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Characterization of callus formation and camptothecin production by cell lines of *Camptotheca acuminata*

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Abstract

Camptotheca acuminata plants contain camptothecin which is a secondary metabolite with strong anti-tumor activity. The induction of callus and cell suspension cultures from *Camptotheca acuminata* stem parts on different media is described. Growth in the original media and in media with different salts and/or vitamins was followed by measuring several growth parameters. Camptothecin was detected and identified by means of TLC, HPLC and GC-MS. The production of camptothecin in cell suspension cultures was followed and compared to the original plant material. Results indicate that high density cultures could be easily established, producing ca 1 mg l^{-1} of camptothecin.

Abbreviations: NAA – naphtalene acetic acid, 2,4-D – 2,4-dichlorophenoxyacetic acid, PCV – packed cell volume, CDW – cell dry weight

Introduction

Camptotheca acuminata Decne. (Nyssaceae) is a tree, originally found in the mainland of China. The stem wood and the bark are known to contain several alkaloids (Poehland et al. 1989). The strongly cytotoxic quinoline alkaloid camptothecin (Fig. 1) was first isolated by Wall and co-workers (Wall et al. 1966). In the Peoples Republic of China camptothecin is widely used in the treatment of cancer. Investigations into the mode of anti-tumor action identified camptothecin as a strong inhibitor of both DNA and RNA synthesis and as an inducer of DNA strand breaks in mammalian cells. Mammalian DNA topoisomerase I has been suggested to be the intracellular target of camptothecin (Hsiang & Liu 1988; Hsiang et al. 1989a,b; Jaxel et al. 1989).

Camptothecin yields after extraction of *C. acuminata* plants vary widely and depend on many factors difficult to control. In general, production in nature can be endangered by pests, climate or political instability in the regions where the plants are grown. Therefore the biotechnological production of camptothecin in bioreactors may be an attractive alternative. In 1974, Sakato et al. reported on the callus and cell suspension growth of *Camptotheca acuminata* and the isolation and identification methods for camptothecin. However, apart from one single value ($2.54 * 10^{-4}$% of CDW was camptothecine), nothing is mentioned about the production of camptothecine with plant cell cul-

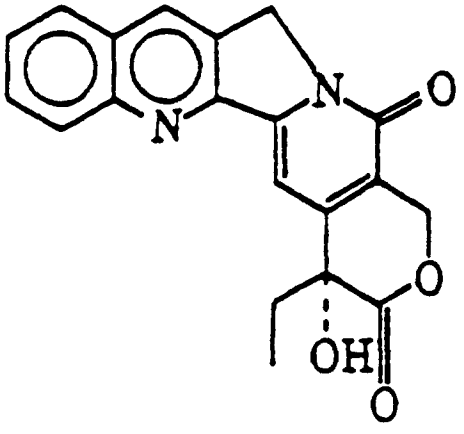

Fig. 1. Structure of 20-(S)-camptothecin.

tures. In this paper, the isolation, identification and production of camptothecin in undifferentiated callus- and suspension-grown cells of *C. acuminata* is described and compared to production in whole plants.

Materials and methods

Plant

Camptotheca acuminata Decne. (Nyssaceae) was obtained from the botanical garden of the Technical University of Delft in the Netherlands.

Culture conditions

Stem parts of *Camptotheca acuminata* were surface sterilized by immersing them for 4 min in a sodium hypochlorite solution (1.75 g l^{-1}). Then the plant material was rinsed five times with sterilized water, once quickly and four times for 10 min. For the induction of callus, stem parts were placed on agar plates with different media. Basic media were MS salt medium (Murashige & Skoog 1962) supplemented with B5 vitamins (Gamborg et al. 1968) or B5 salt medium with B5 vitamins. Supplements were 3 or 4% sucrose and occasionally 3 or 4 mg l^{-1} NAA or 0.4 mg l^{-1} 2,4-D or 2 mg l^{-1} coconutmilk and/or 0.5 mg l^{-1} kinetin. The media are specified in the Results and discussion section. The media were solidified with 0.8% agar. All media were adjusted to pH 6.5 and sterilized by autoclaving during 20 min at 120°C. The callus cultures were grown under a day/night regime (14/10 h) at 27°C, the type of lamp used was Philips TL 8W/33 L8. The light intensity was 1700 Lux. After a period of six weeks of callus growth, cell suspension cultures were initiated by transferring the callus to the same medium without agar. The cell suspension cultures were incubated on a rotary shaker (150 rpm) at 25°C under a day/night regime (14/10 h). Sub-cultures were made by adding 50 ml of an approximately three week old cell suspension culture to 200 ml of fresh medium in a 500 ml Erlenmeyer flask.

The cell lines, obtained in this way, were not only grown in their original media, but also in media with different plant growth regulators and salts. Growth and camptothecin production were followed.

Growth parameters

During growth cells and medium samples were taken. The packed cell volume (PCV) was measured by transfering ca 10 ml of cell suspension to a calibrated conical tube followed by centrifugation for 10 min at 3000 rpm. The precipitated cell volume was divided by the total volume to yield a percentage of packed cell volume. After centrifugation and washing the cells with demineralized water, they were freeze dried and the cell dry weight (CDW) was calculated. The pH and the conductivity of the media were measured. The sugar concentration was checked by means of HPLC and with use of glucose test strips (Medi-Test, Machery-Nagel).

Camptothecin extraction procedure

The freeze dried cells were grounded in a mortar. Fifty mg of the cell mass was transferred to a centrifuge tube and 2 ml methanol was added. After sonification (2 × 30 sec, 20 microns peak to peak), 18 ml water and 20 ml dichloromethane were added and this was mixed vigorously for 5 min on a magnetical stirrer. Centrifugation for 10 min at 2000 rpm yielded two phases. The dichloromethane phase, which was proved to contain the camptothecin, was recovered and evaporated to dryness. The remaining residue was

dissolved in 1 ml chloroform. Several analyses were done with these extracts.

Analyses

Identification of camptothecin absorption spectrum

The absorption spectrum of authentic camptothecin was measured between 200 and 450 nm using an UV-VIS spectrofotometer (Perkin-Elmer Lambda 5) in order to determine the appropriate wavelength for detection by HPLC.

Analysis of camptothecin by HPLC

The HPLC system consisted of a HPLC pump (Waters model 510), a reversed phase column (Chrompack Lichrosorb RP-18 100 mm) and an UV absorbance detector (ABI Analytical Kratos Division, model Spectroflow 757) for the detection of camptothecin at 256 nm. The flow rate was 1 ml min^{-1} and the mobile phase used was acetonitrile/water (25:75). Concentrations were calculated using camptothecin reference solutions (Sigma C 9911).

Analysis of camptothecin by TLC

Cell extracts and camptothecin reference solutions were chromatographed on silicagel 60-F-254 (Merck) plates. The running solvent used was a mixture of $CHCl_3$-EtOH-EtOAc-hexane (20.0:9.5:65.2:5.3 v/v/v/v) (Erdelmeier et al. 1986). The samples were chromatographed over a distance of 14 cm. The spots were detected under UV light of 254 and 366 nm.

Mass spectrometry

Solid probe (GC/MS) analysis of the purified HPLC samples were carried out with a mass spectrometer coupled to a gas chromatograph and a datasystem (Finnigan 4500 GC-MS-DS).

Results and discussion

Extraction, analysis and identification of camptothecin

A small amount of camptothecin, solubilized in chloroform, was used for the determination of the absorption spectrum. Between 200 and 400 nm three peaks of absorption were identified, at 256, 290 and 363 nm. The absorption maximum was found at 256 nm. At this wavelength a concentration of 13 μg ml^{-1} camptothecin showed an absorption of 1.116 (Fig. 2A). As the molecular weight is 348.34 g, the molar absorption coefficient of camptothecin in chloroform at 256 nm is 2.99×10^4 l mol^{-1} cm^{-1}. This wavelength was chosen for the detection and quantification of camptothecin in the HPLC analyses. At 290 and 363 nm, the molar absorption coefficients of camptothecin in chloroform are 6.00×10^3 and 2.02×10^4 l mol^{-1} cm^{-1}, respectively.

The extraction procedure developed (see Materials and methods) was based on a method of Wichers et al. (1990) and showed an efficiency of ca. 94% recovery from an extraction of 50 mg plant biomass, containing no camptothecin, supplemented with 100 μg camptothecin. Extraction of 100 μg of pure camptothecin in methanol yielded a recovery of ca. 99%. This method is substantially faster than the method of Poehland et al. (1989) and gives good results.

TLC analyses of extracts of plant material and camptothecin stock solutions both gave blue/violet spots (R_f-value 0.67), which were visible under UV light of 254 and 366 nm wavelength. Camptothecin solubilized in chloroform and stored at room temperature was stable for several weeks.

Reversed phase HPLC analyses of the extract of the *C. acuminata* cultures yielded chromatograms with camptothecin peaks with a retention time of ca. 4 min. Identification of the proposed

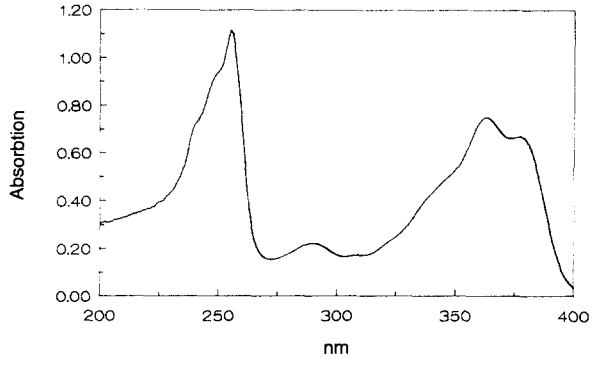

Fig. 2A. UV-VIS absorption spectrum of camptothecin in chloroform between 200 and 400 nm.

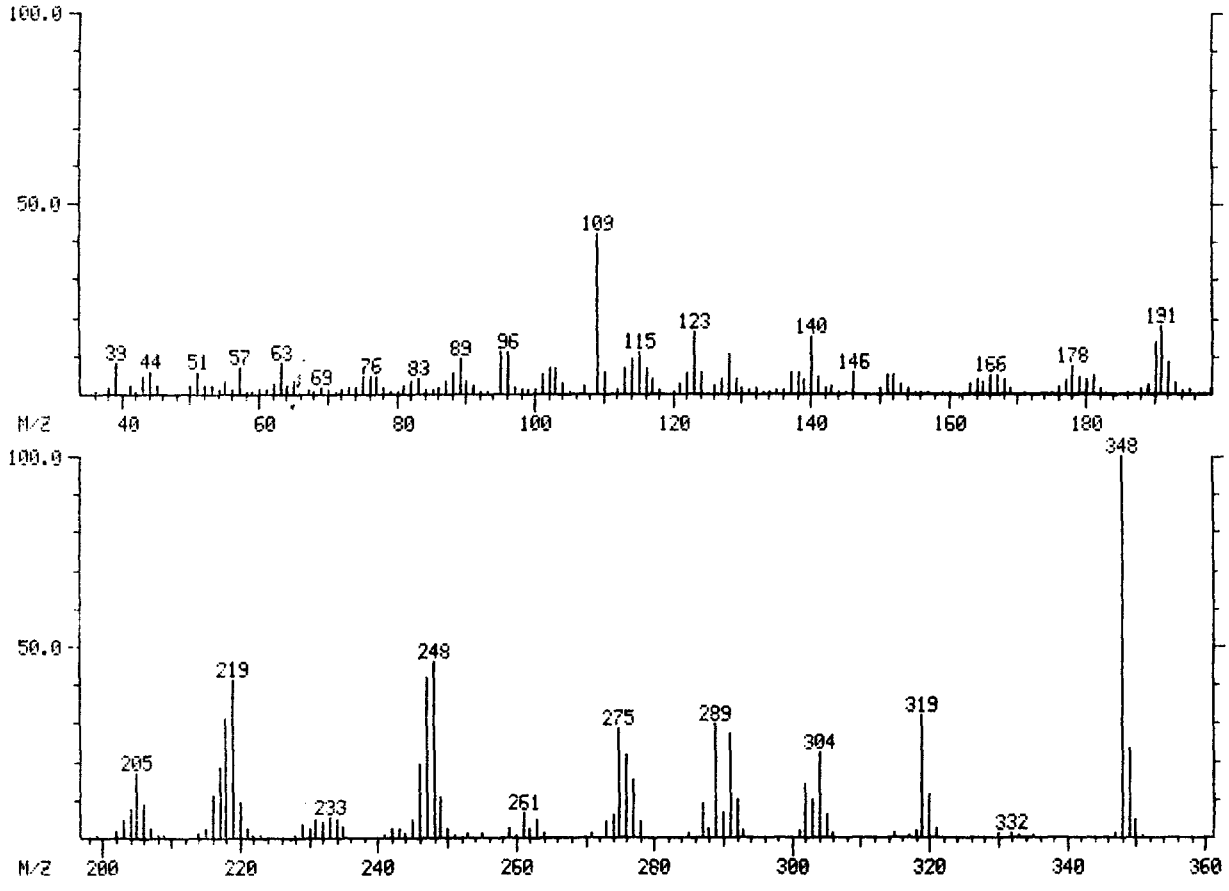

Fig. 2B. EI (= electron impact) mass spectrum of camptothecin at 70 eV.

camptothecin peaks was carried out by means of GC-MS.

After separation by HPLC, the proposed camptothecin peaks were collected, evaporated to dryness and brought into the mass spectrometer. The isolated compound was identified as camptothecin (MW = 348) based on the fragmentation pattern of the reference camptothecin solutions (m/z; relative intensities: 348 (100%), 319 (33%), 248 (47%), 219 (44%), 109 (43%)) (Fig. 2B).

Formation of callus

For the induction of callus, six different types of solid media were used (Table 1). On three of

Table 1. Callus initiation on different media. Successful initiation after 3 weeks is indicated by +, failure by −.

Medium	Salts	Vitamins	Plant growth regulator ($mg\,l^{-1}$)	Carbon source (%)	Callus	Cell line code
A	MS	B5	2,4-D 0.4	sucrose 3	+	CAS-3
B	MS	–	NAA 3	sucrose 3	–	CAS-4
C	MS	B5	–	–	–	–
D	B5	B5	coconut-milk 2	glucose 1 fructose 1	– –	–
E	B5	B5	NAA 4	sucrose 4	+	CAS-1
F	B5	B5	2,4-D 1 kinetin 0.5	sucrose 4	+	CAS-2

these, media B, C and D, no initiation of callus was detected. On the other three media callus was formed. Apparently B5 vitamins and auxins like NAA or 2,4-D are important for the induction of callus from *C. acuminata* stem parts.

Cell suspension cultures

All calli which were introduced in the liquid MS and B5 media formed homogeneous and undifferentiated cell suspension cultures. The diameter of the aggregates was usually smaller than 3 mm. The colour of the cell suspension cultures could change from grey/green into red towards the end of the growth phase. Figures 3A and 3B show the most relevant growth parameters of a typical cell suspension culture grown in different media. The pH of the media showed a minimum at pH 5–5.5 before the start of the exponential growth. Once the exponential growth started the pH rose until pH 6.5 at the start of the stationary phase. Growth stopped when all sucrose was used and resumed when new sucrose was added. The conductivity of the media which gave the best growth declined from 4.5 to 2.3 mS cm^{-1} in the MS media and from 3.6 to 0.6 mS cm^{-1} in the B5 media between day 2 and day 19. When sucrose concentrations were too low, growth stopped and the conductivity increased. Addition of extra sucrose could lead to further growth, and hence a resumed decline of the conductivity. The CDW had increased by a factor 10 to 11 after 14 days of growth in the best growth media. The minor decline in PCV and conductivity at day 34 in Figs 3A and 3B are caused by the dilution of the media with 0.1 volume of a 40% sucrose solution. A further decline of the conductivity and increase of the PCV and CDW of the cell suspension cultures were measured after the addition of this extra sucrose. The conductivity could reach values of 0.4 mS cm^{-1}, whereas the PCV went up to 63% and the maximum CDW was 41 g l^{-1}.

Cell suspension cultures originally initiated on

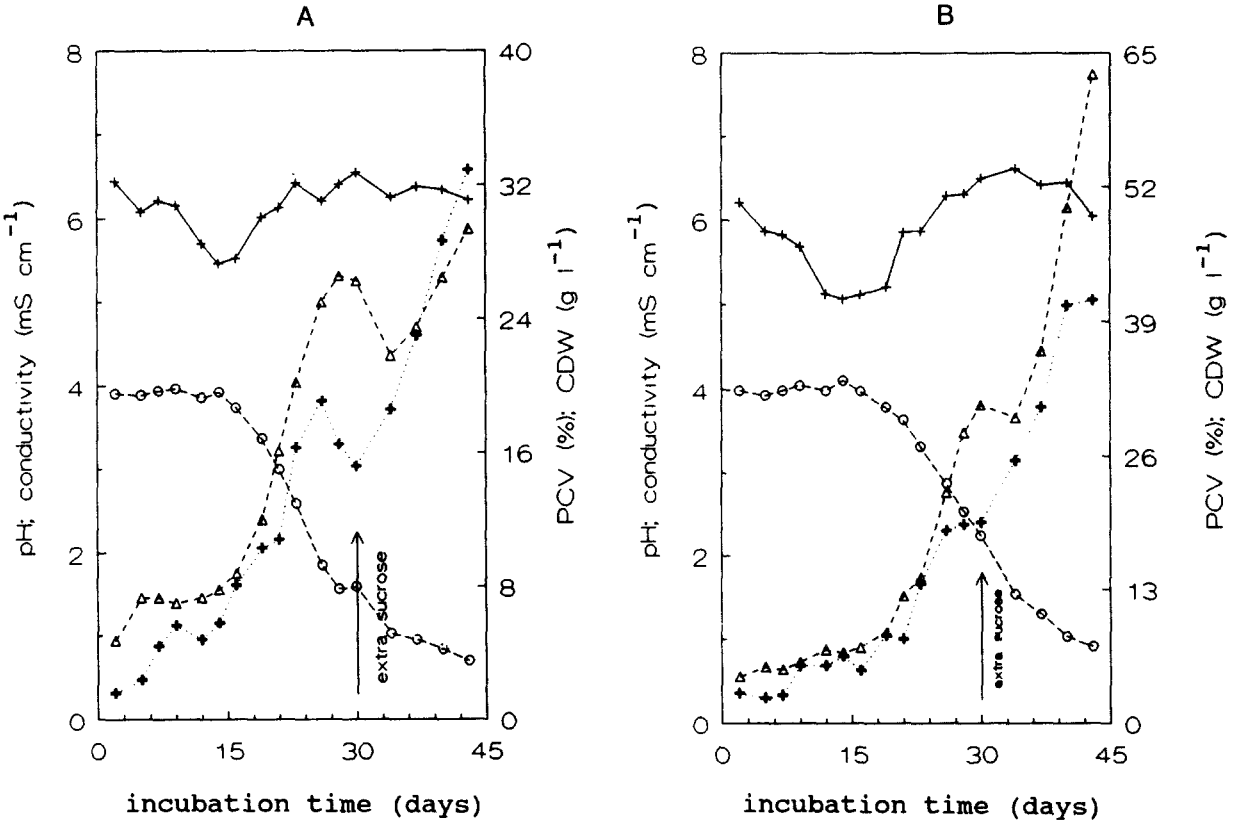

Fig. 3. Growth curves of *C. acuminata* cell suspension cultures. CAS-3 (*A*) in a medium containing B5 salts and vitamins, supplemented with 4 mg l^{-1} NAA; (*B*) B5 salts and vitamins, supplemented with 0.4 mg l^{-1} 2,4-D.
+ = pH △ = PCV (%) ⊖ = conductance (mS cm^{-1}) ···+··· = CDW (g l^{-1}).

solid medium A, showed faster growth in liquid A medium than in liquid E or F medium. A similar observation was made for cell suspension cultures initiated on E or F medium: cell cultures showed fastest growth when they were co-cultivated on the original initiation media.

Camptothecin production

The plant material was extracted and analyzed by TLC and HPLC for camptothecin content. The *C. acuminata* stem parts contained an amount of camptothecin, equivalent to 0.59% of the CDW of the plant material. The highest camptothecin productions by several cell lines in different media are shown in Table 2. Multiplication of CDW and the camptothecin content as a percentage of the CDW in Table 2 does not always give the maximum camptothecin content in $mg\,l^{-1}$, because the production maximum did not always occur at the same time as the maximum CDW. No camptothecin was detected in the media of cell suspension cultures. This indicates that camptothecin is accumulated intracellularly. The maximum camptothecin content in the cell suspension cultures was found to be 0.004% of the CDW. The production in cell suspension cultures is 100 times less than the production in the original plant material. As shown in Table 2, kinetin did not stimulate the production of camptothecin. However, quantitative and qualitative changes of the plant growth regulators might improve the production. Thus, addition of NAA gave the highest production of camptothecin and the MS salt media supplemented with B5 vitamins had an additional positive effect as well. The camptothecin production as percentage of the CDW in CAS-3 and CAS-4 are similar. This is not surprising because both suspension cell cultures are derived from callus that was obtained on the same solid medium. Figure 4 shows the camptothecin production by the CAS-4 cell suspension culture in the different media tested as mentioned in Table 2. The camptothecin content varied with time and was dependent on the growth medium used. The production of camptothecin is positively coupled to the growth of the cells. This is demonstrated by the strong correlation between camptothecin production and the increasing CDW and PCV. The *C. acuminata* cell suspension cultures are apparently able to break down the camptothecin

Table 2. Highest camptothecin production, final cell density and growth rate by several *C. acuminata* cell lines in different media.

Cell line	Medium	Final cell CDW ($g\,l^{-1}$)	Density PCV (%)	Growth rate (day^{-1})	Camptothecin 10^{-3}% of CDW	Content $mg\,l^{-1}$
CAS-1	B5; NAA	18.1	31.5	0.178	3.1	0.89
	MS; NAA	19.6	27.8	0.109	2.6	0.67
	B5; 2,4-D	19.8	36.9	0.206	0.85	0.090
	MS; 2,4-D	19.8	26.9	0.206	1.3	0.39
CAS-2	B5; NAA; K	19.1	33.9	0.217	0.92	0.21
	MS; NAA; K	20.8	24.5	0.115	2.6	0.69
	B5; 2,4-D; K	17.5	31.3	0.230	nd	nd
CAS-3	B5; NAA	19.1	26.6	0.103	2.1	0.49
	MS; NAA	17.3	21.4	0.087	3.6	0.98
	B5; 2,4-D	19.5	31.0	0.119	2.7	0.52
	MS; 2,4-D	20.9	26.9	0.106	1.8	0.53
CAS-4	B5; NAA	21.0	21.8	0.069	1.9	0.35
	MS; NAA	7.4	11.0	0.053	2.4	0.24
	B5; 2,4-D	19.7	25.2	0.071	1.2	0.32
	MS; 2,4-D	20.0	27.0	0.085	1.5	0.29
Plant stem					590	

MS = MS salts and B5 vitamins; B5 = B5 salts and vitamins; NAA = 4 mg l^{-1} NAA; 2,4-D = 0.4 mg l^{-1} 2,4-D; K = 0.5 mg l^{-1} kinetin; CDW = cell dry weight; PCV = packed cell volume; nd = not detected.

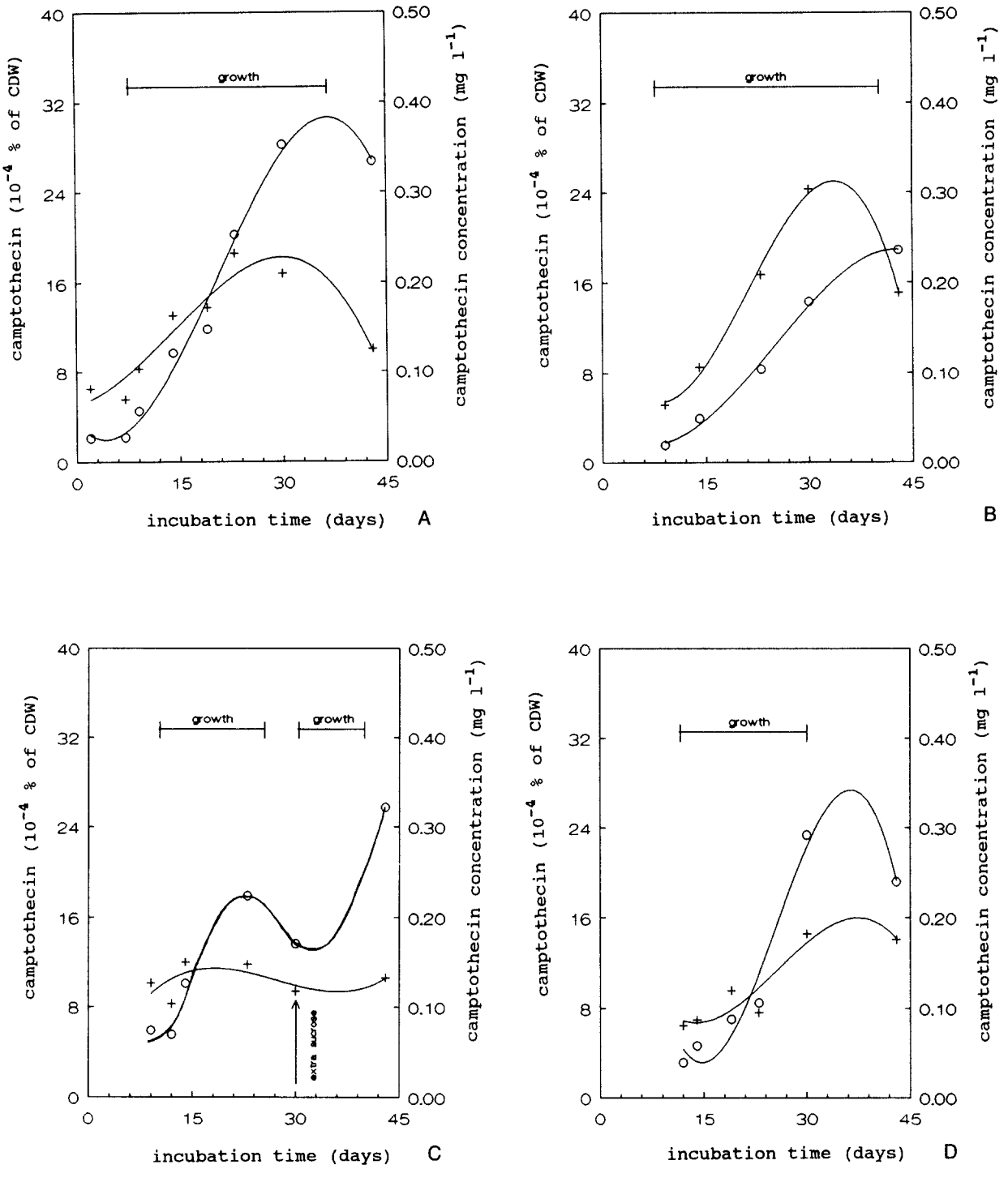

Fig. 4. Camptothecin production in Erlenmeyer shake flask cultures in the time as a percentage of CDW (+) and as a concentration in the culture liquid (○) by CAS-4 *C. acuminata* cell suspension cultures grown in different media: (*A*) in a medium containing B5 salts and vitamins, supplemented with 4 mg l^{-1} NAA. (*B*) MS salts and B5 vitamins, supplemented with 4 mg l^{-1} NAA. (*C*) B5 salts and vitamins, supplemented with 0.4 mg l^{-1} 2,4-D. (*D*) MS salts and B5 vitamins, supplemented with 0.4 mg l^{-1} 2,4-D.

produced when growth stops and CDW declines (Fig. 4). Figure 4C shows that an extra addition of sucrose at day 30 not only leads to further growth, but also to an increase of the camptothecin production. In Figure 4B the camptothecin production expressed in $mg\,l^{-1}$ culture liquid is relatively poor because of the slow growth rate of this cell suspension culture. So optimization of growth might contribute to a higher production of camptothecin per liter culture liquid.

Conclusions

It is possible to generate callus and cell suspension cultures from *C. acuminata* stem parts on different media. The cell suspension cultures acquired were able to grow in media with different salts and plant growth regulators than the original solid callus initiation media. In those different media differences in growth could be observed, the original media causing fastest growth. When cell growth stopped in liquid media, it could be restored by addition of extra sucrose.

Camptothecin could be detected in the original plant as well as in the cell suspension cultures. No camptothecin was detected in the media of cell suspension cultures. This indicates that camptothecin is accumulated intracellularly. The camptothecin production in the cell suspension cultures was dependent on the medium used. The original medium does not necessarily cause the best production, but the production was stimulated by NAA and additionally by MS salts supplemented with B5 vitamins. The camptothecin production period is positively correlated to the growth period. When the CDW and PCV increase, the intracellular camptothecin content as well as the camptothecin production per liter culture rise. *C. acuminata* cell suspension cultures break down camptothecin when growth stops because of sucrose limitation. The camptothecin production of the cells in Erlenmeyer shake cultures might be improved by culturing in bioreactors under controlled conditions, by optimalization of the media, by feeding metabolic intermediates or by genetic transformation of the cells to obtain for example hairy root cultures. Because of a renewed interest in camptothecin, the production of camptothecin in bioreactors might become an attractive way to contribute to the supply of this antitumor drug.

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