

<http://www.pjbs.org>

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSI*net*

Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Production of Podophyllotoxin by Immobilized Cell Cultures of *Juniperus chinensis*

Duangporn Premjet and Sanro Tachibana

Department of Applied Bioscience, Faculty of Agriculture, Ehime University,
Tarumi 3-5-7, Matsuyama, Ehime 790-8566, Japan

Abstract: In the present study immobilized cell cultures of *Juniperus chinensis* were developed using various calcium alginate gel concentrations (1.8, 3 and 6% w/v). The podophyllotoxin productivity of immobilized cell cultures was compared with that for free cell suspension cultures. The free cell suspension cultures accumulate podophyllotoxin in intracellular compartments, while the immobilized cells with calcium alginate excrete marked amounts of podophyllotoxin into the culture media. From the effect of immobilization components on free cell suspension cultures, it was considered that the calcium alginate gel was supposed to be act as an elicitor and forced the cells to release the product into the culture media. The amount of podophyllotoxin excreted depends on the alginate gel concentration used: 3% calcium alginate gel promoted podophyllotoxin production through 28 days of cultivation. The podophyllotoxin production by immobilized cell cultures entrapped with the 3% calcium alginate gel was enhanced to 4 mg g⁻¹ dry weight.

Key words: Immobilized cell cultures, calcium alginate gel, podophyllotoxin, *Juniperus chinensis*

INTRODUCTION

Podophyllotoxin, an aryltetralin lignan, serves as semi-synthetic precursor of etoposide and teniposide^[1]. Podophyllotoxin derivatives like etoposide and teniposide are used clinically as potent chemotherapeutic agents for a variety of tumors including small cell lung carcinoma, testicular cancer and malignant lymphoma. These compounds are derived from a series of modifications, which converted podophyllotoxin from an entity that interacts with tubulin and blocks mitosis to one that induces a block in late S or early G2 phase by interacting with topoisomerase II^[2]. We have been conducting research on the production of podophyllotoxin by tissues and cell cultures for several years in the wake of the discovery of this lignan in *Podophyllum* sp. Podophyllotoxin is extracted from rhizomes of *Podophyllum* sp, a species now at risk of extinction and found that *J. chinensis* extractives contain podophyllotoxin, providing hope that *J. chinensis* may be an alternative to *Podophyllum* sp^[3]. However, the podophyllotoxin was only detected in trace amounts, so cell cultures have been considered a tool to improve yield. Callus cultures and cell suspension cultures of *J. chinensis* have been shown to be able to produce this aryltetralin lignan. The improvement in

podophyllotoxin production on addition of exogenous biogenetic precursors and elicitors has increased target compound accumulation in both callus and cell suspension cultures^[4-6]. However, limitations to long-term production in both systems were found: the podophyllotoxin content declined from sub-culture to sub-culture in callus cultures and free cell suspensions in shake flasks show a slow growth rate and cell shearing. These problems can be overcome by cell immobilization^[7-11]. The major advantages of cell immobilization are that it provides a high cell concentration per unit volume of reactor, better cell-cell contact and more favorable conditions for cell differentiation, resulting in a higher yield of secondary metabolites. In immobilized cell systems, cells release the target product into the medium, so it can be removed from the culture while the cells remain^[12-15]. Podophyllotoxin was released into the medium when *J. chinensis* cells were entrapped in an alginate gel in a preliminary study^[16].

This study will describe a method for the immobilization of *J. chinensis* cells by entrapment at various calcium alginate gel concentrations. The ability of the immobilized cell cultures to produce podophyllotoxin in beads will be investigated in terms of cell growth, the pattern of accumulation in cells and compound released into the culture medium.

MATERIALS AND METHODS

Study area: This research project was carried out in the Faculty of Agriculture, Ehime University, Japan during 2001-2003 in an effort to produce podophyllotoxin from immobilized cell cultures of *Juniperus chinensis*. Authentic podophyllotoxin was purchased from Sigma Chemical Company as well as isolated from the leaves of *J. chinensis*^[3].

Plant materials: Stems of *J. chinensis* were used for callus cultures as described previously^[5].

Cell suspension cultures and incubation conditions: Cell suspensions were developed from the callus cultures prior to immobilization by transferring 20 g of calluses into 100 ml of SH medium supplemented with 3 mg L⁻¹ of NAA and 0.2 mg L⁻¹ of kinetin^[17]. These cell suspensions were maintained by subculturing at 3-week intervals.

Immobilization of cells in calcium alginate beads: For the immobilization of cells of *Juniperus chinensis*, 20 g of the cells was added to 40 ml of SH medium and mixed with 60 ml of 3, 5 and 10% (w/v) sodium alginate made up in the culture medium. The mixture was added drop-wise to 300 ml of a stirred culture medium containing 0.1 M calcium chloride, through a 2 mm tip diameter pipette. Beads formed immediately on contact with the calcium chloride solution. The beads were shaken for 2 h to ensure complete fixation. The beads and medium were poured through a glass filter. Distorted beads and beads containing trapped air bubbles were removed and washed twice with 250 ml of the culture medium by shaking at 120 rpm for 15 min. The medium was drained off. Amount of cells entrapped in beads were determined, 60 beads contained approximately 1 g of fresh cells. The 60 beads were then resuspended in fresh culture medium (10 ml) in 50 ml flasks and cultured. Control experiments were performed by transferred 1 g of fresh cells into 10 ml of SH medium supplemented with NAA (3 mg L⁻¹) and kinetin (0.2 mg L⁻¹). Additional control experiment was performed to observe the influence of immobilization components on free cell suspension cultures by transferred 1 g of fresh cells into SH medium 10 ml follow by addition of 0.1 M CaCl₂ or alginate gel (1 g L⁻¹) at day seventh after subculture.

Each experiment was repeated independently thrice and the results shown, are the average of three measurements, the deviation of each experimental value being within 12%.

Growth measurements: The fresh weight of free cell suspension cultures and immobilized cell cultures were monitored at 7, 14, 21 and 28 days of incubation. The

calcium alginate was dissolved by a sodium citrate solution (4%) and stirred for 30 min at 30 °C. The liberated cells were rinsed with distilled water and their fresh weight determined. Dry weights were measured from freeze-dried cells. Cell viability was determined by fluorescein diacetate (FDA) staining^[18].

Preparation of samples for HPLC analysis: Samples of beads were removed from the immobilized cell cultures at 7, 14, 21 and 28 days and then freeze-dried. These samples were extracted with methanol and water (1:1). The methanol solubles were partitioned between dichloromethane and water (1:1). The dichloromethane layers were collected and evaporated to dryness. The residue was dissolved in methanol. After filtration, the sample was injected into the HPLC apparatus. For the detection of podophyllotoxin accumulated in the culture medium, fresh medium was freeze-dried and extracted with methanol and the same method performed with the cells.

HPLC analysis of podophyllotoxin: Reversed-phase HPLC was used for the separation of these compounds as described in previous work^[4].

RESULTS

Cell growth of free cell suspension cultures and calcium alginate immobilized cell cultures: Polysaccharide matrices generated in gel beads are strongly dependent on several variables like ionic strength and polymer concentrations^[7,18,19]. Thus different matrices, obtained by varying the amount of alginate gel (1.8, 3 and 6%) were used to study the influence of gel concentration on the growth and pattern of release of podophyllotoxin of *J. chinensis* cells. The initial amount of cells in each flask was one-gram fresh weight. The cell growth was measured in term of dry weight at days 7, 14, 21 and 28. As the results show in Fig. 1, free cell suspension cultures had a normal sigmoid growth curve. A slight increase in biomass was detected on days 7 to 14 and maximum growth occurred on day-21 (180 mg/flask). Unlike in control cultures, the growth of immobilized cells in whatever gel concentration was retarded. The immobilized cells in 1.8% alginate gel showed better growth than those of 3 and 6% alginate gel. The maximum cell dry weight (140 mg/flask) was obtained from immobilized cells in 1.8% alginate gel at 21 days of cultivation time. The culture media of the 3 and 6% gels showed a high viscosity over the extended of incubation time, these phenomena might led to the lower biomass of the immobilized beads. The immobilized beads at all calcium alginate gel concentrations were dissolved by sodium citrate solution; the liberated cells were used for measurements of viability. The cells entrapped in 1.8, 3 and 6% calcium alginate gel

Table 1: Podophyllotoxin in immobilized cell cultures of *J. chinensis* at cultivation time 28 days

Alginate gel (%)	Podophyllotoxin (mg g ⁻¹ dry weight)			
	Cells	Medium	Total	Product release (%)
0	0.048	0.0000	0.048	0.00
1.8	0.176	1.2500	1.426	87.60
3	0.130	4.0000	4.130	96.80
6	0.005	0.0525	0.053	99.10

Table 2: Effect of immobilization components on free cell suspension cultures of *J. chinensis* at cultivation time 14 (a) and 21 (b) days

Components	Podophyllotoxin (mg g ⁻¹ dry weight)			
	Cells		Medium	
	a	b	a	b
Normal cells	0.05	0.08	0.00	0.00
Addition of CaCl ₂	0.03	0.04	1.11	2.10
Addition of alginate gel	0.07	0.09	1.84	2.55

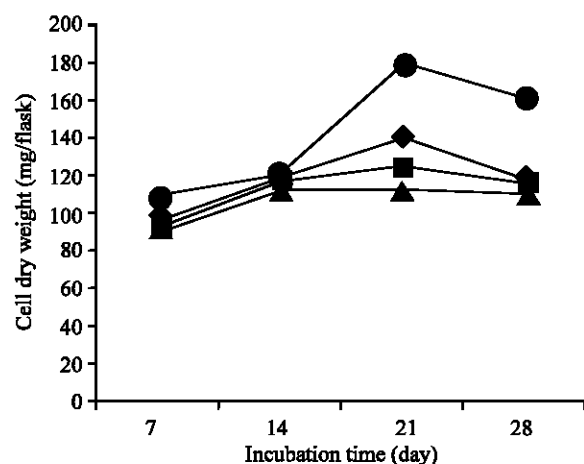


Fig. 1: Relative growth rate of *J. chinensis* cells entrapped in alginate gel at concentrations of 1.8% (◆), 3% (■), 6% (▲) and free cell suspension cultures (●)

retained a viability of about 60, 58 and 10%, respectively through 28 days.

Accumulation of podophyllotoxin in *J. chinensis* cells of immobilized cell cultures and free cell suspension cultures: Cells of *J. chinensis* cell suspension cultures and cells in calcium alginate gel beads were harvested at 7, 14, 21 and 28 days for the determination of podophyllotoxin levels. The results are given in Fig. 2. The podophyllotoxin content of free cell suspension cultures increased slightly from the beginning with a peak at 28 days (0.05 mg g⁻¹ dry weight). Immobilized cell cultures in 1.8% calcium alginate gel showed fluctuations in the accumulation of podophyllotoxin, the level being 5-fold that in the free cell suspension cultures. The cells entrapped in 3% calcium alginate gel produced podophyllotoxin (0.21-0.25 mg g⁻¹ dry weight) at 4-5 folds

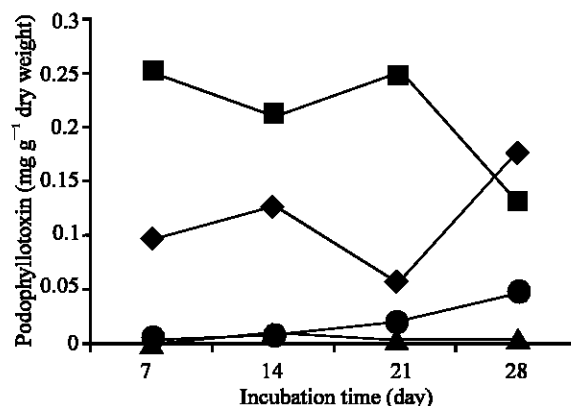


Fig. 2: Podophyllotoxin accumulated in *J. chinensis* cells entrapped in alginate gel at concentrations of 1.8% (◆), 3% (■), 6% (▲) and free cell suspension cultures (●)

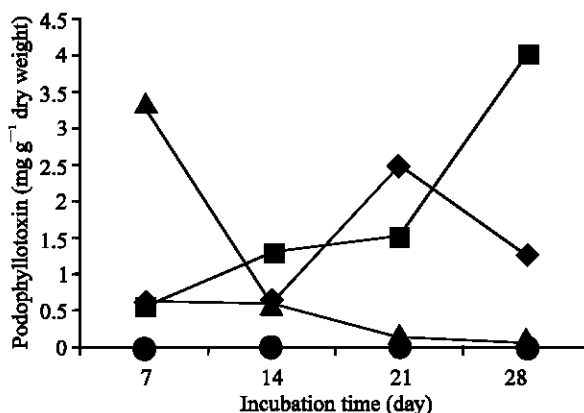


Fig. 3: Podophyllotoxin released into the culture medium by cells entrapped in alginate gel at concentrations of 1.8% (◆), 3% (■), 6% (▲) and free cell suspension cultures (●)

the control level at cultivation time from 7 to 21 days. The lowest podophyllotoxin content was found in 6% calcium alginate gel and small amount of podophyllotoxin was detected from the cells after 14 days of incubation.

Podophyllotoxin released into the culture medium: Patterns of release of podophyllotoxin into cell culture medium from calcium alginate immobilized cells and free cell suspension cultures are shown in Fig. 3. Almost no podophyllotoxin was detected in the medium of free cell suspension cultures. Excretion patterns of podophyllotoxin were observed as a function of time. The cells entrapped in 1.8% calcium alginate gel exhibited a maximum release of podophyllotoxin (2.45 mg g⁻¹ dry weight) at 21 days, while those entrapped in 3% alginate gel showed a peak (4 mg g⁻¹ dry weight) at 28 days. A

calcium alginate gel concentration of 6% caused a definite loss of podophyllotoxin productivity after 14 days. In the calcium-alginate immobilized cells, 87-99% of all the podophyllotoxin production can be released into the medium (Table 1).

Effect of immobilization components on free cell suspension cultures of *J. chinensis*: The role of the individual components of the immobilization matrix was examined as results shown in Table 2. Podophyllotoxin production was enhanced when cells exposure to 0.1 M CaCl_2 or uncross linked alginate and the product was excreted into the culture medium. The cells grown in medium with uncross linked alginate produced superior podophyllotoxin content, it was 1-1.4 times over than that of the control, while cells exposed to CaCl_2 exhibited lower yield to control. Addition of CaCl_2 or uncross linked alginate to the medium of free cell suspension cultures substantially enhanced the releasing podophyllotoxin into the medium. The product recovery from medium was 96-98% of that found in cells and treated cells produced total product 22-36 times larger than that of the control.

DISCUSSION

The results of this study clearly demonstrated that the production of podophyllotoxin is enhanced in cells and greater amounts released into the culture medium by immobilized cell cultures of *J. chinensis* than free cell suspension cultures. Entrapped cells in calcium alginate matrix attenuated cell growth in this study the result was likely to the cell cultures of *Solanum chrysotrichum* and *S. aviculare*^[19,20]. The podophyllotoxin production tended to depend on gel concentration. The concentrations of 1.8 and 3% calcium alginate used for the immobilization led to improve podophyllotoxin synthesis, alginate gel concentration 1.8 and 3% gave a maximum yield of 2.45 mg g⁻¹ dry weight and 4 mg g⁻¹ dry weight, respectively. The optimum alginate gel concentration for immobilization of *J. chinensis* cells was 3%. The entrapment by 3% gel was not favorable for increases in cell growth, but was suitable for the production of podophyllotoxin. The viability of immobilized beads entrapped in 3% alginate gel can be preserved for 28 days. Secretion of the desired product into the culture medium could be enhanced to 96-98% of total product. Podophyllotoxin production by immobilized cell cultures with the flask system offered an operation period of one month while an extended period with a lack of nutrients occurred.

Matrices, polysaccharides in alginate gel were supposed to be act as an elicitor in the *J. chinensis*

immobilization system and force cells to release the product into the culture medium as the individual components of matrix showed that they have the effect on promote yields in free cell suspension cultures. It has been reported that uncross linked alginate can act as an elicitor of secondary metabolite^[21] and also encouraged product release to culture medium of *Lithospermum erythrorhizon* cell cultures^[14]. Present result showed interesting impact of CaCl_2 on podophyllotoxin synthesis; it enhanced the podophyllotoxin production to nearly the same amount as alginate treated cells. Addition of CaCl_2 to *Plumbago rosea* cells promoted plumbagin production only 2 times to the control^[22]. The immobilization of *J. chinensis* cells in optimum gel concentration displayed a secretion of desired metabolite into surrounding medium superior to 97% of the total podophyllotoxin production. This property of entrapment offers the opportunity to increase the production of podophyllotoxin. The results obtained in this study, with immobilized *J. chinensis* cells in 3% calcium alginate gel will pave the way for the production of podophyllotoxin by immobilized cell cultures on a large-scale in a bioreactor.

ACKNOWLEDGMENTS

We thank Mr. Tadashi Watanabe for providing *J. chinensis* for this study.

REFERENCES

1. Ayres, D.C. and J.D. Loike, 1990. Lignans; Chemical and Biological and Clinical Properties, Cambridge University Press, Cambridge, pp: 113-132.
2. Botta, B., G.D. Monache, D. Misiti, A. Vitali and G. Zappia, 2001. Aryltetralin Lignans: chemistry, pharmacology and biotransformations. Current Med. Chem., 8: 1363-1381.
3. Miyata, M., K. Itoh and S. Tachibana, 1998. Extractive of *Juniperus chinensis*; Isolation of podophyllotoxin and yatein from the leaves of *J. chinensis*. J. Wood. Sci., 44: 397-400.
4. Muranaka, T., M. Miyata, K. Itoh and S. Tachibana, 1998. Production of podophyllotoxin in *Juniperus chinensis* callus cultures treated with oligosaccharides and a biogenetic precursor. Phytochem., 49: 491-496.
5. Premjet, D., K. Itoh and S. Tachibana, 2002a. Stimulation of the production of podophyllotoxin by biogenetic precursors and an elicitor in *Juniperus chinensis* stem-derived callus cultures. Pak. J. Biol. Sci., 5: 313-316.

6. Premjet, D., K. Itoh and S. Tachibana, 2002b. Enhancement of podophyllotoxin production by biogenetic precursors and elicitors in cell suspension cultures of *Juniperus chinensis*. Pak. J. Biol. Sci., 5: 1267-1271.
7. Brodelius, P. and K. Nilsson, 1980. Entrapment of plant cells in different matrices. FEBS Lett., 122: 312-316.
8. Brodelius, P., 1983. Immobilized plant cells. Mattiason, B., Ed. In: Immobilized cells and organelles, CRC Press, Boca Raton, pp: 27-55.
9. Lindsey, K., M.M. Yeoman, G.M. Black and F. Mavituna, 1983. A novel method for the immobilization and culture of plant cells. FEBS Lett., 155: 143-149.
10. Stephane, C., F. Gillet, M.L. Villarreal, J.N. Barbotin, M.A. Fliniaux and J.E. Nava Saucedo, 2000. Immobilization of *Solanum chrysotrichum* plant cells within Ca-Alginate gel beads to produce an antimycotic spirostanol saponin. Plant Physiol. Biochem., 38: 875-880.
11. Gillet, F., C. Roisin, M.A. Fliniaux, A. Jacquin Dubreuil, J.N. Barbotin and J.E. Nava Saucedo, 2000. Immobilization of *Nicotiana tabacum* plant cell suspensions within calcium alginate gel beads for the production of enhanced amounts of scopolin. Enzyme and Microbial. Technol., 26: 229-234.
12. Brodelius, P. and K. Nilsson, 1983. Permeabilization of immobilized plant cells, resulting in release of intracellularly stored products with preserved cell viability. Eur. J. Appl. Microb. Biotech., 17: 275-280.
13. Nilsson, K., S. Birnbaum, S. Flygare, L. Linse, U. Schroder, U. Jeppson, P. Larsson, K. Mosbach and P. Brodelius, 1983. A general method for the immobilization of cells with preserved viability. Eur. J. Appl. Microbiol. Biotech., 17: 319-326.
14. Dong, J.K. and H.N. Chang, 1990. Enhanced shikonin production from *Lithospermum erythrorhizon* by *in situ* extraction and calcium alginate immobilization. Biotechnol. Bioengineer., 36: 460-466.
15. Seki, M., C. Ohzora, M. Takeda and S. Furusaki, 1997. Taxol (Paclitaxel) production using free cell and immobilized cells of *Taxus cuspidata*. Biotechnol. Bioengineer., 53: 214-219.
16. Premjet, D., K. Itoh and S. Tachibana, 2002c. Immobilization of cultured cells from *Juniperus chinensis* for production of podophyllotoxin. In: Abstracts of the 52nd Annual Meeting of the Japan Wood Research Society, Gifu, pp: 391.
17. Schenk, R.U. and A.C. Hildebrandt, 1972. Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can. J. Bot., 50: 199-204.
18. Mohamed, T. Z. and H.S. Alan, 1990. Plant cell immobilization in alginate and polyurethane foam. Jeffrey, W.P., John, M.W., Eds. In: Methods in molecular biology. Humana press, New Jersey, pp: 525-536.
19. Nava Saucedo, J.E., C. Roisin and J.N. Barbotin, 1996. Complexity and heterogeneity of microenvironments in immobilized systems. R. Wijffels *et al.* Eds., In: Immobilized Cells. Basics and Applications. Elsevier Science, Amsterdam, pp: 39-46.
20. Roisin, C., F. Gillet-Manceau, J.E. Nava Saucedo, M.A. Fliniaux and A. Jacquin Dubreuil, 1997. Enhanced production of scopolin by *Solanum aviculare* cell immobilized within Ca-alginate gel beads. Plant Cell Rep., 16: 549-553.
21. Johnson, T.S., G.A. Ravishankar and L.V. Venkataraman, 1991. Elicitation of Capsaicin production in freely suspended cells and immobilized cell cultures of *Capsicum frutescens* Mill. Food Biotechnol., 5: 197-205.
22. Komaraiah, P., S.V. Ramakrishna, P. Reddanna and P.B. Kavi Kishor, 2003. Enhanced production of plumbagin in immobilized cells of *Plumbago rosea* by elicitation and *in situ* adsorption. J. Biotechnol., 101: 181-187.