0.31±0.01a	0.11±0.02bc	0.79±0.12b	0.09±0.01a	0.33±0.04b

Values are expressed as mean ± standard deviation (S.D.). Means within a column followed by the same letter are not significantly different (P = 0.05) according to Duncan's test.

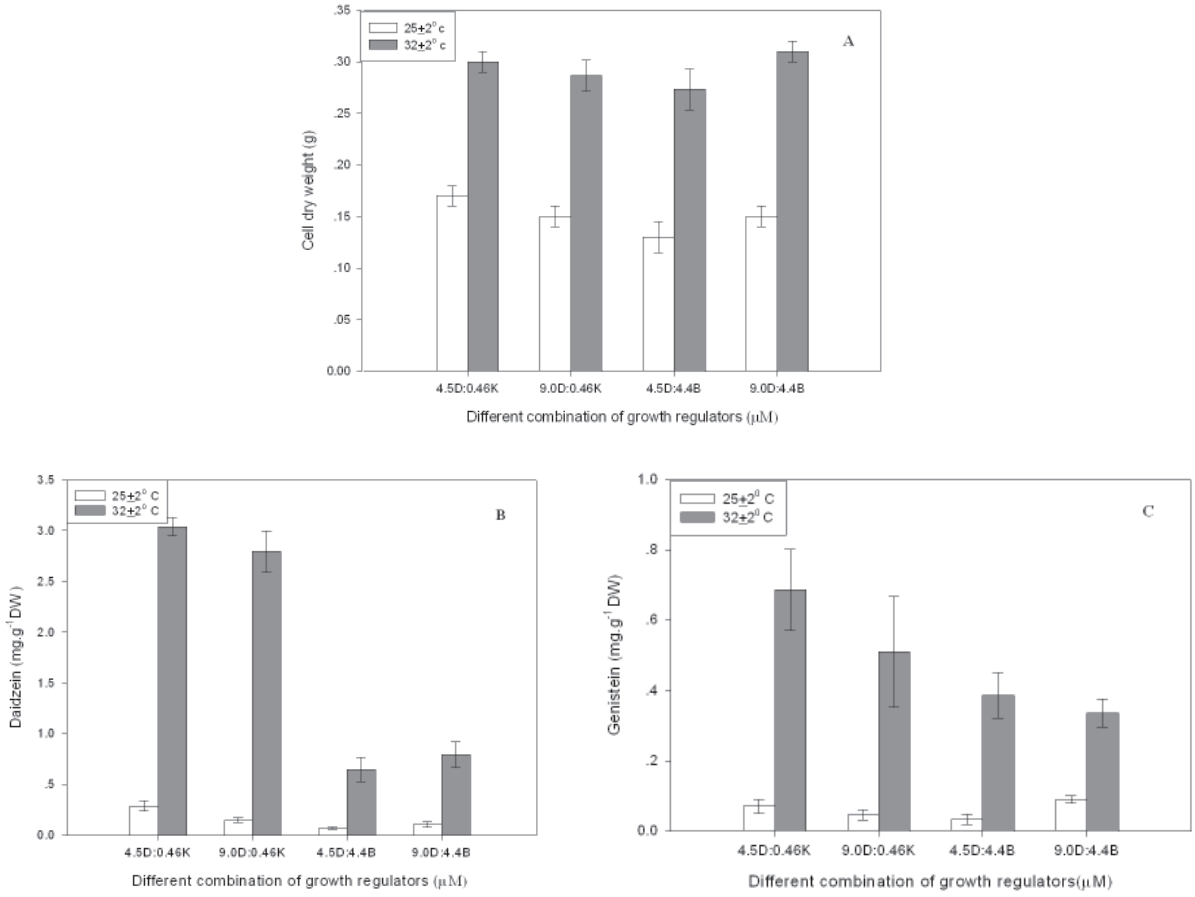

Figure 1. Growth and isoflavones production in calli of *P. candollei* as affected by various combination of certain growth regulators at 25±2ºC and 32±2ºC; callus growth (A), daidzein (B) and genistein (C) production (D = 2,4-D, K = Kinetin, B = BAP). The vertical lines represent standard deviation for three replications.

showed that the medium supplemented with 4.5 µM 2,4-D and 0.46 µM kinetin resulted in the highest cell dry weight and isoflavone contents at both temperatures. The callus obtained from this supplementation condition was friable, creamy-yellow, fast-growing with no tendency for morphogenesis and capable of forming homogenous suspension when scaling up in liquid medium. Although supplementation of 2,4-D and BAP at other combinations could stimulate the callus growth but the callus appeared to be both friable and compact.

For temperature effect, Table 1 and Figure 1A showed that, cell dry weight of the callus obtained when cultured at 32±2ºC was significantly higher than that obtained from the control temperature (25±2ºC).

The accumulation of isoflavones observed in all the combination of growth regulators were also enhanced at 32±2ºC in comparison to the control temperature at 25±2ºC. The medium supplemented with 4.5 µM 2,4-D and 0.46 µM kinetin resulted in the highest isoflavone accumulation when compared to other combinations, both at 25±2ºC and 32±2ºC (Table 1, Figures 1B and 1C). However, the amount of daidzein accumulated in every condition was remarkably higher than the genistein accumulated.

Effect of different parts of explants on callus growth and isoflavone accumulation

The growth and isoflavone production of callus cells originated from cotyledons, stems, leaves and shoots were compared to those from the roots. Calli were cultured in MS medium supplemented with 4.5 µM 2,4-D and 0.46 µM kinetin at 32±2ºC for 28 d. The results in Figure 2A show that the growth profile from various parts of explants did not show significant difference in both cell mass and growth rate. For all parts, a lag phase of about 4 d was observed and followed by rapid growth thereafter. In all cases, except for the roots, growth attained its maximum on day 16.

Regarding the isoflavone accumulation, the daidzein and genistein contents were highest in the callus originated from the stems on day 24 (Figures 2B and 2C). The accumulated amounts were 5.12 and 2.77 mg/g, DW for daidzein and genistein, respectively. The isoflavone accumulation began at the middle of the growth phase (day 8) and continued even with no further increase in biomass.

The comparison of isoflavone contents between the callus cultures of *P. candollei* var. *mirifica* and the intact plant is presented in Table 2. Both daidzein and genistein contents found in calli originated from stems were approximate 34 times higher than in tuberous roots.

Discussion

Growth regulators have an important role in callus cultures of *P. candollei* var. *mirifica* in enhancing isoflavone production. The suitable supplemented level of 2,4-D for isoflavone production was found to be 4.5 µM, although a higher concentration of 9.0 µM could stimulate callus growth but reduction in isoflavone contents was noticed at higher levels of 2,4-D. Cherdshewasart *et al*. (2003) reported that callus originated from shoot of this plant and cultured in MS medium supplemented with various concentration of NAA and BAP, could grow rapidly but the accumulation of some isoflavones were less than the intact plants. For cytokinin effect, a concentration of 0.46 µM kinetin was found suitable for both callus growth and isoflavone accumulation while BAP only stimulated callus growth. Similar observations were reported for callus cultures of *Pueraria lobata* (Takeya and Itokawa, 1982; Theim, 2003). Callus cultures of *Psoralea* sp. and *Genista* sp. also showed high accumulation of isoflavones with the addition of 2,4-D and kinetin (Bougue *et al*., 1998; Luczkiewicz and Glod, 2003).

The higher cultivation temperature of 32± 2ºC showed the better response for the callus growth and the accumulation of daidzein and genistein compared to the control temperature (25±2ºC). At higher temperature, there is a higher metabolic activity of *P. candollei* var. *mirifica* cells with increased growth rates accompanied by an increase in isoflavone accumulation. Similar

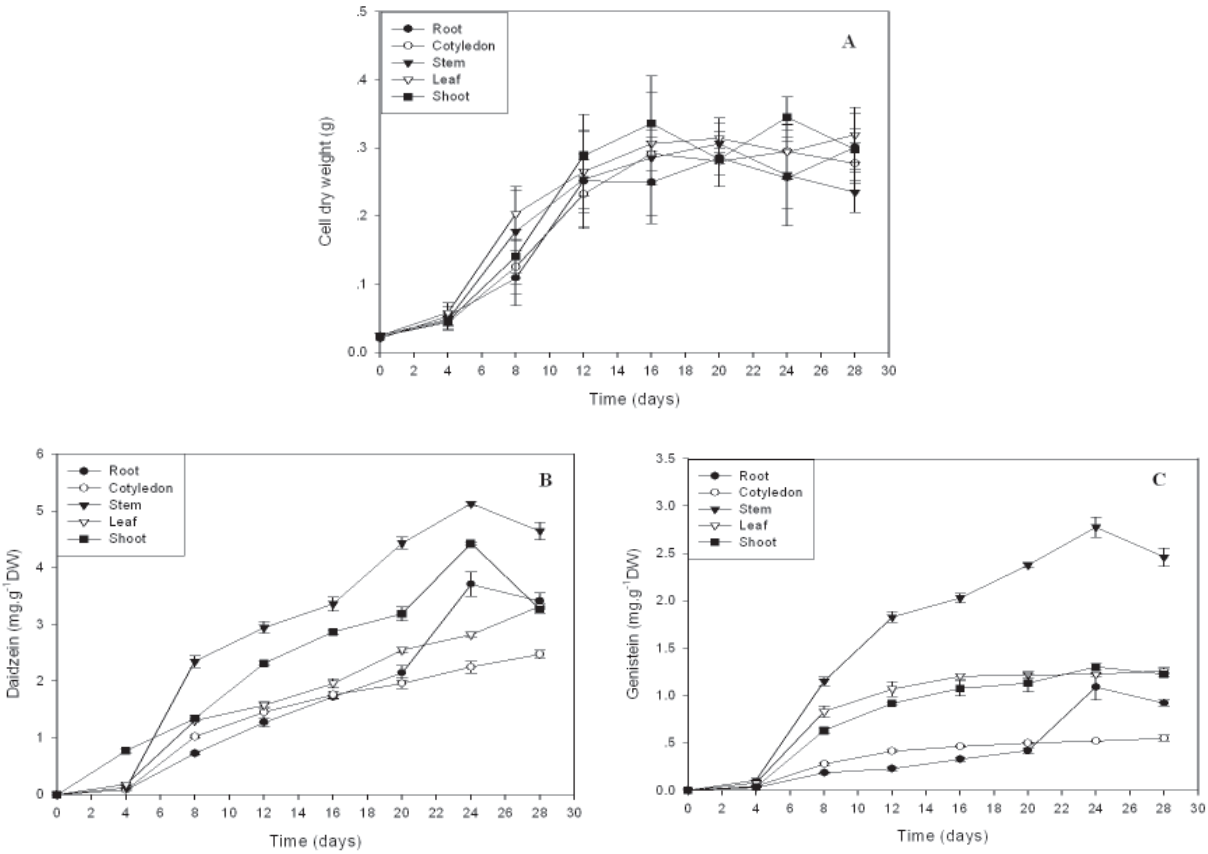

Figure 2. Time course of growth and isoflavone production of callus originated from different parts of explants cultured on MS medium supplemented with 4.5 µM 2,4-D and 0.46 µM kinetin at 32±2ºC for 28 days; (A) cell dry weight; (B) daidzein content; (C) genistein content.

Table 2. Comparison of isoflavone contents accumulated in callus cultures and the intact plants.

Sources	Isoflavone contents (mg/g, DW±S.D.)	
	Daidzein	Genistein
Callus originated from stem (24-day old)	5.12±0.04	2.77±0.10
Tuberous roots (16-month old)	0.15±0.07	0.08±0.02

Mean of three replicate analyses ± standard deviation (S.D.)

effects have been observed with anthocyanin biosynthesis by *Daucus carota* cells at 30ºC (Narayan *et al*., 2005). These results may lead to the possibility of using this parameter as a tool for manipulation of isoflavone production by *in vitro* culture of plant cells. It is not clear at present how the temperature affects the cellular metabolism. One may assume that the activities of enzyme involved in isoflavone biosynthesis such as isoflavone synthase (Hagmann and Grisebach, 1984)

and 2-hydroxyisoflavanone dehydratase (Hakamatsuka *et al*., 1998) are strongly affected by culture temperature. However, Zhao *et al*. (2001) found that jaceosidin production in callus culture of *Saussurea medusa* increased when cultured at 25ºC and decreased at 20 and 35ºC. Therefore, the optimal temperature for cell growth and metabolite accumulation is dependent on the species, and even for the same species temperatures which are suitable for cell growth and metabolites production may be different (Georgiev *et al*., 2004). No other investigations on the effect of temperature on isoflavone accumulation in callus cultures of any plant species have been reported, to the best of our knowledge.

Biosynthesis of daidzein and genistein in *P. candollei* var. *mirifica* callus cultures was closely related with the growth of the biomass. During the growth phase, the accumulation of daidzein and genistein took place slowly. The accumulation became particularly intensive during the stationary phase. This confirms literature reports of intensive accumulation of secondary metabolites in the stationary phase (van der Plas *et al*., 1995).

Callus cultures of *P. candollei* var. *mirifica* initiated from different parts of explants affected the isoflavone accumulation. The highest daidzein and genistein contents were found in callus originated from stem. Although, isoflavones accumulated in tuberous roots of intact plant were usually higher than the other organs (Ingham *et al*., 1986), the metabolic differences in different organs of intact plants may be different *in vitro* (Bouque *et al*., 1998). The higher accumulation of daidzein and genistein in callus culture as compared to the intact plant was also observed in *Psoralea species* (Bouque *et al*., 1998), *Maackia amurensis* (Fedoreyev *et al*., 2000) and *Glycine max* (Federici

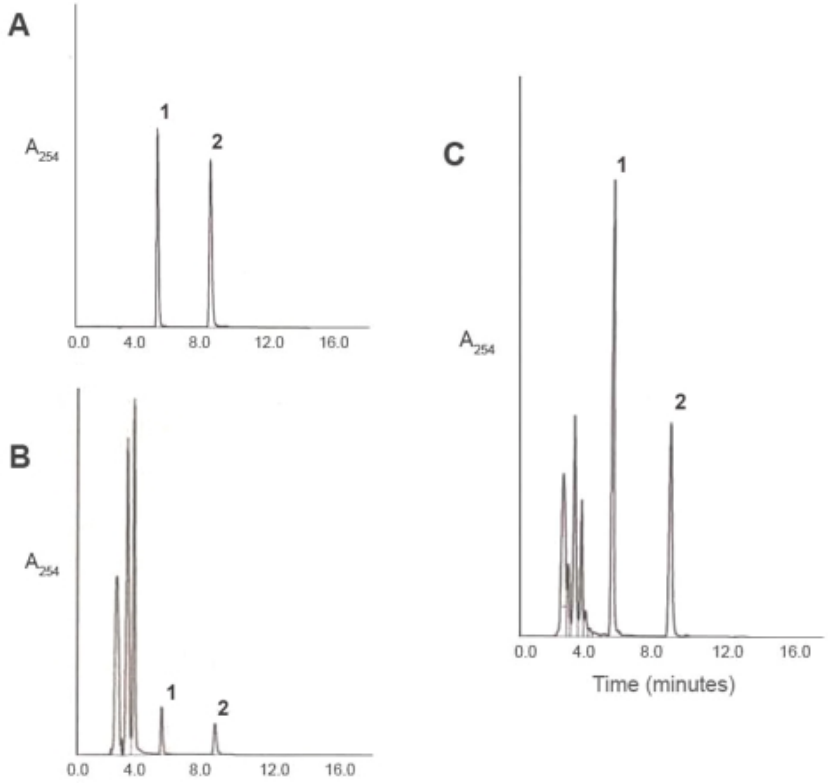

Figure 3. HPLC chromatograms of isoflavone standards (A) and extracts from tuberous roots (B) and callus originated from stem (C) monitored at 254 nm. Peak identification: 1, daidzein and 2, genistein.

et al., 2003). The suggestion was made that plant tissue cultivation *in vitro* represents an unorganized, differentiated stage in which the cells present a high variability in gene expression (Stöckigt *et al*., 1995)

In conclusion, this paper has described, for the first time, a procedure for initiation and establishment of callus cultures of *P. candollei* var. *mirifica*, which was able to accumulate high level of isoflavones of phytoestrogenic activity. The results demonstrated the importance of the components of growth regulators in growth medium, the cultivation temperature and parts of explants on the callus growth and daidzein and genistein production. However, the production of isoflavones in callus cultures was limited in the scale-up for commercial production. Therefore, the parenchymatic callus cultures, which have a potential to retain the capacity to produce high level of isoflavones and growth rates, are now being applied for further research in order to develop a homogenous suspension culture. In the future, we will attempt to produce cell suspension cultures from our callus collection and investigate the production of daidzein and genistein in the liquid medium, with the addition of elicitors to enhance the synthesis.

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