Biotechnol. Bioprocess Eng. 2001, 6: 51-55

Production of 8-*epi*-Tomentosin by Plant Cell Culture of *Xanthium strumarium*

Jae Sung Park[1], Gi Hwan Yi[3], Min Hee Nam[3], and Sun Ho Park[1,2]*

[1] Faculty of Engineering, Keimyung University, Daegu 704-701, Korea
[2] Biotechnology Research Institute, Bicosys Co. Ltd., Daegu 704-701, Korea
[3] National Yeongnam Agricultural Experiment Station, RDA, Milyang 627-130, Korea

Abstract This study was conducted to establish a plant cell culture system for the production of medically important secondary metabolites from *Xanthium strumarium*. The effects of plant growth regulators including NAA, 2,4-D, kinetin, and ABA were examined in terms of callus induction, maintenance of callus and suspension cultures. It was shown that callus was induced upon treatment with NAA while embryo was induced after treatment with 2,4-D. Callus formation was further improved by treatment with ABA and NAA. The level of callusing increased by 17-29% for the seed case, cotyledon, leaf, and hypocotyl and by 96% in the case of the root. Suspension cell lines were established using calli produced from cotyledon, hypocotyl and root and cultured at 25°C under light conditions. The cells grew up to 15 g/L with NAA 2 ppm, BA 2 ppm, and ABA 1 ppm treatment. Supernatants of suspension cultures of cell lines derived from coyledon and hypocotyl produced some distinctive secondary metabolites, one of which was identified as 8-*epi*-tomentosin, which belongs to the xanthanolides. The amounts of 8-*epi*-tomentosin produced by the cotyledon- and hypocotyl-derived cell lines were 13.4 mg/L and 11.0 mg/L, respectively.

Keywords: induction of callus, plant growth regulators, suspension cell culture, 8-*epi*-tomentoin

INTRODUCTION

Plants have played an important role as a source of useful compounds to mankind. In particular, some herbs have recognized important roles in Chinese medicine and throughout Asia, moreover, compounds derived from these plants have been used in medical treatments for human diseases. However, plants known in Chinese medicine only represent a small part of the plant kingdom, so tremendous unknown opportunities remain to be discovered amongst the plant compounds. For this reason, plants are being recognized, as they were in the past, for the diversity of biomedicines they offer, in spite of the production of numerous new organic compounds. More than 20,000 biochemicals have been derived from plants, which are mainly secondary metabolites, and these have been used as medicines, spices, fragrances, dyes, pesticides, and chemicals with high purity. In addition, it has been reported that more than 1,600 of biochemicals are investigated each year [1].

Xanthium strumarium belongs to the genus Compositae and is found in Korea, Japan, Europe, and the United States [2]. Its dried seeds and leaves are used as a raw material in Chinese medicine for fever, diaphoresis, pain, paralysis, ulcerative skin disease, neuralgia, smallpox, and malign tumor [3,4]. It is also known to have antibacterial effects against typhoid bacillus, dysentery bacillus, and yellow staphylococcus [5,6]. The roots and stems are used in Chinese medicine as well as its seeds and leaves. In particular, the roots are used to induce robustness, for hypothyroidism and as an anti-tumor agent and the leaves are reported to reduce blood sugar levels [7-8].

Although *X. strumarium* has been widely used in many clinical applications, the secondary metabolites have not been extensively studied. Moreover, the traditional procedure used for extraction and purification of the most important compounds from intact plants was not successful in an effort to identify its active metabolites [9]. The plant cell culture system might offer a better alternative to field plants for the production and identification of the secondary metabolites of *X. strumarium* [10,11].

With the aim of elucidating clinically important compounds in *X. strumarium* and developing an economic production system, this study successfully established cell lines of *X. strumarium* by cell suspension culture. Having met this requirement, the influence of plant growth regulators on callus induction was investigated. Secondly, cell lines with high productivity were selected from several subcultures using the liquid cul-

*Corresponding author
Tel: +82-53-580-5457 Fax: +82-53-633-4929
e-mail: park@kmucc.kmu.ac.kr

ture method. Finally, major clinically important compounds excreted from the cultures were purified and identified.

MATERIALS AND METHODS

Germination of Seed

The seeds of *X. strumarium* collected in Sungju, Kyungsangpook province and were used for this study. They were trimmed by removing the seed coats and immersed in 95% ethanol for 2 min. The seeds were then surface-sterilized with 4%(v/v) sodium hypochlorite solution containing Tween 80 (2-3 drops/100 mL) for 1 h in a vacuum chamber. After sterilization, they were rinsed with distilled water 3-4 times and excess water was removed from the seeds using sterilized filter paper, and germinated in MS medium with 30 g/L of sucrose, and 5 g/L of agar (at pH 5.7) in the dark. Cotyledon, leaves, hypocotyl, roots from seedlings and the seeds themselves were used as the initial plant materials.

Callus Formation from Different Tissues

Cotyledons, leaves, hypocotyls, and roots from seedlings and seed were separately used for callus induction. Plant growth regulators, 2,4-D, NAA, IAA, and kinetin were employed to find the best condition for callusing independently and in combination; (1) 2,4-D 1, 2 ppm and NAA ranging from 0.5 to 1.5 ppm were examined independently. (2) 2,4-D ranging from 0.1 to 1 ppm and kinetin 2 ppm were tried as a mixture. (3) A mixture of NAA ranging from 0.5 to 2 ppm and IAA 0.5 ppm was also examined. MS medium with added 30 g/L of sucrose and 5 g/L of agar, pH 5.7 was used as a basal medium for callus induction. Plant materials were planted on the solid medium containing different plant growth regulators and incubated at 25°C in the dark. In addition, the influence of ABA on callusing was investigated. One ppm of ABA was added to the MS basal medium containing 2 ppm of NAA and the cultures were incubated using the culture conditions described above.

Selection of Cell Lines and Suspension Cultures

Well-developed calli obtained from different plant tissues were inoculated into 20 mL of MS liquid medium in 100-mL flasks. For liquid culture, 2.0 g/L of casein acid hydrolysates (CH), 1.15 g/L of L-proline, and 30 g/L of D-sorbitol were additionally added to the MS basal medium, though in this case 20 g/L of sucrose was used instead of 30 g/L. Callus clumps with sizes approximately one third that of the liquid medium volume were used, and all cultures were incubated in a gyratory shaker at 110 rpm and 25°C in a light room. Throughout this procedure, a cell line growing vigorously in liquid was selected. To determine optimal condition for the selection of suspension cultures, auxin type growth regulators, 2,4-D and NAA, and the cytokinin type, kinetin and BA were examined. In addition, ABA, a cell growth stimulator, was selected and its influence on the growth of the suspension cultures examined.

Production and Structure Determination of Secondary Metabolites

HPLC or TLC techniques were used to purify and identify secondary metabolites, excreted by the selected cell lines [9]. Samples were collected from the upper phase of suspension cultures after cultivating *X. strumarium* for 8 days, and extracted using chloroform. HPLC was performed using a Bondapak C18 column (Hitachi L-4250). The mobile phase consisted of H_2O : MeOH (6 : 4) and the eluent was monitored by measuring the absorbance at UV 254 nm. In case of TLC, plates of silica gel 60 (Merck) was used. To structurally identify the purified compounds we used an IR spectrometer (Shimadzu IR-470) and a NMR spectrometer (Unit plus 500). IR spectrometer samples were diluted 10 folds with KBr and the solvent used for the NMR was $CDCl_3$.

RESULTS AND DISCUSSION

Optimal Induction Condition for Callus Formation

Callus was induced from the different parts of *X. strumarium* in the MS basal medium containing growth regulators. This experiment was conducted in triplicate and the results are summarized in Table 1. The highest amounts of callus induced from cotyledons, leaves, and seeds were 3 g, 3.23 g, and 4.44 g, respectively, which were obtained by treating with 2 ppm of NAA. In the cases of the hypocotyl and root, treatment with 2 ppm of NAA and 0.5 ppm of IAA resulted in the highest callusing and were 5 g and 3.11 g, respectively. Consequently, the best callusing of *X. strumarium* was obtained using the hypocotyl tissue treated with a combination of 2 ppm of NAA and 0.5 ppm of IAA.

Callus induction appeared to be stimulated by increasing the NAA concentration up to 2 ppm, regardless of the type of plant tissues used. Interestingly, after NAA treatment, the production of hairy roots was observed in all plant tissues. This was particularly the case for roots treated with 0.5 ppm of NAA and 0.5 ppm of IAA. Callusing from seed occurred only after development to the hypocotyl and root stage; a thick and short hypocotyl was produced when the NAA concentration increased to 2 ppm.

The effects of 2,4-D and kinetin concentrations on callus induction are demonstrated in Table 2. This treatment resulted in a distinctive tendency to induce somatic embryo formation rather than callus induction. Somatic embryo was formed from callus tissue when the 2,4-D concentration was below 0.5 ppm. But, when the 2,4-D concentration exceeded 0.5 ppm, the embryo was formed directly from the plant tissues without call

Table 1. Effect of plant regulators on callus growth of *Xanthium strumarium*. The callus was cultured at 25°C for 45 days.

Plant regulator		Seed	Cotyledon	Leaf	Hypocotyl	Root
NAA (ppm)	IAA (ppm)	(g)	(g)	(g)	(g)	(g)
-	-	1.24	0.14	0.41	0.35	0.17
0.5	-	2.96	1.02	2.17	2.45	0.94
1	-	0.80	1.64	1.75	3.58	2.69
1.5	-	3.14	1.95	2.39	2.62	1.83
2	-	4.44	3.23	3.00	4.24	2.90
0.5	0.5	3.92	0.94	1.40	2.37	1.64
1	1	4.00	1.50	1.87	4.16	1.60
1.5	1.5	4.10	1.67	2.09	3.00	1.62
2	2	4.22	1.94	2.17	5.00	3.11

Table 2. Effect of plant regulators on callus and embryo growth of *Xanthium strumarium*. The callus was cultured at 25°C for 45 days

Plant regulator		Seed	Cotyledon	Leaf	Hypocotyl	Root
2,4-D (ppm)	Kinetin (ppm)	(g)	(g)	(g)	(g)	(g)
-	-	1.24	0.14	0.41	0.35	0.17
1	-	2.62	0.77	2.13	0.64	0.78
2	-	2.54	0.55	2.35	1.05	0.74
0.1	0.5	3.19	1.07	2.02	0.89	0.86
0.2	0.5	3.44	1.08	2.13	0.77	0.80
0.5	0.5	2.72	0.34	0.32	1.14	0.72
1	0.5	2.04	0.67	2.06	0.93	0.76

using. In direct somatic embryogenesis, the tissue first swelled and the embryo formed at the inner side of the swollen parts. In particular, root tissues appeared to be the most appropriate for the induction of somatic embryos, in terms of both quality and quantity. Fig. 1 shows calluses and embryos formed by treatment with NAA or 2,4-D. From this result, it is believed that NAA and 2,4-D, the auxin type growth regulators, can give rise to different plant responses, although all the other culture conditions remain unchanged. Thus, the optimal condition for callus induction of *X. strumarium* as determined in our study seems to be different from that of Ellis and Champer [10]. However, 2,4-D has a harmful effect on living organisms, and therefore, NAA might be a more desirable growth regulator in food and health related applications.

In addition, ABA greatly influenced the level of callus induction, though this was dependent upon the plant tissue type (Table 3). Cotyledon, leaf, and hypocotyl upon treatment with 1 ppm of ABA and 2 ppm of NAA showed 30% increases in callus induction, whereas roots receiving the same treatment showed a 96% increase in callusing. This result of increased callusing by ABA treatment is in good agreement with Ammirato's report [12], which claimed that ABA remarkably stimulated cell and callus growth.

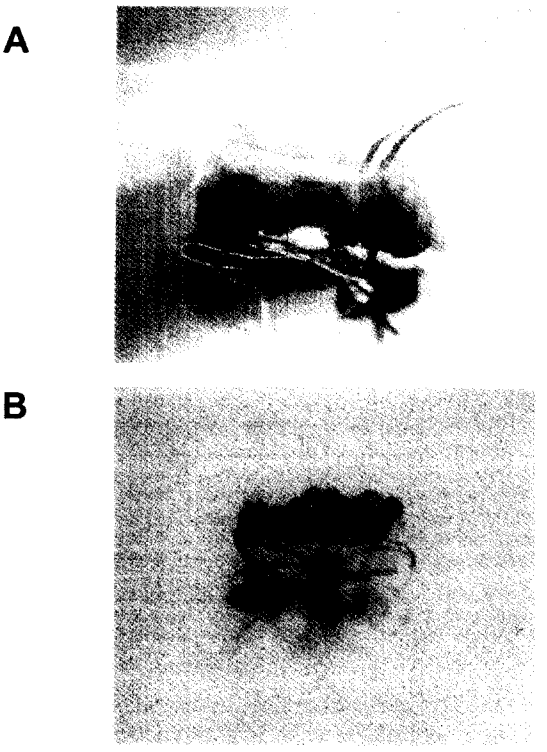

Fig. 1. Induction of callus and embryo from hypocotyl(A) and root(B) of *Xanthium strumarium*. The callus was cultured for 45 days at 25°C in the dark.

Table 3. Effect of plant regulator(ABA) on callus growth of *Xanthium strumarium*. The callus was cultured at 25°C for 21 days

Explant	NAA 2 ppm	NAA 2 ppm + ABA 1 ppm	Increased ratio after treatment with ABA(%)
Cotyledon	0.31 g	0.40 g	29
Leaf	0.79 g	1.01 g	28
Hypocotyl	1.25 g	1.46 g	17
Root	0.55 g	1.08 g	96

Selection of Cell Lines and Suspension Cultures

Suspension cell lines were selected by repeated subculture from cotyledon, hypocotyl, and the root of *X. strumarium*. Typical cell lines from cotyledon and root among those selected are shown representatively in Fig. 2. In case of *X. strumarium*, cells grew more actively under light than in the dark. No cell growth was detected after the single treatment of 2,4-D or NAA, or the combined treatment with 2 ppm of 2,4-D, 2 ppm of kinetin, and 1 ppm of ABA. However, some cell aggregation occurred. In case of treatment with the 2 ppm of NAA, 2 ppm of kinetin, and 1 ppm of ABA combination, partial cell growth and more cell aggregation were observed. Better results were obtained from the following three treatments; (1) 2 ppm NAA, 5 ppm kinetin, and 1

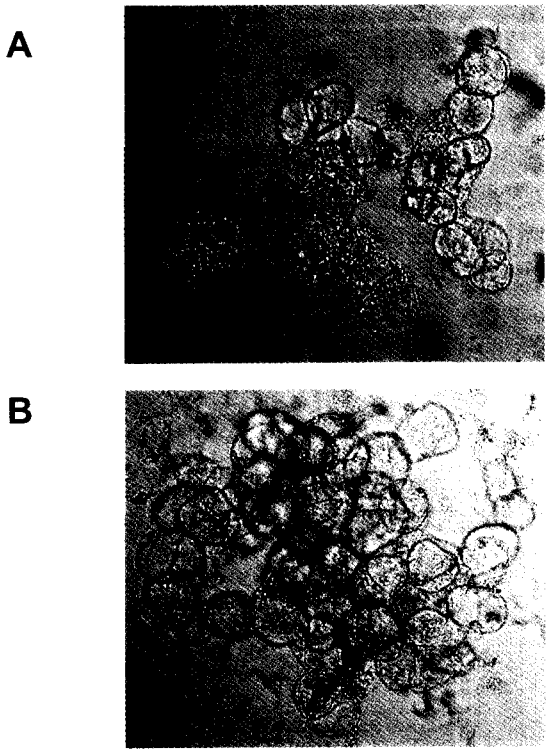

Fig. 2. Suspension cell lines were derived from induced callus of cotyledon(A), and root(B) of *Xanthium strumarium*. The cells were cultured at 25℃ and 110 rpm under illuminated conditions.

ppm ABA, (2) 2 ppm NAA, 1 ppm BA, and 1 ppm ABA, and (3) 2 ppm NAA, 2 ppm BA, and 1 ppm ABA. Further investigations revealed that treatment with 2 ppm NAA, 2 ppm BA, and 1 ppm ABA gave the best hormone usage for these cell lines, since this treatment caused less cell aggregation and better growth than others (data not shown). The dry cell weights of the different plant tissues were almost the same (15 g/L).

Production and Identification of Secondary Metabolites

Fig. 3 shows typical HPLC chromatograms purified from suspension culture supernatants of different *X. strumarium* tissues. As can be seen in Figs. 3(b) and 3(c), the HPLC retention time of 8-9 min of the main one peak from cotyledon and hypocotyl were distinguishable from those of the other cell lines and this fraction was structurally confirmed by IR and NMR. As shown in Figs. 4 and 5, the main peaks of the IR spectrum (Fig. 4) of the isolated product show that it possesses a sesquiterpene lactone ring and with an aid of a HMQC NMR spectrum (Fig. 5) it was identified as 8-*epi*-tomentosin. The molecule has one benzene ring supplemented with a lactone ring as shown in Fig. 6. By TLC, it was found that 8-*epi*-tomentosin has an R_f value of 0.58 (data not shown). The amounts of

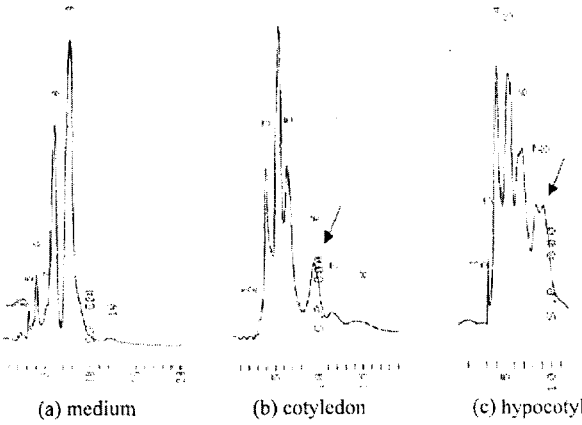

Fig. 3. Chromatogram of secondary metabolites excreted by cotyledon and hypocotyl of *Xanthium strumarium* by HPLC (Hitachi L-4250, C18 Bondapak column, H_2O:MeOH = 6:4, UV 254 nm).

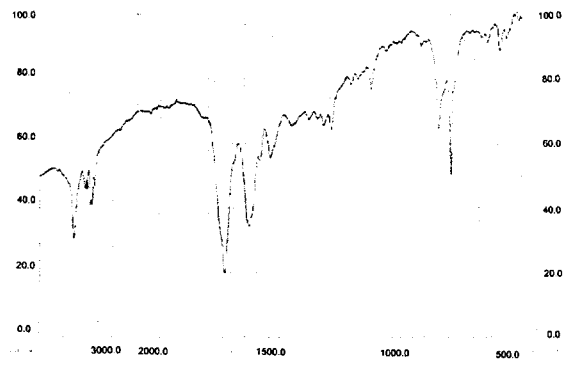

Fig. 4. IR spectrum for secendary metabolite of *Xanthium strumarium*.

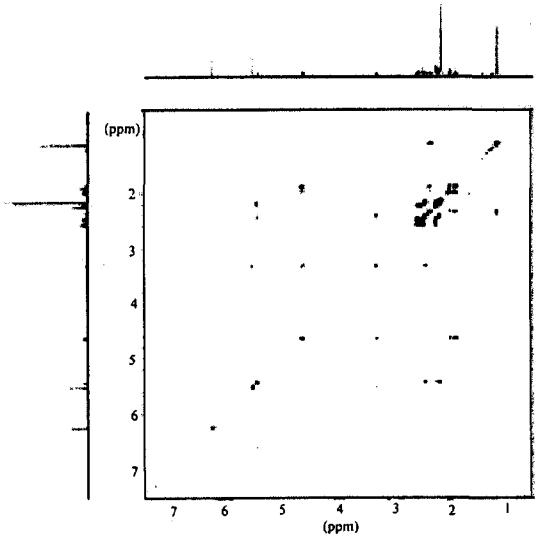

Fig. 5. HMQC NMR spectrum for secondary metabolite of *Xanthium strumarium*.

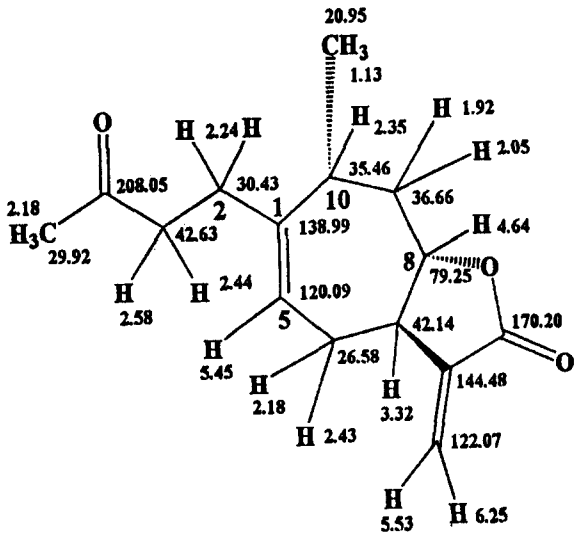

Fig. 6. Structure of 8-*epi*-tomentosin.

8-*epi*-tomen-tosin produced by cotyledon- and hypocotyl-derived cell lines were estimated as 13.4 mg/L and 11 mg/L, respectively.

Meanwhile, seed, leaf and root-derived cell lines produced negligible amounts of 8-*epi*-tomentosin. Sesquiterpene lactones, such as xanthatin, 8-*epi*-xanthatin, and 8-*epi*-tomentosin have been reported to be cytotoxic [13] and have antimalarial activity [14]. However, further examination from a biomedical viewpoint is needed to better understand the physiologic effects, and in particular, its degree of cytotoxicity in various human cancer cell lines. The cytotoxicity and antimicrobial activity of this material are currently under investigation.

REFERENCES

[1] Wink, M. (1998) Plant breeding: Importance of plant secondary metabolites for protection against pathogens and herbivores. *Theor. Appl. Genet.* 75: 225-233.

[2] Agata, I., S. Goto, T. Hatano, S. Nishibe, and T. Okuda (1993) 1,3,5-tri-O-Caffeoylquinic acid from *Xanthium strumarium*. *Phytochem.* 33: 508-509.

[3] Chopra, R. N., I. C. Chopara, K. L. Handa, and L. D. Kapur (1958) *Indigeous Drugs of India*. 2nd ed. U. N. Dhar and sons, Calcutta, India.

[4] Lee T. B. (1979) *Illustrated Flora of Korea*. p. 733, Academy Press, Seoul, Korea.

[5] Talakai, T. S., S. K. Dwivedi, and S. R. Sharma (1995) *In vitro* and *in vivo* antitrypanosomal activity of *Xanthium strumarium* leaves. *J. Ethnopharm.* 49: 141-145.

[6] Lee, D. B. (1974) Illustrated encyclopedia of fauna of Korea. *Economic Plants* 15: 423.

[7] Mukherji, S., A. K. Banerjee, and B. M. Mitra (1970) Plant antitumour agents. *Indian J. Pharmacy* 32: 48-49.

[8] Dhar, M. L., M. M. Dhar, B. N. Dhawan, B. N. Mehrotra, and C. Ray (1968) Screening of indian plants for biological activity: Part I. *Indian J. Exp. Biol.* 6: 232-247.

[9] Kim, H. S. and J. O. Shin (1997) Isolation and antimicrobial activity of *Xanthium strumarium* L. *Kor. J. Appl. Microbiol. Biotechnol.* 25: 183-188.

[10] Ellis, J. P. and N. D. Camper (1994) *In vitro* culture of *Xanthium strumarium* (Cocklebur). *Plant Cell Tiss. Org.* 36: 369-372.

[11] Ellis, J. P. and N. D. Camper (1995) *In vitro* cultured cocklebur (*Xanthium strumarium* L.) response to dimercaptopropanesulfonic acid and monosodium methanearsonate. *J. Plant Growth Regulation* 14: 9-13.

[12] Ammirato, P. V. (1983) *Handbook of Plant Cell Culture, Technique for Propagation and Breeding* Vol. 1. D. A. Eavns, W. R. Sharp, P. V. Ammirato and Y. Yamada (eds.). MacMillan Pub. Co. NY, USA.

[13] Ahn, J. W., Z. S. No, S. Y. Ryu, O. P. Zee, and S. K. Kim (1995) Isolation of cytotoxic compounds from the leaves of *Xanthium strumarium* L. *Natural Product Sci.* 1: 1-4.

[14] Joshi, S. P., S. R. Rojatkar, and B. A. Nagasampag (1997) Antimalarial activity of *Xanthium strumarium*. *J. Med. Plant Sci.* 19: 366-368.

[Received January 30, 2001; accepted February 12, 2001]