

Examination of camptothecin and 10-hydroxycamptothecin in *Camptotheca acuminata* plant and cell culture, and the affected yields under several cell culture treatments

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ABSTRACT: Camptothecin and its derivatives are monoterpenoid indole alkaloids exhibiting significant anti-tumor actions. With the aim of improving the production of these pharmaceuticals, the contents of camptothecin and 10-hydroxycamptothecin in different tissues including roots, stems, leaves, young flower buds, opening flowers, fading flowers and seeds from *Camptotheca acuminata*, were investigated. The young flower buds had the highest alkaloid concentrations (camptothecin, 2.46 mg/g of dry weight; 10-hydroxycamptothecin, 1.41 mg/g of dry weight). Callus showed lower concentrations but it should also be considered as a potential source of these pharmaceuticals. In the present study, the growth rate of *Camptotheca acuminata* cells in culture did not correlate with contents of camptothecin and 10-hydroxycamptothecin. Alkaloid accumulation by cells under various treatments (heavy metal ions, UV-B), methyl-jasmonate, abscisic acid, salicylic acid and hydrogen peroxide was examined, and the most notable effects appeared in the cells induced by UV-B light (which showed an 11-fold increase in camptothecin concentration) and by salicylic acid (which showed a 25-fold increase in 10-hydroxycamptothecin concentration). These results are significant in the context of the production of both pharmaceuticals.

Introduction

Camptothecin is a modified monoterpene indole alkaloid, first isolated from the extract of a Chinese medicinal tree *Camptotheca acuminata* Decne. (Nyssaceae) (Wall *et al.*, 1966). *C. acuminata* is a deciduous tree native to China, and has been extensively used in traditional Chinese medicine. Various organs of

the species contain camptothecin and its derivatives (Wiedenfeld *et al.*, 1997).

Camptothecin shows a strong antineoplastic activity, the sodium salt of its 10-hydroxy isomer shows less toxicity but similar activity (Wiedenfeld *et al.*, 1997). Nowadays, the camptothecin derivatives are used throughout the world for the treatment of various cancers, and over a dozen more camptothecin analogues are currently at various stages of clinical development (Lorence and Nessler, 2004; Wiedenfeld *et al.*, 1997).

Due to the pharmacological aspects of these alkaloids and the rapid growth of the market, many biotech-

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nological means have been used for raising the production of the alkaloids (Lorence and Nessler, 2004). In the early 1980's, it was shown that plant cells (callus) could grow indefinitely and culture conditions for its growth could be easily controlled. This technology has great potential for producing useful metabolites on an industrial scale. Therefore, the production of valuable secondary metabolites using plant cell culture (callus) technology is widely investigated (Bourgau *et al.*, 2001; van der Heijden *et al.*, 2004; Vanhengel *et al.*, 1992).

Furthermore, the terpenoid-indole alkaloids in plant cells can be induced by both abiotic and biotic elicitors. It has been shown that ORCA3 (octadecanoic-responsive *Catharanthus* AP2-domain protein 3) regulates jasmonate-induced terpenoid-indole alkaloids biosynthesis in *Catharanthus roseus* cells (van der Fits and Memelink, 2000; van der Heijden *et al.*, 2004). So far, the studies have showed that camptothecin can be induced by abiotic and biotic elicitors including drought (Zhao *et al.*, 2000), heat-shock (Zu *et al.*, 2003), high NaCl salinity (Abdala *et al.*, 2003), acetylsalicylic acid (Halitschke *et al.*, 2004), methyl-jasmonate and jasmonic acid (Song and Byun, 1998), benzyladenine and naphthalene acetic acid (Abdala *et al.*, 2003).

In the present study, the contents of camptothecin and 10-hydroxycamptothecin in various organs (including roots, stems, leaves, young flower buds, opening flowers, fading flowers and seeds) from *C. acuminata* in Fudan University campus (Shanghai, China) were investigated. The contents of the two compounds in *C. acuminata* callus during its growth period were also studied. With the effects of diverse elicitors used in the treatments of callus, the contents of camptothecin and 10-hydroxycamptothecin were further examined comparing with control to unveil the most effective elicitors.

Materials and Methods

Plant materials and culture conditions

Camptotheca acuminata tissues (roots, stems, leaves, young flower buds, opening flowers, fading flowers and seeds) were collected in the Fudan University campus (Shanghai, China).

Callus cells were obtained in cultures initiated from young leaves and maintained in Petri dishes containing Murashige and Skoog's (1962) solid medium supplemented with 5 mg/L naphthalene-acetic acid, 0.5 mg/L 6-benzyladenine, 0.3 mg/L 2,4-dichlorophenoxy-acetic acid and 30 g/L of sucrose. The medium pH was ad-

justed to 5.8 before autoclaving. The leaves were incubated at $25 \pm 2^\circ\text{C}$ during 4 weeks in the dark and the obtained callus cells were then cultured in fresh medium during 0, 5, 10, 15, 20, 25 and 30 days. Growth curves were determined from experiments initiated by inoculating 1.5 to 2.0 g (fresh weight) of *C. acuminata* callus cells onto fresh medium. Fresh weight of the grown cells was measured on those days, and the percent change from the weight recorded 5 days before each measurement was made.

Treatments

The culture medium was drained from triplicate samples of *C. acuminata* callus cells that had been cultured for 15 days, and then the cells were placed on fresh Murashige and Skoog's medium in petri dish and soaked during 3 days in either (1) distilled water, (2) 100 μM abscisic acid, (3) 100 μM methyl-jasmonate, (4) 100 μM salicylic acid, (5) 100 μM Cu^{2+} , as CuCl_2 , (6) 100 μM Pb^{2+} , as PbCl_2 , (7) 100 μM Mn^{2+} , as MnCl_2 , or (8) 10 mM hydrogen peroxide by adding the corresponding solutions to the petri dish. Also, after being drained, some cell triplicates were either placed on fresh culture medium and soaked in distilled water or they were merely placed on fresh culture medium before being exposed to 5 $\mu\text{mol}/\text{m}^2/\text{s}$ ultraviolet-B light for 3 days. All these treatments were carried out at $25 \pm 2^\circ\text{C}$ in the dark. After the treatments, all the samples were subjected to analysis.

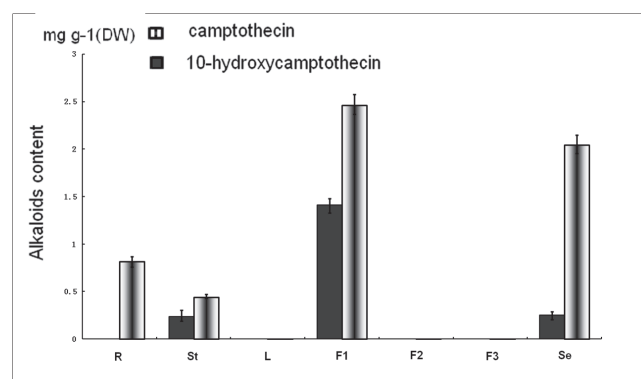


FIGURE 1. Alkaloids concentrations in different organs of *Camptotheca acuminata*. Camptothecin and 10-hydroxycamptothecin in roots (R), stems (St), leaves (L), young flower buds (F1), opening flowers (F2), fading flowers (F3) and seeds (Se). Values are mean \pm SEM, $n=3$. Camptothecin was undetectable in leaves and in opening and fading flowers, while 10-hydroxycamptothecin was also undetectable in roots.

Alkaloids extraction and analysis

Either plant tissues or callus cells were oven-dried at 60°C before extraction for alkaloid determinations. The dried samples were dipped for 24 h in a Soxhlet apparatus with dichloromethane. The extracts were evaporated to dryness and the residues were resolved in 4 ml of acetonitrile:methanol (1:1). The solutions were directly analyzed by high performance liquid chromatography (HPLC Model 2690; Waters, Milford, MA, USA) as described by Wiedenfeld *et al.* (1997).

Results

Alkaloid concentrations in different tissues of *Camptotheca acuminata*

The concentration of camptothecin and 10-hydroxycamptothecin differed in tissues of *C. acuminata* (Fig. 1). Camptothecin was found in all studied tissues except leaves and opening and fading flowers, and the highest concentration was found in young flower buds and in seeds; 10-hydroxycamptothecin was only found in stems, seeds and young flower buds, and the highest concentration was found in the latter.

Correlative *C. acuminata* cell growth rate and alkaloid content

Growth rate of callus cells in culture (relative to the preceding day) was maximal on day 15 and dropped thereafter (Fig. 2A). The mean concentration of both camptothecin and 10-hydroxycamptothecin was between 1 and 2 µg/g of cell dry mass on day 0 of culture. Camptothecin concentration reached a peak on day 10 and decreased later, while that of 10-hydroxycamptothecin decreased during culture (Fig. 2B).

Effect of various treatments on alkaloids content in *C. acuminata* cell culture

As indicated in Fig. 3, three heavy metal ions (Cu^{2+} , Pb^{2+} , Mn^{2+}) induced respective content of alkaloids in *C. acuminata* cells. The ions exhibited separate effects to the accumulations of camptothecin and 10-hydroxycamptothecin. Cu^{2+} and Pb^{2+} significantly intrigued camptothecin accumulation while Mn^{2+} showed no inducement rather than inhibition. Soak appeared to be another important factor in this process, as the com-

binated treatment with UV-B and water-soak markedly induced 10-hydroxycamptothecin yield and elicited no increase of camptothecin. Contrarily, UV-B alone induced dramatic accumulation of camptothecin which was 11-fold above control but exhibited no effects on 10-hydroxycamptothecin accumulation. Methyl-jasmonate did not elicit camptothecin and 10-hydroxycamptothecin yields in *C. acuminata* cells, abscisic acid exhibited a little of inducement effect, salicylic acid dramatically stimulated 10-hydroxycamptothecin yield with 25-fold above control.

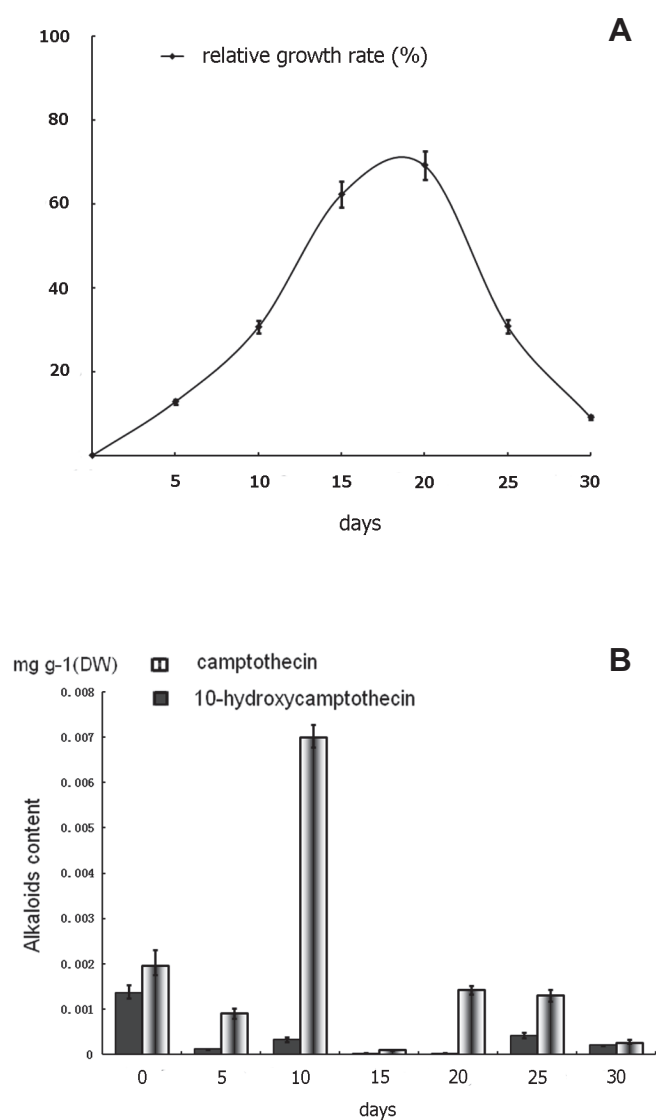


FIGURE 2. A. Growth rate (% mass change relative to the preceding day) of *Camptotheca acuminata* cultured callus cells. B. Camptothecin and 10-hydroxycamptothecin concentration in different culture days. Values are mean \pm SEM, $n=3$.

Discussion

It was previously reported that camptothecin content in young leaves of *Camptotheca acuminata* was 1.5-fold higher than that in seeds (Lopez-Meyer *et al.*, 1994), but camptothecin content in the flowers had rarely been examined before. Summarily, camptothecin content seemed to be related with plant development as it was the most abundant in the early developmental stages of both leaves and flowers, and remarkably declined in the latter stages. Considering both the concentration of pharmaceuticals and quantity of materials, seed was the most advisable part to be harvested as medicinal materials.

Callus was an alternative source for secondary metabolites. During the development of callus, it didn't show that the greater growth rate was achieved when the camptothecin content was lower. It was also reported that camptothecin accumulation started at the end of the growth phase and the maximum content was observed 10 days after inoculation, with time course changes of cell growth and camptothecin production (Song and Byun, 1998).

Many of the alkaloids could be induced by abiotic stresses including heavy metal ions and UV light. The results of this investigation indicated that there might be different mechanisms in the inducements of camptothecin and 10-hydroxycamptothecin. The terpenoid-indole alkaloids production in *Catharanthus roseus* cells could be induced by methyl-jasmonate (van der Heijden *et al.*, 2004), and increasing acetylsalicylic acid concentration increases leaf camptothecin concentration (Halitschke *et al.*, 2004). Methyl-jasmonate and

jasmonic acid could increase camptothecin production 6 and 11 times in *C. acuminata* cells, respectively (Song and Byun, 1998). In the present study, methyl-jasmonate didn't show a similar effect as well. Despite that camptothecin and 10-hydroxycamptothecin were also terpenoid-indole alkaloids, the results of methyl-jasmonate inducement were not consistent with what had been reported. Herein it was hypothesized that: (1) methyl-jasmonate showed inhibition at the early stage and subsequently exhibited inducement, and the time course in this present study was so short as only to cover the early stage; (2) methyl-jasmonate had another affection mechanism on the yields of camptothecin and 10-hydroxycamptothecin in *C. acuminata* compared with that on the yield of terpenoid-indole alkaloids in *Catharanthus roseus*.

Furthermore, the combined use of metabolic engineering and physiological modulation in transgenic and wild-type plants would provide the sustainable and rational supply of these bioactive compounds (Pasquali *et al.*, 2006).

In conclusion, the accumulations of camptothecin and 10-hydroxycamptothecin in *C. acuminata* were significantly correlated with the different organs of the plant, and the distinct developmental stages of both plant flowers and callus. Seed acted as the most advisable part as medicinal materials. Although the contents of camptothecin and 10-hydroxycamptothecin in callus were not equal as in plant seeds, UV-B and salicylic acid showed dramatic effects on the yields of the two pharmaceuticals, indicating the potential breakthrough in the production of camptothecin and 10-hydroxycamptothecin.

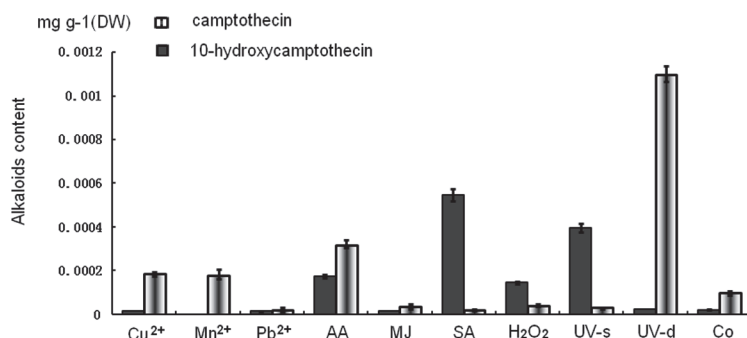


FIGURE 3. Alkaloids concentrations (camptothecin and 10-hydroxycamptothecin) in *Camptotheca acuminata* control cells (Co) and after being exposed to phytohormones (abscisic acid, AA; methyl jasmonate, MJ; salicylic acid, SA), heavy metals (Cu²⁺; Mn²⁺; Pb²⁺), hydrogen peroxide (H₂O₂) and ultraviolet-B light (ultraviolet light-exposed cells, UV-d; water-soaked, ultraviolet light-exposed cells, UV-s). Values are mean ± SEM, n=3.

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