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Production of puerarin and isoflavones in cell suspension cultures of *Pueraria lobata* (Willd.) : Effects of medium supplementation with casein hydrolysate and coconut milk

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Abstract: *Callus induced from leaf explants of Pueraria lobata seedlings were suspended in Gamborg B5 medium supplemented with 1 mg l^{-1} 2,4-dichlorophenoxyacetic acid, 1 mg l^{-1} naphthalene acetic acid, 0.5 mg l^{-1} kinetin and 30 g l^{-1} sucrose. The effects of coconut milk and casein hydrolysate (CH) on cell growth and yields of puerarin and isoflavones in cells suspension were studied. The contents of total isoflavones and puerarin in suspension cultures were determined by spectrophotometry and HPLC. Coconut milk (10%, filter sterilized) decreased the growth of cell cultures and the accumulation of total isoflavones, while 0.2% CH promoted the growth of cell cultures and the accumulation and release of puerarin and total isoflavones. The total yield of puerarin and isoflavones were 34% and 40.8% higher than in the control, respectively. The optimum medium for cell cultures of leaves of P. lobata seedlings was B_5 liquid medium supplemented with 2% sucrose, 1.0 mg l^{-1} 2,4-D, 1.0 mg l^{-1} NAA, 0.5 mg l^{-1} kinetin and 20 mg l^{-1} CH. The procedure use is a potentially useful for the production of isoflavones .*

Key words: *Coconut milk, Casein hydrolysate, Pueraria lobata (Willd.), Suspension cell culture, Puerarin, Isoflavone.*

Introduction

Pueraria lobata (Willd.) Ohwi (Fabaceae), is one of the oldest medicinal plants in traditional Chinese medicine, and is widely used presently also in China and other East Asian countries. The dried roots of *P. lobata* have been used as the main ingredient of a traditional prescription, *Puerariae Radix*, (called 'Gegen' in China), for the treatment of early symptoms of the common cold and as an antipyretic, antidiarrhetic, diaphoretic, and antiemetic agent (Keung and Vallee, 1998; Rong *et al*., 1998). Ethanolic extracts yielded flavonoids, coumarins and total isoflavones, which include puerarin, daidzein and daidzine-4',7-diglucoside (Cao *et al*., 1999). The main active and pharmacological constituents of Puerariae Radix are isoflavones, among which puerarin, daidzein, and daidzine are the most important (Zhong *et al.,* 1992). These have been shown to stimulate cerebral and coronary blood circulation (Huang *et al.,* 2001). Increasing interest in these isoflavones led us to develop a possible production method using *in vitro* plant culture.

Callus and suspension cultures from cotyledons of *P. lobata* produce isoflavones in the cells and medium and Gamborg B5 medium (Liu and Li, 2002) was suitable for cell suspension culture. The authors found the production of puerarin to be linked with cell growth and puerarin yield reached a maximum after 8 d. However, the accumulation pattern of total isoflavones was different from that of puerarin. The yield of the latter reached a maximum after 10 d. The yield of total isoflavones in cell suspension was higher than in the wild plant while the yield of puerarin was lower (Liu and Li, 2002).

The addition of natural products, such as coconut milk (CM), casein, casein hydrolysate (CH), lactoprotein hydrolysate, banana juice and apple juice, to the *in vitro* medium could meet the needs of growth and development of excised tissues and promote cell growth as well as production of secondary metabolites (Zhang *et al.,* 1994; Zhao and Li, 2000). CH and CM contain a mixture of various organic compounds and amino acids. CM also contains phytohormones. Extracts of corn powder and CH increased cell growth of *Panax quinquefolium* (Tang *et al.,* 1994). Soybean powder extract decreased cell growth during the early stages of cell culture, but promoted it in the latter stages (Yuan and OuYang, 1994). The promotive effect of lactoprotein hydrolysate on growth of suspension cells in *Panax ginseng* was found to be higher than that of CH, while CH was better than LH in *Taxus yunnanensis* to increase the growth rate of the cell suspension (Zhang *et al*., 1997). Taxol content could be promoted by increasing supplementation of CM, CH and LH; while CM promoted cell growth of the cell culture (Gan *et al.,* 1997).

The ready availability and low cost of CM and CH prompted us to investigate their potential as broad spectrum supplements to promote the cell culture of many plants (Zhang *et al.,* 1997; Gan *et al.,* 1997). The aim of this study was to determine the effects of CM and CH on cell growth and on isoflavone levels in suspension cell cultures of *P. lobata* leaves.

Materials and Methods

Plant material: Unripe (green) pods of *P. lobata* growing in the Botanical Garden (South China Normal University) were sequentially rinsed in water, 70% EtOH for 1 min and 0.2% $HgCl_2$ for 20 min, and finally rinsed three times in sterile water. Seeds were germinated in the dark for 48 hr. The leaves focus seedlings with four leaves developed after 8 days were excised and placed on Murashige and Skoog medium (Murashige and

Skoog, 1962) supplemented with 1mg l^{-1} naphthalene acetic acid, 0.5 mg l^{-1} 4-PU (N-(2-chloro-4-pyridyl)-N'-phenylurea) and 30 gl^{-1} sucrose to induce callus formation. Cultures were maintained under a 16 hrs photoperiod regime with fluorescent light at 25ºC without light .After 5 days, white yellowish-colored callus clumps were formed at the edge of the explants. Pieces of callus were excised and transferred to 0.8% agar gel with Gamborg B5 medium (Liu and Li, 2002) supplemented with 1 mg l^{-1} 2,4-D, 1 mg l^{-1} NAA, 0.5 mg l^{-1} kinetin and 30 gl^{-1} sucrose for culture. The callus was subcultured every 15 days. After a sequence of 6 subcultures, a light-yellowish coloured, friable callus was obtained. This was used for initiation of suspension cultures. Callus (1.5 g fresh wt) was transferred to a 150 ml flask containing 50 ml of liquid Gamborg B5 medium supplemented with 1 mg l^{-1} 2,4-D, 1 mg l^{-1} NAA, 0.5 mg l^{-1} kinetin, and 30 gl^{-1} sucrose. To study the roles of casein acid hydrolysate or coconut milk, some media also contained either 20 mg l^{-1} CH (sigma, 70% free amino acids) or 10% (v/v) fresh CM (mature fruit boiled, then filtered by filter paper and finally filter sterilized).

The friable callus rapidly disintegrated to single cells and developed into small cell aggregates after incubation at 25ºC on an orbital shaker (130 rpm). The cell cultured with the optimal initial density of 2 g l^{-1} dry mass, shielded from light, at 25°C. Suspension cell cultures were subcultured every 7 days and the medium (about 1/3 of the total volume) was added to fresh medium. The resulting suspension consisted of spherical or elongated single cells and small cell clumps, the latter being quite homogeneous during the early stages of growth, becoming larger with time.

To determine callus fresh weight, the entire callus (scraped off the medium) was transferred to a pre-weighed boat and the fresh weight was determined. Fresh weight of cell cultures was measured by transferring the flask to a pre-weighed graduated centrifuge tube. The tube was centrifuged at 200 g for 5-10 min. When the supernatant was free from cells, the culture medium was removed and the fresh weight was determined. The dry mass of callus and suspension cell cultures were measured after the samples were dried at 60°C until the dry mass was stabilized.

Measurement of total isoflavones and puerarin: Total isoflavones and puerarin were extracted and determined from callus and suspension cultures as described earlier (Meng *et al.,* 1996; Zhong *et al.,* 1992). Dry powder (25 mg) was blended with 30 ml 95% EtOH and placed in a water bath at 70°C for 6 hr. After cooling, the crude extract was diluted with 95% EtOH to 50 ml. Samples (1.0 ml) of the extract were diluted with distilled H_2O upto 25.00 ml. The extinction of samples at 250 nm was determined by a UV-VIS spectrophotometer (UV-2100, shimadzu, Japan). The linear regression equation y=0.0685x+0.0064, where x is the concentration of total isoflavonold (mg l^{-1}), y is E_{250}, was determined by measuring a series of concentrations of standard puerarin samples (Sigma). For HPLC analysis, 100 mg dry powder samples were extracted in 1.0 ml absolute

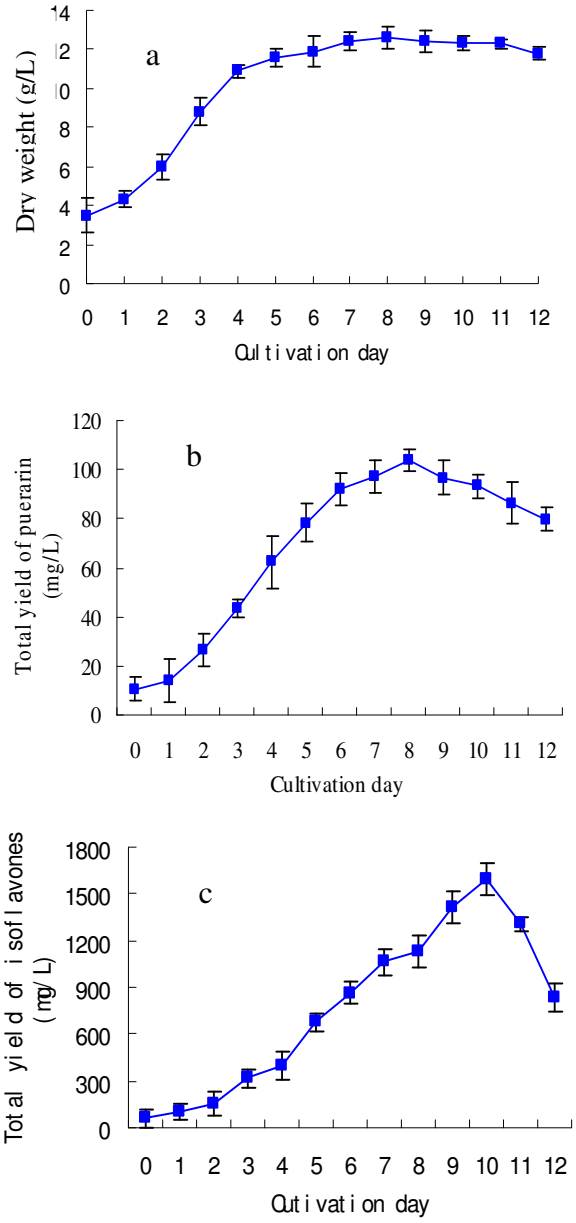

Fig. 1: The cultivation time course of a. cell growth, b. total yield of puerarin and c. total isoflavones in cell suspension cultures of *P. lobata*. The data points are the average of three determinations.

EtOH for 24 h. Extracts were combined, filtered, and diluted with absolute EtOH to 5 ml. The HPLC (Gold System, Beckman) was used as follows: column, Ultrasphere ODS (4.6 mm × 250 mm); eluant, MeOH: H_2O (50 : 50); flow rate, 1.0 ml/min; detection, E 248 nm. The concentration of puerarin was calculated according to the linear regression equation y=1584.3x+0.3569, where y is the peak area, x is the concentration of puerarin. The equations were calculated using the standard puerarin (Sigma, St Louis, MO, USA). The results presented are the means of three independent experiments.

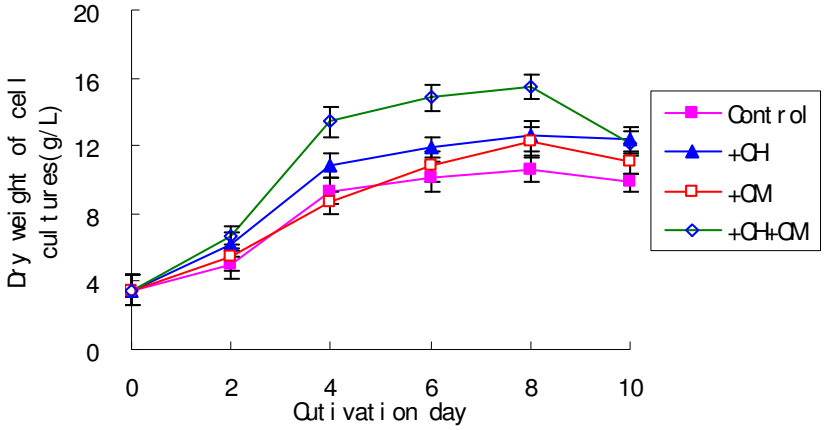

Fig. 2: Effects of media supplementation with casein hydrolysate (CH) and coconut milk (CM) on the growth of suspension cell cultures of *P. lobata* . Data points the average of three determinations.
Control: Unsupplemented liquid medium
+CH : Supplemented with 20 mg l^{-1} CH
+CM: Supplemented with 10% CM
+CH+CM: Supplemented with 20 mg l^{-1} CH and 10% CM

Results and Discussion

The time course of *P. lobata* cell growth in B5 liquid medium supplemented with 2,4-D, NAA as well as KT, cell growth is typically "S" shaped, consisting lag, logarithmic growth and stationary phases (Fig. 1a). For suspension cultures, the cell growth was maximum (dry mass) reached on day 8. Puerarin was present in suspension cultures from the early stages but the maximum product accumulation coincided with the stationary phase of growth (Fig. 1b) after 8 days. The maximum yield of isoflavones in suspension culture occurred after 10 days (Fig. 1c). These results show that the production of puerarin was strongly correlated with cell growth under these conditions. Further formal analysis of the data would be required to show that the highest levels of total isoflavones were reached during the stationary phase.

The influence on cell growth in suspension culture of medium supplementation with CH and CM was investigated (Fig. 2) . CH 20 mg l^{-1}, added alone or combination with CM, increased the rate of cell growth during the logarithmic phase. The enhancement of cell growth by CH combination with CM was better than CH treatment alone. The effect of CM alone was not significantly different during the rate of logarithmic phase growth. Both supplements alone (CH+CM) increased the yield of cells in stationary phase, and to a further extent when added together. The colour of callus grown in medium supplemented with CM was light-brown, and then was sepia with prolonged time. The enhancement of cell growth by CH is likely to have been due to the effects of one or more amino acids in this protein hydrolysate. No conclusion can be drawn about CH combination with CM responsible for increment cell growth.

Supplementation of culture medium by CH promoted the accumulation of puerarin over the control on both a cell dry weight basis (Fig. 3a) and total yield (Fig 3b). Both of these were inhibited by CM (Fig. 3a, b). When CH and CM were added together, puerarin content and total yield of puerarin was greater than that of CH alone or CM alone up to 6 days, but lower thereafter.

The puerarin concentration was also determined in the culture media after removal of the cells (Fig. 3c). In the control, the proportion of puerarin in the medium was less than 5% of the total. This was elevated by CH, but not by CM, while the presence of both supplements enhanced the concentration of puerarin in the medium further up to day 4 but not after longer cultivation times. This indicates that CH or CM have some effect on puerarin release in the early growth stage in suspension cell culture. Little is understood with respect to stimulation of puerarin production by CH treatment, except that recovery of puerarin from the liquid medium can be excluded as a production method on account of the low concentrations of total isoflavones and puerarin released by cells to the liquid medium, which will be collected puerarin or isoflavones from the liquid medium.

Less than 2% of isoflavones were present in the liquid medium, showing that these compounds were also contained intracellularly. The effects of CH and CM on production of isoflavone in suspension cell culture (Fig. 4a, 4b) and accumulation in the liquid medium (Fig. 4c) were similar to their effects on puerarin content (Fig. 3). CM inhibited the production of isoflavones in suspension culture throughout the experimental period, although it promoted isoflavone release to the liquid medium (Fig. 4c). CH might make the cell membranes more permeable and stimulated total isoflavone production, resulting in a 40.76% increase over the control after 10 days. However, it did not affect the release of isoflavones in the liquid medium. CH in combination with CM promoted the production of

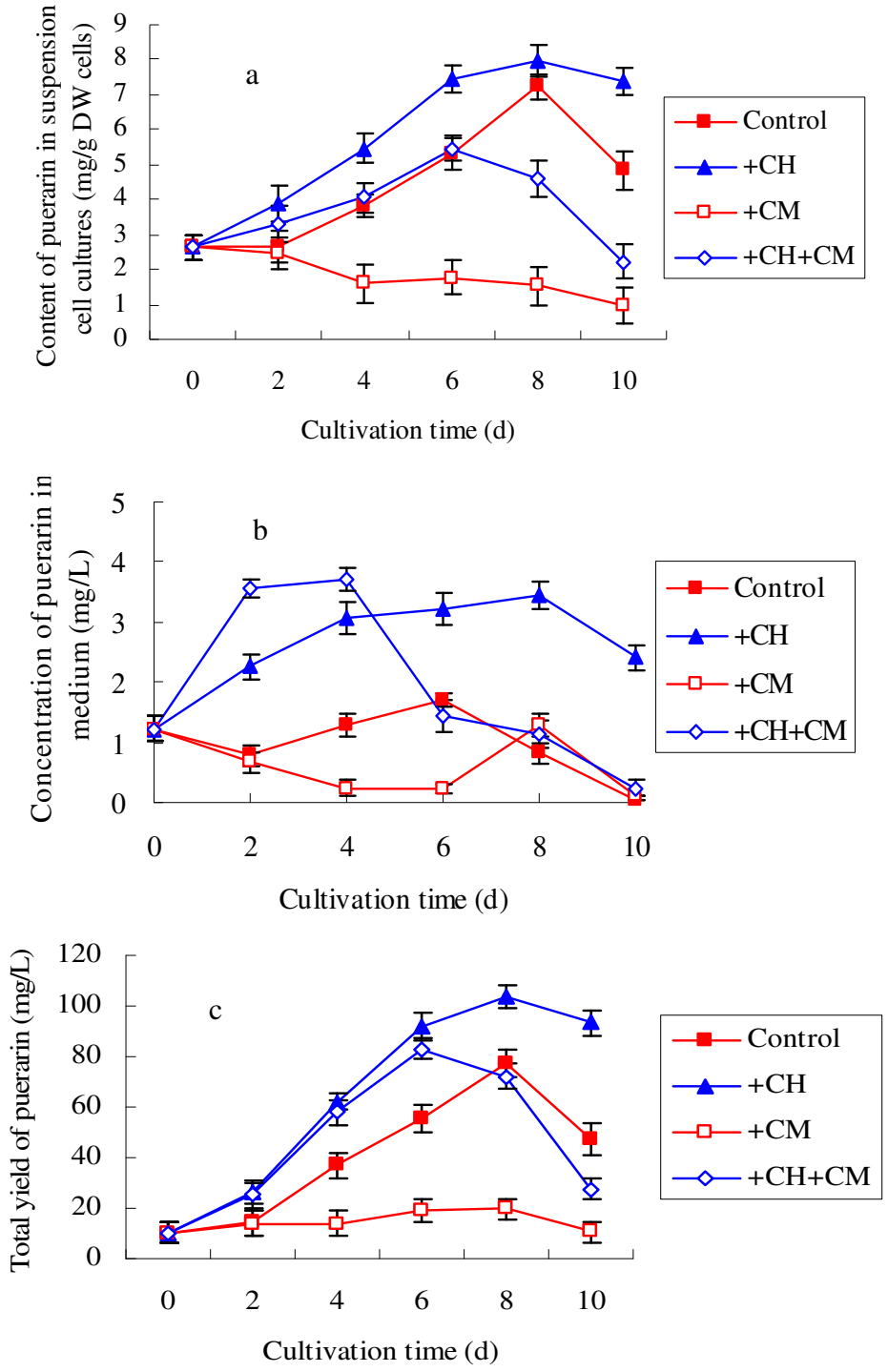

Fig. 3: Effects of CM and CH on the yield of puerarin in suspension cell cultures of *P. lobata*. (a): puerarin content on a cell mass basis; (b): concentration of puerarin in the medium;(c): total prerarin concentration in cultures . Data points are the average of three determinations.
Control: Unsupplemented liquid medium
+CH : Supplemented with 20 mg l^{-1} CH
+CM: Supplemented with 10% CM
+CH+CM: Supplemented with 20 mg l^{-1} CH and 10% CM

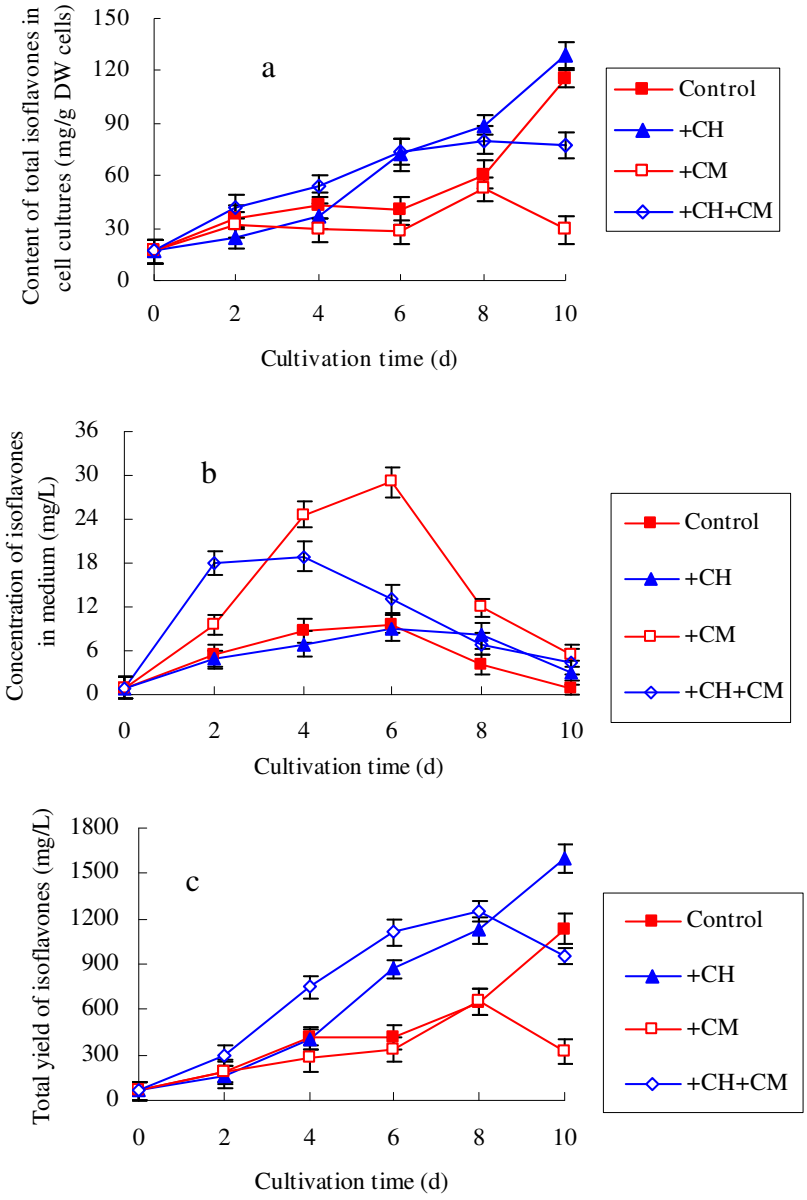

Fig. 4: Effects of CM and CH on the total yield of isoflavones in cell cultures of *P. lobata* . Data points are the average of three determinations.
Control: Unsupplemented liquid medium
+CH : Supplemented with 20 mg l^{-1} CH
+CM: Supplemented with 10% CM
+CH+CM: Supplemented with 20 mg l^{-1} CH and 10% CM

isoflavones both in suspension culture and in medium, which will be used to isoflavones production.

Liu and Li (2002) found that total isoflavones and puerarin produced in suspension cell culture of *Pueraria* cotyledons were lower than in suspension cell culture of leaves. It is possible that the accumulation of secondary metabolism isoflavones and puerarin by suspension cell culture are related to organic resource and organization of cellular tissue.

The higher content of puerarin in natural roots, compared to that in callus and suspension cells, is perhaps due to better cell to cell contact, ageing, or the limited cellular differentiation in the callus and cell culture. It has been shown that the degree of differentiation and organization of cellular tissue are positively correlated with accumulation of secondary compounds (Flores, 1992; Yu *et al*., 2002). On the other hand, an increased secondary metabolite may be correlated with a

slow cell division rate in cell suspension culture (Lindsey and Yeoman, 1983). However, in our system suspension cells grow much faster than callus and its rate is markedly lower than that of callus, which may further account for the higher production.

We conclude that the better medium for cell cultures of *P. lobata* seedlings leaves was B_5 liquid medium supplemented with 2% sucrose, 1.0 mg l^{-1} 2,4-D, 1.0 mg l^{-1} NAA, 0.5 mg l^{-1} kinetin and 20 mg l^{-1} CH . Filtered CM resulted in increase the production of isoflavones and puerarin in the medium. Further studies will focus on improving isoflavones concentrations in suspension cells with elicitors and bioreactor cultivation of cells to assess the possibility of increasing production in cultures.

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