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Characterization of Cell Growth and Camptothecin Production in Cell Cultures of *Camptotheca acuminata*

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Abstract Studies were made to elucidate the cell growth and the production of camptothecin and its derivatives in cell cultures of *Camptotheca acuminata*. High resolution HPLC chromatograms to analyze camptothecin and 10-hydroxycamptothecin in lactone and carboxylate forms were obtained with a fluorescence detector. Calli inductions were optimized with the young stem of explant on Schenk and Hildebrandt (SH) medium supplemented with 5 mg/l α-naphthaleneacetic acid (NAA), 0.2 mg/l 6-benzylamino purine (BAP), 2.0% sucrose, and 0.5% agar. The hybrid medium, a mixture of SH and Murashige and Skoog (MS) salts, was developed for homogeneous suspension cultures without large cell aggregates. The optimum phytohormone concentrations for successful suspension cultures were 1.0 mg/l of 2,4-D and 0.5 mg/l of kinetin. The highest growth in suspension cultures was observed when 49.7% (w/w) of the cells was composed of small aggregates which were below 0.1 mm in diameter. Time course changes of cell growth and camptothecin production showed that camptothecin accumulation was started at the end of the growth phase and the maximum content was obtained 10 days after inoculation. Yeast extract elicitor increased camptothecin accumulation 4 times. Methyl jasmonate and jasmonic acid also increased camptothecin production 6 and 11 times, respectively.

Key words: Camptothecin, *Camptotheca acuminata*, elicitor, jasmonic acid

Camptothecin is an antitumor alkaloid first isolated from *Camptotheca acuminata*, a native tree of China. In China, camptothecin has been widely used in the treatment of cancer. In 1958, Monroe Wall and Jonathan Hartwell discovered the antitumor activity of extracts from this plant in the course of a screening project for a natural source of steroids to make cortisone. In 1966, Dr. Wall and Mansukh Wani successfully isolated the active ingredient, camptothecin, from bark extracts [1]. Clinical trials began in the early 1970s, but the drug proved too toxic for clinical use. Following these discouraging results, camptothecin research continued slowly and efforts focused on discovering how the drug killed cells. Camptothecin is a strong inhibitor of both DNA and RNA synthesis. It was found that camptothecin strongly inhibited topoisomerase I [2, 3, 4]. Topoisomerase I exists in both normal and tumor cells and appears to take advantage of the growth rate difference between them. There are two possible explanations for the mechanism of action. Topoisomerase I is most vulnerable in cells at a stage of the cell cycle known as the 'S' phase. In this phase, cells copy their DNA while preparing to divide. Therefore, the target enzyme is present more frequently and at a higher concentration. Since rapidly-proliferating tumors have a greater percentage of cells in the 'S' phase compared to normal, slow-growing cells, tumors are more susceptible to the action of topotecan. Another explanation might be that rapid metabolic processes of tumor cells enable a greater influx of the drug.

Efforts have been made to reduce the toxicity of camptothecin for clinical use. 10-Hydroxycamptothecin, having less toxicity, is so far one of the promising derivatives of camptothecin. Topotecan is an analogue of 10-hydroxycamptothecin [5]. SmithKline Beecham Pharmaceuticals discovered topotecan and markets it as Hycamtin (R). It has been approved by the FDA for refractory ovarian cancer. Daiichi Pharmaceutical Co. Ltd. discovered CPT-11 (irinotecan) [6], another analogue of 10-hydroxycamptothecin, which is approved in Japan for refractory ovarian cancer. In a series of clinical trials, topotecan showed activity against a variety of solid tumors including ovarian cancer, small-cell lung cancer, and others. In later Phase III trials, examining topotecan as a second-line therapy against cancers, response rates were 10 to 15% among patients who had not previously responded to standard treatment and 25 to 30% among patients who had responded to first-line therapy. In one

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study, comparing topotecan and taxol for the treatment of advanced, recurrent ovarian cancer, topotecan showed a 20% response rate, whereas taxol had a 12% response rate [7]. Topotecan is as significant as taxol in terms of a unique mechanism of action and novelty of approach [8].

Camptothecin and its derivatives can be isolated from *C. acuminata*. Production in nature, however, can be limited by several factors of instability in the regions where the plants are grown. Therefore, the biotechnological production by plant cell culture can be an attractive alternative. Sakato *et al.* [9, 10] induced callus from the stem of *C. acuminata* on MS medium containing 0.2 mg/l 2,4-D and 1 mg/l kinetin. Giberellin, L-tryptophan and conditioned medium stimulated the cell growth. After 15 days of suspension culture, the concentration of camptothecin in the cells was 0.0025% on dry weight basis, which was about 1/20 of the level in the intact plant. Hengel *et al.* [11] also established the suspension culture system of *C. acuminata*. The highest level of camptothecin accumulated in the cells harvested in MS medium containing 4 mg/l NAA. In previous studies, however, the cell growth rates were much lower than in normal plant suspension cultures and their suspension cells were very heterogeneous with cell aggregates. Stable suspension cultures with fine cells were required to increase the cell growth and camptothecin production. In this paper, culture methods and growth conditions for stable callus and suspension culture are described. Also, production of camptothecin and 10-hydroxycamptothecin in cultured cells of *C. acuminata* is identified and manipulated.

MATERIALS AND METHODS

Cell Cultures

Seeds of *C. acuminata* were obtained from the Cheolipo Botanical Garden in Taean, Korea and propagated. Various parts of *C. acuminata* were surface sterilized by immersing them for 5 min in a sodium hyperchlorite solution (20% v/v) containing 0.1% of Tween 20. The plant material in the solution was sonicated for 30 sec and sterilized 10 more min. Then, the plant material was rinsed five times with sterilized distilled water. For callus induction, various parts of the plant were cut and placed on agar plates with different media. Basic media were hybrids of MS (Murashige and Skoog) and B5 (Gamborg) salt medium (1:1) supplemented with 2,4-D (5 μM), kinetin (0.5 μM), and 25 g/l of sucrose as a carbon source. 0.5% (w/v) of agar was added to prepare the solid medium for callus maintenance. The pH was adjusted to 5.8 with 1 N KOH. For the maintenance of the suspension culture, 16 g of cells (fresh cell weight) was transferred into 200 ml medium in a 500-ml Erlenmeyer flask every 8 days. Callus subculturing was carried out every 40 days by transferring a spoonful of healthy callus on 40 ml of solid medium. 125-ml Erlenmeyer flasks containing 50 ml of growth medium were used for experimental batch cultures on a gyrotory shaker at 180 rpm. The temperature of the culture room was 26°C.

Chemicals

Camptothecin and 10-hydroxycamptothecin were supplied from Sigma Chemical Co. (St. Louis, U.S.A.). Solvents used for HPLC such as acetonitrile, chloroform, methanol, and water were bought from Fisher Scientific Co. (Rochester, U.S.A.). Tetrabutylammonium phosphate as an ion-pairing agent and all the medium salts were supplied from Sigma Chemical Co. All other chemicals used in this study were of reagent grade.

Cell Growth Measurements

Suspension cells were filtered through Whatman No. 1 filter on a Buchner funnel under slight vacuum. The cells were washed with distilled water and the water was removed by draining fully under vacuum until no water drops appeared. Fresh cell weight (FCW) was determined by measuring washed cells on a balance as quickly as possible. After measuring fresh cell weight, the cells on a preweighed aluminum tray were dried in an oven at 60°C to evaluate dry cell weight.

Analysis of Camptothecin and 10-Hydroxycamptothecin

Cells were harvested by vacuum filtration and the filtrate was collected for the analysis of extracellular alkaloids in the medium. For the measurement of intracellular camptothecin and 10-hydroxycamptothecin concentration, 5.0 g of cells (FCW) was extracted with 30 ml methanol and the suspension was sonicated at 125 W for 10 min. 40 ml water and 70 ml chloroform were added and then mixed vigorously. After centrifugation for 10 min at 3,000 rpm, the chloroform phase was recovered and evaporated to dryness. The remaining residue was dissolved in methanol and filtered through 0.45 μm membrane filters for HPLC analysis. For extracellular alkaloids analysis, 10 ml medium filtrate was added to 10 ml chloroform and mixed vigorously. After centrifugation for 10 min at 3000 rpm, the chloroform phase was recovered and evaporated to dryness. The remaining residue was dissolved in methanol and filtered for analysis. The HPLC analysis was made with an HPLC system (Spectra System, Thermo Separation Products, Co., U.S.A.) with a reversed phase column (Vydac C-18, 250×4.6 mm, 10 μm, 90 Å) and a fluorescence detector (FL2000, Thermo Separation Products, Co., U.S.A.). The flow rate was 1.2 ml/min. The gradient variable mobile phase composition was used for better resolution. For the first 15 min, the ratio

of buffer solution (0.075 M ammonium acetate with 1 mM tetrabutylammonium phosphate, pH 6.4) to acetonitrile was maintained constant as 78:22 (v/v). Then, the ratio was increased from 78:22 to 50:50 in 5 min in a linear gradient mode. For the last 10 min the ratio was maintained constant at 50:50. The excitation wavelength of the fluorescence detector was 350 nm and the emission wavelength was optimized.

Sugar Analysis

The HPLC system was also used for the simultaneous analysis of sucrose and its hydrolyzed products, glucose and fructose. An amino column (Supelcosil LC-NH_2, 250×4.6 mm, Supelco Inc.) was used with a refractive index (RI) detector (Perkin-Elmer Corp., Wilton, U.S.A.). The mobile phase was 75% acetonitrile and 25% water and the flow rate was 2 ml/min with constant flow mode at the ambient temperature.

RESULTS AND DISCUSSION

Analysis of Camptothecin and 10-Hydroxycamptothecin

For the detection of camptothecin and 10-hydroxycamptothecin which were separated in the HPLC system with a C-18 column, the fluorescence detector was more sensitive and specific than the UV detector. Figure 1 shows that the fluorescence detector was not only more sensitive than the UV detector but specific to detect camptothecin and its derivatives. The wavelength of excitation was 364 nm [12]. The emission wavelength was optimized as 450 nm for camptothecin and 550 nm for 10-hydroxycamptothecin.

Most of the natural camptothecin is in the lactone form. It can also exist in the carboxylate form, an opening lactone ring structure [13]. The carboxylate form shows less antitumor activity than the lactone form but it has 200 times higher affinity to human serum albumin. The carboxylate form can be converted to the lactone form. The standard lactone form of camptothecin was made in a mixture of methanol and 0.01 M citric acid (pH 3.0) (50:50, v/v). The standard carboxylate form was prepared with a solution of methanol and 0.01 M sodium tetraborate (pH 9.0) (50:50, v/v). Neither lactone nor carboxylate forms of camptothecin were well separated by C-18 column because of similar polarities. Tetrabutylammonium phosphate was added into the mobile phase buffer as an ion pairing agent and its concentration was optimized as 1 mM. The mobile phase composition with ion paired 0.075 M ammonium acetate buffer (pH 6.4) and acetonitrile was optimized to yield high resolution chromatograms of camptothecin and 10-hydroxycamptothecin in lactone and carboxylate forms (Fig. 2).

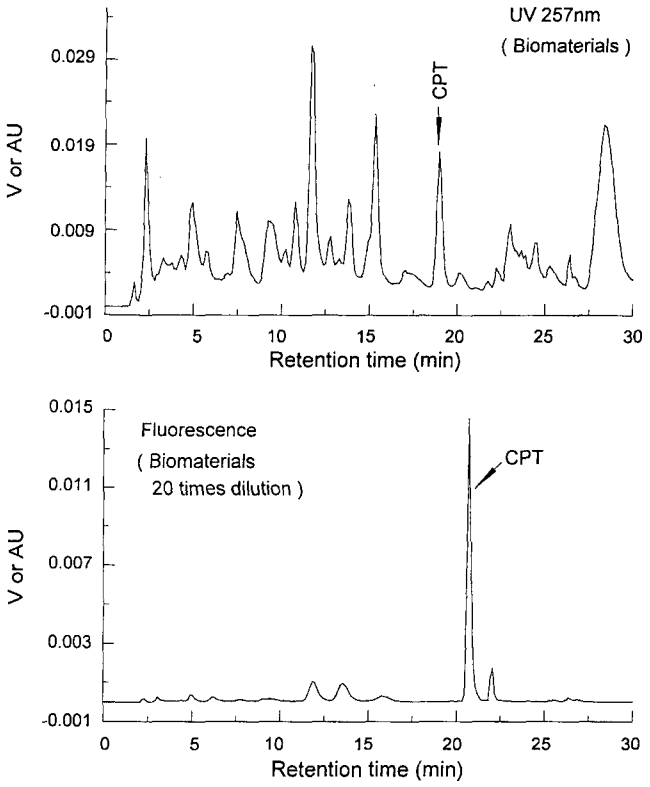

Fig. 1. Chromatogram of methanol extract of *C. acuminata* biomaterials measured with UV/Vis detector and fluorescence detector.

CPT: camptothecin.

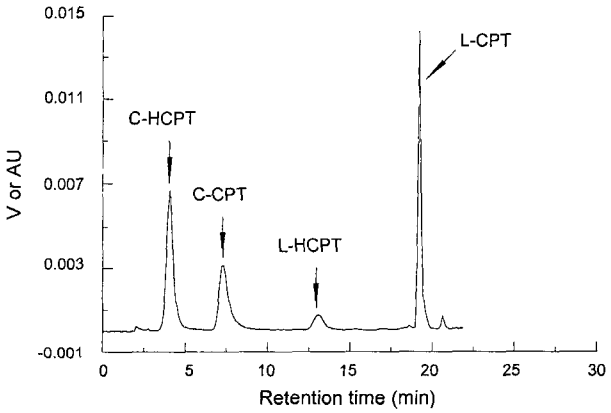

Fig. 2. Chromatogram of camptothecin (0.0625 ppm lactone form, carboxylate form) and 10-hydroxycamptothecin (2.5 ppm lactone form, carboxylate form). Fluorescence detection wave lengths were set at 364 nm (excitation) and 450 nm (emission).

CPT: camptothecin, HCPT: 10-hydroxycamptothecin, L: lactone form, C: carboxylate form.

Callus Induction and Cell Line

Various parts of *C. acuminata* were used to induce calli. The induction medium was Schenk and Hildebrandt (SH) medium supplemented with 5 mg/l α-naphthaleneacetic acid (NAA), 0.2 mg/l 6-benzylamino purine (BAP), and 2.0%

Table 1. Callus initiation from various plant parts.

Plant Part	Cell Line	Time (day)									
		7	8	9	10	11	12	13	14	15	16
Leaf	1				+	+	+	++	++	++	++
	2			+	+	+	++	++	+++	+++	+++
	3				+	+	+	+	++	+++	+++
	4				+	+	+	+	+	++	++
Stem	1		+	+	+	+	++	++	++	++	+++
	2		+	++	++	++	++	+++	+++	+++	+++
	3	+	+	+	++	++	++	+++	+++	+++	+++
	4		+	++	++	++	+++	+++	+++	+++	+++
Leaf with Stem	1		+	+	+	+	+	++	++	++	+++
	2			+	++	++	++	+++	+++	+++	+++
	3			+	+	+	++	++	++	++	++
	4	+	+	+	+	++	++	++	++	+++	+++
Shoot	1		+	+	+	+	+	++	++	++	++
	2	+	+	+	+	++	++	++	++	++	++
	3		+	+	+	++	++	++	+++	+++	+++
	4			+	+	++	++	++	+++	+++	+++

Degree of callusing: +, limited, usually at the cut surface; ++, localized; +++, abundant.

sucrose as a carbon source. Surface sterlized explants from four different parts of the plant were placed on the solid medium containing 0.5% agar. After 7 days from initiation, the calli formations were observed in petri dishes containing stem, leaf with stem, and shoots of the plant. Throughout the callus initiation period, the stem was the best part of the plant for callus induction and subsequent growth (Table 1). Morphological changes of callus cells were observed after several weeks from induction. When the callus was induced first, its color was ivory and its surface was soft. Afterwards, the color became white and the surface of the callus became hard.

Three different types of solid media were used to find out the most suitable medium for callus initiation. Surface sterilized young stems cut into 1-cm lengths were placed on three different salt media of SH, B5, and MS supplemented with 5 mg/l NAA, 0.2 mg/l BAP, 2.0% sucrose, and 0.5% agar. The culture condition for callus induction was 25°C and some of them were grown under a day/night regime (14/10 h). The first calli formations were observed after 7 days from initiation on SH medium (Table 2). On B5 medium, the first callus was seen after 8 days from initiation, whereas it took 11 days from initiation for MS medium. Among culture media tested, the SH medium was the most suitable, not only for callus initiation but for callus growth. It was true for different culture conditions tested with or without light illumination. Few calli were induced and grown under light conditions on B5 and MS medium. On SH medium, however, the calli induction and growth were not affected by light illumination. In spite of the high chance

Table 2. Callus initiation on different media and light condition.

	Cell Line	Light*	Time (day)									
			7	8	9	10	11	12	13	14	15	16
SH	1	D		+	+	+	++	++	+++	+++	+++	+++
	2	D		+	+	+	+	++	++	+++	+++	+++
	3	D					+	+	+	++	++	++
	4	D	+	+	+	+	++	++	++	+++	+++	+++
	5	D			+	+	+	+	+	++	++	++
	6	D		+	+	+	++	++	++	+++	+++	+++
	7	D	+	+	++	++	++	++	++	+++	+++	+++
	8	D							+	+	+	+
	9	D							+	+	++	++
	10	D				+	+	++	++	++	+++	+++
	11	L/D			+	+	+	++	++	++	++	+++
	12	L/D	+	+	+	+	+	+	++	++	++	++
	13	L/D		+	+	+	+	+	+	++	++	++
	14	L/D						+	+	+	++	++
	15	L/D										
B5	1	D										
	2	D		+	+	++	++	++	++	++	++	++
	3	D			+	+	+	+	+	+	+	++
	4	D					+	+	+	+	+	++
	5	D										
	6	D					+	+	+	+	+	+
	7	D										
	8	L/D										
	9	L/D										+
	10	L/D										
MS	1	D					+	+	+	+	+	+
	2	D										
	3	D					+	+	+	+	+	++
	4	D						+	+	+	+	+
	5	D										
	6	D										
	7	D										
	8	L/D								+	+	+
	9	L/D										
	10	L/D										

Degree of callusing: +, limited, usually at the cut surface; ++, localized; +++, abundant.
*D, dark condition; L/D, light/dark (14/10 h) condition.

of callus induction and suitability for callus growth, the SH medium was not perfect for calli maintenance. Calli grown on SH medium for several months became hard on their surface. A hybrid medium developed for suspension culture was applied to get rid of the surface hardness and was used for cell line maintenance.

Cell Suspension Cultures

Calli which were introduced in the liquid SH medium formed undifferentiated cell suspension cultures. Suspension cultures with various media were made to elucidate the optimum suspension culture media. Basic SH, B5, MS, White's medium supplemented with 0.5 mg/l kinetin, 0.5 mg/l 2,4-D, and 2.0% sucrose were tested. Five grams of

fresh cells were inoculated into 50 ml of liquid media in 125-ml Erlenmeyer flasks and cultured in a shaking incubator at 125 rpm at 25°C. Subcultures were repeated 4 times with each culture media. The cell growth was the highest in SH medium. The doubling time was 6.5 days in SH medium and 7.0 days in B5 medium. In SH and B5 medium, cell aggregates appeared and grew bigger throughout the repeated subcultures. The doubling time was 7.5 days for MS medium. Cell aggregates, however, did not appear in this high salt medium. The doubling time was the lowest in White's medium at 13 days. It was necessary to make a hybrid medium for homogeneous suspension cultures with high growth. The mixture of half SH and MS salts supplemented with phytohormones and sucrose was adequate for fast cell growth without large cell aggregates. This hybrid medium was applied throughout the studies.

Phytohormones are important for the cell growth of *C. acuminata*. Among various phytohormones tested, 2,4-D and kinetin were the most efficient for suspension cultures as auxin and cytokinin (data not shown). Figure 3 shows the optimum concentration of 2,4-D in suspension

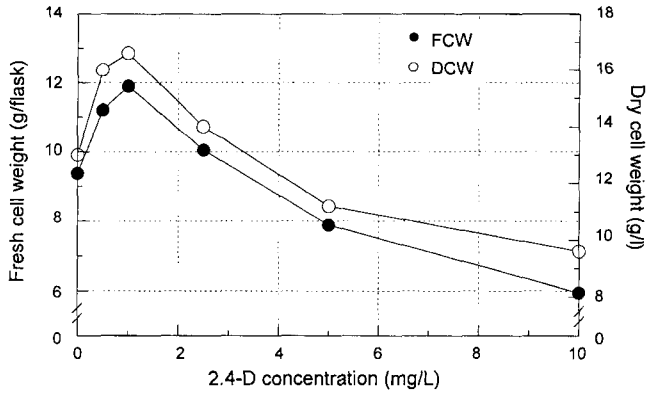

Fig. 3. Effect of 2,4-D on cell growth in suspension cultures of *C. acuminata*.

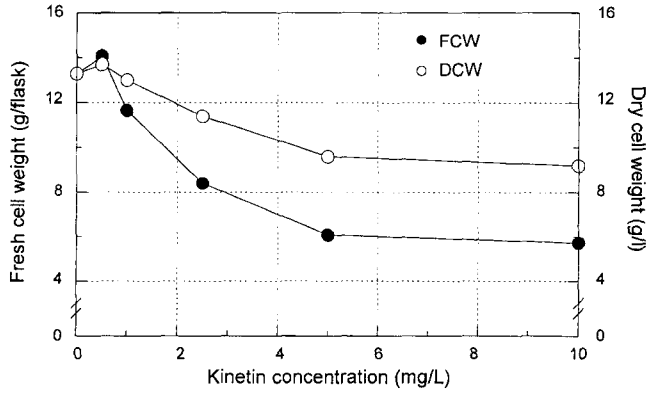

Fig. 4. Effect of kinetin on cell growth in suspension cultures of *C. acuminata*.

cultures. The highest cell growth was observed at 1.0 mg/l of 2,4-D. The optimum kinetin concentration was 0.5 mg/l as shown in Fig. 4.

C. acuminata cells were prone to aggregate in suspension cultures. Packed cell volume (PCV, 10 min) showed 68% of whole cells was aggregated cells which were 0.1 mm or higher in diameter. When a mixture of single cells and small aggregates (below 0.1 mm in diameter) was inoculated in the suspension medium, cells started to aggregate and 10% of whole cells were aggregated cells (0.1 mm or higher) after 7 days. Repeated subcultures increased the portion of aggregated cells and 68% of whole cells were aggregated cells after 5 consecutive subcultures (Fig. 5). The growth rate of suspension cells was affected by the size composition of aggregated cells. Effects of various cell aggregates were observed. Suspension cells were divided into three groups according to their size. The large group was composed of cell aggregates distributed between 1.5~2.5 mm in diameter. The middle group was for the cells between 0.1~1.5 mm. The small group was for the cells of below 0.1 mm. Three different groups of cells were inoculated into the same suspension media with the same amout of fresh cells (5 g). Cells were harvested after 7 days and re-inoculated into fresh media. The same procedure was repeated two times. At the first culture, only the cells in the small group grew slowly. This low growth, however, was recovered at the second culture (Fig. 6). At the third culture, the cell growth of the small group was higher than the large and middle groups of cells. The growth of suspension cells

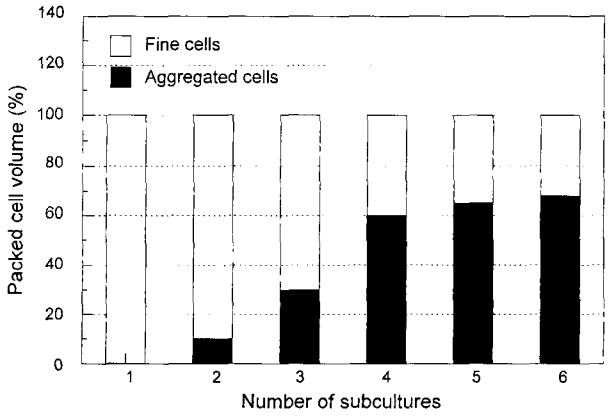

Fig. 5. Composition change of cell aggregates in suspension cultures of *C. acuminata*.

Table 3. The weight and size distribution of suspension cells of *C. acuminata*.

Aggregated all group	Weight %	Size distribution (mm)
Large	17.5	1.5~2.5
Middle	32.8	0.1~1.5
Small	49.7	below 0.1

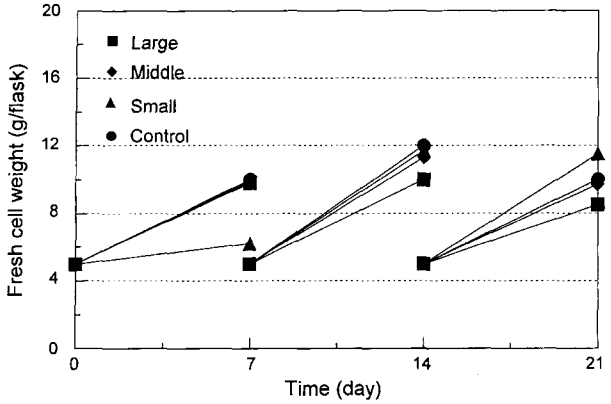

Fig. 6. Cell growth of various cell aggregates in suspension cultures of *C. acuminata.*

was affected by cell size distribution. The mixture of single cells and aggregates resulted in better cell growth than homogeneous cells of narrow size distribution. The size distribution of suspension cells used to be determined by various factors, culture medium, shear generated by shaking, and the degree of aggregation for cell to cell communication. The best cell size distribution for suspension cultures was not determined, but the size distribution of suspension cells grown with culture conditions applied in this study is shown in Table 3.

Camptothecin Production

The basic kinetics of cell growth and camptothecin production were observed. Five grams of fresh cells were inoculated into 50 ml of hybrid medium supplemented with 0.5 mg/l kinetin, 0.5 mg/l 2,4-D, and 2.5% sucrose and cultured in a shaking incubator of 125 rpm at 25°C. The cell growth and alkaloid production were monitored for 20 days. Figure 7 shows the cell growth and camptothecin production. The lag phase was 3 days. The exponential growth phase was between 4 days and 9 days from inoculation. The maximum amount of cells grown was observed on the 10th day with 12.8 g/l of dry cell weight (DCW). After the stationary phase, from day 9 to day 12, the DCW continued to decrease. The specific growth rate (μ) at the exponential growth phase was 0.24 day^{-1} and the corresponding doubling time was 2.9 days. The camptothecin production was not observed until 7 days from inoculation. 10-Hydroxycamptothecin production was not observed. The maximum camptothecin was obtained at the 10th day and the camptothecin content decreased. After the 13th day, camptothecin production was not observed anymore. The pattern of camptothecin production was the typical non-growth associated. Almost at the end of the growth phase it started to be synthesized. There was no evidence that camptothecin was the end product of the metabolic pathway in *C. acuminata* because it was not accumulated after biosynthesis. It could be degraded by cell lysis or it could be converted to other metabolites. No further information is known so far. Figure 8 shows the time course changes of sugar consumption in basic kinetics. The sucrose supplied was hydrolyzed into fructose and glucose in 4 days. Between fructose and glucose, any preference for consumption by suspension cells was not observed. At the end of the stationary phase of cell growth, the sugar was completely consumed.

Some secondary products function as so-called post-infection defensive substances within the plant's defensive system against attack by microorganisms. These phytoalexins are characteristics of certain cultures [14]. The signals triggering the formation of phytoalexins are elicitors. In the widest sense, these are molecules inducing a reaction in plant cells assumed to be characteristics of its defensive response. The uniformity of the cell's response to different elicitors and the parallel occurrence of an elicitor effect and marked changes in concentration of particular intracellular components may indicate an indirect elicitor effect [15]. Such effects of external signals via second messengers are known in culture systems. In

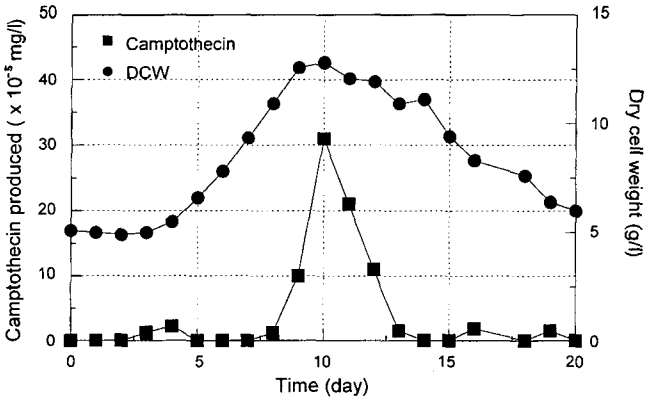

Fig. 7. Time course changes of cell growth and camptothecin production during batch suspension cultures of *C. acuminata.*

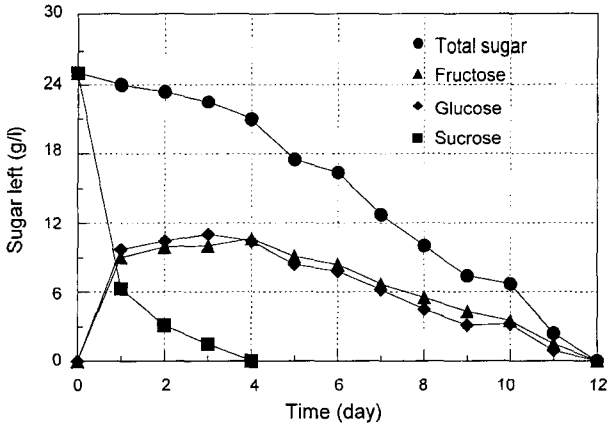

Fig. 8. Hydrolysis of sucrose and sugar consumption during batch suspension cultures of *C. acuminata.*

plant cells, polyamines, Ca^{2+}, cAMP, and jasmonic acid are being discussed as intracellular reaction messengers [16].

Several elicitors and intracellular reaction messengers were tested to camptothecin production in suspension cultures of *C. acuminata*. Yeast extract was used as a biotic elicitor and cupric sulfate and ferulic acid were applied as abiotic elicitors. Methyl jasmonate and jasmonic acid were tested as intracellular reaction messengers. Chlorocholine chloride was used as a metabolic pathway modifier. These were applied at a concentration of 100 μM in 50 ml of suspension cultures 4 days after inoculation. Samples were harvested 2 days after elicitation. Figure 9 shows the responses to 6 different elicitors. Yeast extract elicitor increased camptothecin production 4 times. Methyl jasmonate and jasmonic acid also increased camptothecin production 6 and 11 times. The concentrations of camptothecin elicited were less than the maximum concentration in basic kinetics (Fig. 7). It was due to the difference in cell mass in suspension cultures. The maximum concentration in basic kinetics was obtained from a cell mass of 12.8 g/l, whereas the cell mass of elicitation experiments was 7.1 g/l. Jasmonic acid and its methyl ester, methyl jasmonate, have been proposed as key signal compounds in the process of elicitation leading to the production of various secondary metabolites [17]. The jasmonates have been shown to induce anthocyanin accumulation in *Glycin max*, and to control a number of plant development processes [18, 19]. They have also been reported to play an important role in a signal transduction process that regulates defence genes in plants [20, 21]. In this experiment, yeast extract played a role in increasing camptothecin accumulation as an elicitor. We also believe that jasmonic acid could be an integral part of the signal transduction system regulating inducible defence genes followed by the increase of camptothecin accumulation. The optimum dose concentrations of yeast extract, methyl jasmonate, and jasmonic acid to induce maximum accumulation of camptothecin were not determined in this experiment. Other culture conditions with these elicitors could affect camptothecin production and further studies to elucidate them are required.

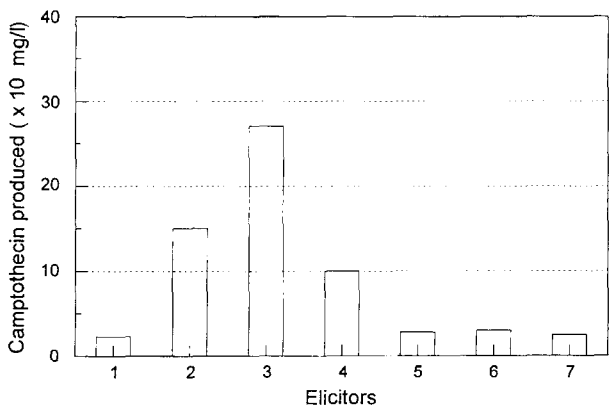

Fig. 9. Response to various elicitors in suspension cultures of *C. acuminata*.

1, Control; 2, Methyl jasmonate; 3, Jasmonic acid; 4, Yeast extract; 5, Ferulic acid; 6, Cupric sulfate; 7, Chlorocholine chloride.

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