Biotechnology and Bioprocess Engineering **2003**, 8: 32-36 ©KSBB

Light-susceptibility of Camptothecin Production from *In Vitro* Cultures of *Camptotheca acuminata* Decne

Young Goo Park[1], Mee Hee Kim[1], Jae Kyung Yang[2], Young Gwan Chung[2], and Myung Suk Choi[2,3]*

[1] Dept. of Forestry, Kyungpook National University, Daegu 702-701, Korea
[2] Division of Forest Science, Gyeongsang National University, Jinju 660-701, Korea
[3] Institute of Agriculture and Life Sciences, Gyeongsang National University, Jinju 660-701, Korea

Abstract Production of camptothecin (CPT) from callus cultures of *Camptotheca acuminata* Decne was affected by light and culture conditions. Among the culture media tested, modified B5 medium containing 3% (w/v) sucrose, 2 mg/L 2,4-D, 2 times of MS medium vitamins, 500 mg/L casein hydrolysate, 250 mg/L myo-inositol, 0.05% (w/v) activated charcoal, and 0.15% (w/v) gelite was used for callus induction. The highest cell growth and CPT production were obtained in dark and green light condition, respectively. Photoperiod has no effect on cell growth and CPT production. Both cell growth and CPT production were also influenced by combination ratio of red and blue light. Cell growth and CPT production were the highest in the ratio of red and blue light, 90:10.

Keywords: *Camptotheca acuminata*, camptothecin, callus cultures, light

INTRODUCTION

The strong cytotoxic quinoline alkaloid, CPT was first isolated by Wall *et al.* [1]. Investigation into the mode of anti-tumor action identified CPT as a strong inhibitor of both DNA and RNA synthesis and as an inducer of DNA strand breaks in mammarian cells [2].

Recent advance in plant cell culture system allowed us to establish callus and suspension cultures of useful plants. The differentiated cells in suspension cultures could produce useful secondary metabolites. Plant cell cultures have been used as a source for a number of anti-tumor compounds, *e.g.* vinblastine, vincristine, tripdiolides and podophyllotoxines [3]. However, concentrations of the secondary metabolites are 1% to 5% which are significantly lower than those of intact plants [4].

In order to overcome the low production yield, studies in plant cell culture have been focused on the selection of cell lines with suitable genetic biochemical and physiological characteristics, and the optimization of culture environments [5].

Among the culture condition, light illumination affects accumulation of useful secondary metabolites [6]. However, little is known about the effect of light illumination on production of secondary metabolites. Although there are some reports of light illumination effect on production of flavonoids such as anthocyanin [7] and terpenoid [8]. The illumination effect on production of other compounds including alkaloids has not been reported. In this study, effect of light sources and intensity on CPT production from the cell cultures of *C. acuminata* was studied.

*** Corresponding author**
Tel: +82-55-751-5493 Fax: +82-55-753-6015
e-mail: mschoi@nongae.gsnu.ac.kr

MATERIALS AND METHODS

Plant Materials

Camptotheca trees growing at Chun Li Po arboretum in Korea were collected, dried and used for analysis of comptothecin content. High CPT containing trees were selected through the analysis and used as source for callus cultures.

Induction and Proliferation of Callus Cultures

Stem explants of *C. acumunata* were collected during actively growing period of May to June. Surface sterilized stems were cut into small pieces with a scalpel and cambials tissues, and then cultured onto the medium horizontally. The medium for callus induction was modified B5 [9] medium containing 3% (w/v) sucrose, 2 mg/L 2,4-D, 2 times of MS medium vitamins, 500 mg/L casein hydrolysate, 250 mg/L myo-inositol, 0.05% (w/v) activated charcoal, and 0.15% (w/v) gelite. The medium pH was adjusted to 5.8 prior to autoclaving. All cultures were maintained in a culture room of complete dark condition at 25± 1°C. It was subcultured every four weeks on the modified B5 medium. Various culture media such as mB5, MS [10], GD [11], SH [12], White [13] and WPM [14] were tested to optimize nutritional requirement for callus proliferation and

maintenance. To find out effective cytokinin types and concentrations, callus tissues cultured on mB5 medium supplemented with 0.01 to 5.0 mg/L of concentration of 2,4-D, NAA (naphthalenecetic acid), BA (benzyiadenine), and 2iP (N6-iso-pentyl amino purine).

Illumination on Callus Cultures

For elucidation of light sources effect on CPT production and cell growth, callus was maintained in LED incubator (Jeiotech, Korea) which illumination condition could be controllable. One gram fresh callus was cultured under blue, red, green, and fluorescent light, respectively. Photoperiod varying from 4 h to 24 h were associated. Cultures for investigation of the light effect were carried out in culture room at 25 ± 1°C with 16/8 hr light/dark cycle condition. Light intensities from various colored lamps were 2,500 to 3,500 lux. Light intensity was measured by using Lux meter (SU10, Jeiotech) just under the light source. Dry weight in cultured cells was determined after 4 weeks of cultures. Cell growth was represented with growth index which was calculated by following equation.

Growth index = (final dry cell weight - initial dry cell weight)/ initial dry cell weight

Each numerical value represents the mean and standard error from 6 replications. Means followed by different letters are significantly different at α = 0.05 following Duncan's multiple range test.

Extraction and Analysis of CPT

Dried cells (50 mg each) were extracted with 10 mL of hexane, and then 10 mL chloroform. When mixtures were difficult to separate by partitioning, the chloroform layer was centrifuged and dried in vacuo at 40°C. The chloroform extracts were dissolved in 100 ppm concentration of acetonitrile. All extracts were filtered through a 0.2 μm FH-type Millipore filter, and the resulting solution analyzed.

For TLC analysis, the chloroform extracts were analyzed by using aluminium-backed silicagel plates of 250 um layer, UV 254 (Whatman Lts, Maidstone, Kent, UK) with co-chromatography. The plates were developed with chloroform:methanol (24:1 v/v) and detection under UV light.

The HPLC (Dionex BioLC) system was used with a Curosil B column (3.2 × 250 mm, 5 μm, Phenomenex) with a C18 guard column and UV detector at 245 nm. A mobile phase mixture of acetonitrile : water (25 : 75) at a flow rate of 0.6 mL/min was used. Using the conditions described above, linear standard curves were obtained 10 to 1,500 μg of CPT. The samples were analyzed three time independently to measure the deviation among analysis.

MS Analysis of CPT

After separation by HPLC, the proposed CPT peaks were collected, and then analyzed by EI mass spectrometry (JMS-AX505, WA, USA).

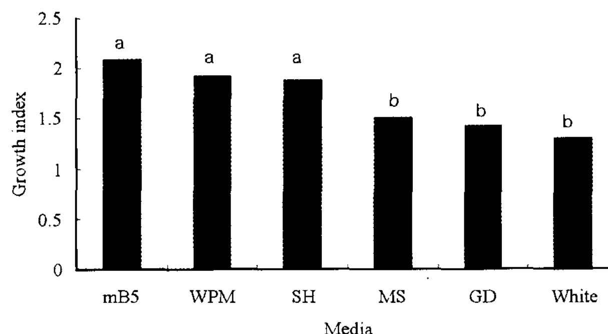

Fig. 1. Effects of various culture media on callus growth of *C. acuminata* Decne. Growth index was calculated by the following equation : (final dry cell weight – initial dry cell weight)/ initial dry cell weight. Each numerical value represents the mean and standard error from 6 replications after 4 weeks of cultures. Means followed by different letters are significantly different at α =0.05, following Duncan's multiple range test.

RESULTS AND DISCUSSION

Significant variation of CPT content was observed depending on individual trees. It suggests that the genetic diversity of the tree and various environmental factor affect CPT synthesis. CPT content of stem tissue was higher than those of bark, leaf and root (data not shown). Callus from cambial tissues of *C. acuminata* trees contains high amount of CPT. Callus originated from the side of the cambium and callus induction time took 7 and 21 days. Induced callus showed gray color from light to dark with no evidence of differentiation.

Various culture media such as mB5, MS, GD, SH, White and WPM were tested to optimize nutritional requirement for callus proliferation (Fig. 1). Among the media tested, mB5, WPM and SH media gave better result and their growth index represented 2.007, 1.923 and 1.885, respectively.

To find out effective growth regulators and its concentrations, various auxins such as 2,4-D, NAA (naphthalenecetic acid) were preliminarily examined. Among the auxins tested, 2,4-D gave vigorous callus growth. When the two type of auxins at different level were associated in culture media, IBA revealed as slightly more effective than 2,4-D in their growth index. The best result was obtained using 0.01 mg/L 2,4-D treatment and their growth index represented 1.692 (Table 1).

Hegel et al. [4] reported that 0.4 mg/L 2,4-D and 4 mg/LNAA containing 0.5 mg/L kinetin were better than single dose of auxin for *C. acuminata*. Sakato and Misawa [15] also reported that 0.1 mg/L NAA and 1.0 mg/L 2,4-D containing 0.1 mg/L kinetin were better than single dose of auxin. These results seem to be partly due to the physiological and/or genetical difference in the explant source or culture stages of the callus tested.

Table 1. Effect of exogenous auxins and cytokinins on callus proliferation and camptothecin (CPT) from cambium culture of *C. acuminata* Decne

	Auxins (mg/L)	Dry cell weight (mg)	Growth index	CPT (ug/g dry wt)
2.4-D	0.01	70[1]	1.692[a2]	>10[3]
	0.10	64	1.462[b]	>10
	1.00	59	1.269[b]	>10
	3.00	52	1.000[c]	>10
	5.00	51	0.962[c]	>10
NAA	0.01	61	1.346[c]	-
	0.10	69	1.654[a]	-
	1.00	66	1.538[ab]	>10
	3.00	63	1.423[bc]	>10
	5.00	61	1.346[c]	>10
BAP	0.01	571	1.192[ab2]	-
	0.10	58	1.231[a]	>10
	1.00	54	1.077[bc]	>10
	3.00	52	1.000[cd]	-
	5.00	49	0.885[d]	26.3
2ip	0.01	55	1.115[a]	>10
	0.10	56	1.154[a]	20.6
	1.00	55	1.115[a]	43.0
	3.00	58	1.231[a]	29.4
	5.00	49	0.885[b]	-

[1] Each numerical value represents the mean from 6 replications after 4 week of culture.
[2] Means followed by different letters are signficantly different at $\alpha=0.05$, following Duncan's multiple range test.
[3] T represents trace.

Among the cytokinins incorporated to this experiment, zeatin gave vigrous callus growth (Table 2). When various types of cytokinin at different level were associated in culture media, zeatin was more effective than any other cytokinins tested. The best result was obtained with 1.0 mg/L zeatin treatment and growth index represented 1.577. For this experiments, callus of 1 g fresh weight (initial inoculation dry cell weight 26 mg) was inoculated on each plastic Petri dish.

The comparison of CPT content and growth of callus were made under various auxins and/or cytokinins in static cultures using visually selected callus. The results are summarized in table 1 and table 2. Callus growth with either of 0.01 mg/L 2,4-D and 1.0 mg/L IBA with 0.1 mg/L BA was quit good, but CPT contents revealed just a trace.

Influence of cytokinins in growth was excellent in the level of 1.0 mg/L zeatin with 0.01 mg/L 2,4-D, but CPT contents was higher in 2ip than zeatin. It was observed that the combination of 2ip and 2,4-D is lower in growth index and higher in CPT content than that of zeatin with 2,4-D.

TLC analysis of extract showed for the presence of CPT as indicated by spots with the same Rf values as authentic samples. These showed blue fluorescence under UV light. The HPLC analysis equipped photo diode array detector further confirmed their presence as this spot had the same retention times (3.457 min) and same UV absorption maxima as authentic sample for CPT (254 nm). CPT isolated by HPLC was qualitatively confirmed by mass spectrometry. The mass spectrum of CPT from the extracts of callus was identical to that of an authentic sample with molecular (M+H, m/z 348) basal ion fragments (data not shown).

Table 2. Effect of various light source on callus proliferation and CPT production from callus culture of *C. acuminata* Decne

Light sources	Light intensity (Lux)	Dry cell weight (mg)	Growth Index[1]	CPT content (μg/g dry wt)
Blue	3,267	70 ± 0.7[2]	2.5[ab3]	-[4]
Red	2,522	65 ± 0.8	2.3[b]	>10
Fluorescent	3,161	73 ± 0.4	2.7[a]	>10
Green	2,511	63 ± 0.8	2.2[b]	45.6 ± 3.5
Dark	0	75 ± 3.8	2.8[a]	-

[1] Growth index was calculated by the following equation : (final dry cell weight - initial dry cell weight)/initial dry cell weight.
[2] Each numerical value represents the mean and standard error from 6 replications after 4 weeks of cultures.
[3] Means followed by different letters are significantly different at $\alpha=0.05$, following Duncan's multiple range test.
[4] Not determined.

CPT production was stimulated by light illumination, but cell growth was suppressed (Table 2). Among the light condition tested, dark condition gave the highest value on cell dry weight and growth index, whereas the highest result on CPT yield was obtained in green light. CPT was not produced from callus in dark condition. Impulse of particular wavelength of light triggers morphological or biochemical differentiation process. Plant pigments such as phytochrome and cryptochrome were absorbed their particular corresponding wavelength of light. There is also possibility of light activation of some genes, which are not expressed in dark condition [16]. A key step in the terpenoid indole alkaloid (TIA) pathway is catalysed by tryptophan decarboxylase (TDC) and gene coding TDC is expressed during plant development. Moreover, expression of TDC gene and gene coding TDC is induced by external stress signal of UV light [17]. Light illumination might be able to act as a stress signal for enhanced production of CPT. Kurata et al [18] also reported that expression of genes related to purine alkaloid production was induced by light after a lag-time of 1 day. Light illumination also changed callus from yellowish to green-wish. Illumination can be the increased of chlorophyll content. Chlorophyll may be carry with precursors of secondary metabolites biosynthesis. When cells were cultured on dark conditions, the energy partitioned to the synthesis of CPT was lower in the dark than higher light illumination [19].

The photoperiod had no effect on cell growth and

Table 3. Effect of various photoperiod of green light on callus proliferation and CPT production of *C. acuminata* Decne

Photoperiod (h)	Dry cell weight (mg)	Growth index[1]	CPT content (μ g/g dry wt)
24	71 ± 1.1	2.6[ab3]	>10
16	67 ± 1.8	2.4[b]	>10
12	73 ± 0.9	2.7[a]	>10
8	74 ± 0.6	2.7[a]	>10
4	67 ± 0.3	2.4[b]	>10
0	75 ± 3.8	2.8[a]	-[4]

[1] Growth index was calculated by the following equation : (final dry cell weight - initial dry cell weight)/initial dry cell weight.
[2] Each numerical value represents the mean and standard error from 6 replications after 4 weeks of cultures.
[3] Means followed by different letters are significantly different at α =0.05, following Duncan's multiple range test.
[4] Not determined.

Table 4. Effect of combination of blue and red light on the callus proliferation and CPT production of *C. acuminata* Decne

Ratio (R : B)	Dry cell weight (mg)	Growth index[1]	CPT content (μ g/g dry wt)
100:0	65 ± 0.8	2.3[c3]	>10
95:5	75 ± 0.7	2.8[a]	-[4]
90:10	75 ± 1.5	2.8[a]	49.7± 3.5
80:20	66 ± 2.1	2.3[bc]	21.1± 6.6
70:30	65 ± 0.7	2.3[c]	44.0± 7.6
0:100	70 ± 0.7	2.5[b]	>10

[1] Growth index was calculated by the following equation : (final dry cell weight - initial dry cell weight)/initial dry cell weight.
[2] Each numerical value represents the mean and standard error from 6 replications after 4 weeks of cultures.
[3] Means followed by different letters are significantly different at α =0.05, following Duncans multiple range test.
[4] Not determined.

CPT yield (Table 3). This may be because photoperiod was favorable to CPT biosynthesis. Biosynthesis of CPT will take short time. This results are in agreement with the ones obtained by Banthorpe and Njar [20].

It was investigated to observe the effect of combination of blue and red light on CPT production and cell growth (Table 4). The combination ratio of blue and red light was changed from 100:0 to 0:100 supplied by red light. Growth index and CPT content in ratio of 90:10 were 2.75 and 49 μg per g dry weight, respectively, which were the highest among the combination ratios of blue and red light tested.

Previous studies on the effect of light on secondary metabolite production have been carried out with constant illumination and a single light source. However, the effect of a combination of light sources on metabolite production has not yet been reported. Accordingly, the current study investigated the effect of a combination of light sources on both the cell growth and CPT production of callus. The CPT production with a blue and red light ratio of 90:10 was 5 times higher than with a single blue or red light source. It is possible that the genes related to CPT production were highly induced with the optimal blue and red light ratio, thereby maximizing the enzyme activities for CPT production. The cell growth with the blue and red light ratio of 90:10 was similar to that under dark conditions, whereas the other light conditions inhibited cell growth. Consequently, the current results indicate that the production of secondary metabolites can be stimulated and enhanced by optimizing the illumination conditions, such as the light quality and intensity and combination of light sources.

REFERENCES

[1] Wall, M. E., M. C. Wani, C. E. Cook, K. H. Palmer, A. T. Mcphail, and G. A. Sim (1966) Plant antitumor agents: I. The isolation and structure of camptothecin, a novel alkaloids leukemia and tumor inhibitor from *Camptotheca acuminata*. *J. Am. Chem. Soc.* 88: 3888-3890.

[2] Hsiang, Y. H. and L. F. (1988) Identification of mammalian DNA topoisomerase I as an intracellular target of the anticancer drug camptothecin. *Cancer Res.* 48: 1722-1726.

[3] Misawa, M. and T. M. Nakanishio (1988) Antitumor compounds: Production by plant cell culture. pp. 199-207. In: Y. P. S. Bajaj (ed.). *Biotechnology in Agriculture and Forestry, Medicinal and Aromatic Plants* Vol. 4, Springer-Verlag, Berlin, Germany.

[4] Van Hegel, A. J., M. P. Harkes, H. J. Wichers, P. G. M. Hesselink, and R. M. Buitelaar (1992) Characterization of callus formation and camptothecin production by cell lines of *Camptotheca acuminata*. *Plant Cell Tiss. Org. Cult.* 28: 11-18.

[5] Zhong, J. J., J. T. Yu, and T. Yoshida (1995) Recent advances in plant cell cultures in bioreactors. *World J. Microbiol. Biotechnol.* 4: 461-467.

[6] Endress, R. (1994) *Plant Cell Biotechnology*. pp 121-142. Springer-Verlag, Berlin, Germany.

[7] Senger, H. (1987) Blue light responces: Phenomenona and occurrence in plant and microorganisms. pp. 135-138, CRC Press, Boca Raton, USA.

[8] Siefermann-Harms, D. (1994) Light and temperature control of season-dependent changes in the α - and β - carotene content of spruce needles. *J. Plant Physiol.* 143: 488-494.

[9] Gamberg, O. L., R. A. Miller, and K. Ojima (1968) Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell. Res.* 50: 148-151.

[10] Murashige, T. and F. Skoog (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant.* 15: 473-497.

[11] Gressoff, P. M. and C. H. Doy (1972) Development and

differentiation of haploid *Lycopersicon esculetum* (Tomato). *Planta* 107: 161-170.
[12] Schenk, R. U. and A. C. Hildebrandt (1972) Medium and techniques for induction and growth of monocotyledonous and dicotyle donous plant cell cultures. *Can. J. Bot.* 50: 199-204.
[13] White, P. (1934) Potentially unlimited growth of excised tomato root tips in a liquid medium. *Plant Physiol.* 9: 585-600.
[14] Lloyd, G. B. and B. H. McCown (1981) Commercially feasible micropropagation of mountain aurel (*Kammia latifolia*) by use of shoot tip culture. *Prop. Int. Plant Proc. Soc.* 30: 421-427.
[15] Sakato, K. and M. Misawa (1974) Effect of chemical and physical conditions on growth of *Camptotheca acuminata* cell cultures. *Agri. Biol. Chem.* 38: 491-497.
[16] Aerts, R. J. and V. de Luca (1992) Phytochrome is involved in the light regulation of vindoline biosynthesis in *Catharanthus roseus. Plant Physiol.* 100: 1029-1032.
[17] Ouwerkerk, P. B. F., T. O. Trimborn, F. Hilliou, and J. Memelink (1999) Nuclear factors GT-1 and 3AF1 interact with multiple sequences within the promoter of the Tdc gene from Madagascar periwinkle: GT-1 is involved in UV light-induced expression. *Mol. Gen. Genetics* 261: 610-622.
[18] Kurata, H., S. Matsumura, and S. Furusaki (1997) Light irradiation causes physiological and metabolic changes for purine alkaloid production by a *Coffea arabica* cell suspension culture. *Plant Sci. Limerick* 123: 1-2.
[19] Zavala, J. A. and D. A. Ravetta (2001) Allocation of photoassililates to biomass, resin and carbohydrates in *Grindelia chiloensis* as affected by light intensity. *Field Crops Res.* 69: 143-149.
[20] Banthorpe, D. V. and V. C. O. Njar (1984) Light-dependent monoterpene synthesis in *Pinus radiata* cultures. *Phytochem.* 23: 295-299.

[Received October 25, 2002; accepted December 30, 2002]