Biotechnology Letters **26:** 1745–1748, 2004.

Improvement of growth and camptothecin yield by altering nitrogen source supply in cell suspension cultures of *Camptotheca acuminata*

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Received 15 July 2004; Revisions requested 30 July 2004; Revisions received 17 September 2004; Accepted 20 September 2004

Key words: ammonium, *Camptotheca acuminata*, camptothecin, nitrate, two-stage culture

Abstract

Nitrate at 70 mM gave the highest biomass of *Camptotheca acuminata* in suspension culture in MS medium, but a NH_4^+/NO_3^- molar ratio of 5:1 (giving a total of 40 mM N) gave the maximum camptothecin yield. A two-stage flask culture system was established to improve culture efficiency; cell dry weight, camptothecin content and yield was increased by 30%, 280% and 340%, respectively when compared with those of control, reaching up to 36 g l^{-1}, 0.36 mg g^{-1}, and 12.8 mg l^{-1}, respectively.

Introduction

Camptothecin, a water-insoluble monoterpene-derived indole alkaloid, first isolated from the Chinese tree *Camptotheca acuminata* (Wall *et al.* 1966) and later from *Ophiorrhiza pumila* and *Nothapodytes foetia*, inhibits growth of tumor cells by blocking the topoisomerase I (Hsiang *et al.* 1985). It is also active against the human immunodeficiency virus (HIV) (Hung *et al.* 2001). At present, two semi-synthetic camptothecin analogues, topotecan and irinotecan, are being used in the clinic for treatment of cancers throughout the world and several other analogs, such as 10-hydroxycamptothecin, 9-nitrocamptothecin and lurtotecan, are currently under clinical development (Du 2003). The rapid increasing pharmaceutical market and economic value of these alkaloids have prompted efforts to produce them with plant cell cultures but, until now, only a few studies addressing this possibility have been carried out (van Hengel *et al.* 1992, Wiedenfeld *et al.* 1997, Ciddi & Shuler 2000, Fulzele *et al.* 2002, Thengane *et al.* 2003). Although untransformed root (Fulzele *et al.* 2002) and hairy roots (Sudo *et al.* 2002, Lorence *et al.* 2004) can accumulate comparatively high content of camptothecin, scale-up still remains a problem.

In plant cell or tissue culture, the nitrogen source may significantly affect cell growth and the formation of many alkaloids. In different plant cell cultures, an optimal medium formula for cell growth and secondary metabolism is dependent on the species and thus a detailed investigation for each case is necessary (Moreno *et al.* 1995). As far as we know, there is no report of the effect of the nitrogen source on the biosynthesis of camptothecin in cell suspension cultures of *C. acuminata*. Therefore, the aim of our study was to modify the nitrogen supply to enhance camptothecin yield in *C. acuminata* cell suspension cultures.

Materials and methods

Plant cell cultures

A cell line (HP-22) screened from callus, which was induced from hypocotyls of *Camptotheca acuminata* seeds gathered from the trees in the campus of Wuhan University, Hubei Province, China, was used and grown on MS media supplemented with

0.1 mg 2,4-dichlorophenoxyacetic acid l^{-1}, 0.5 mg naphthaleneacetic l^{-1}, 0.5 mg N^6-benzylaminopurine l^{-1} and 40 g sucrose l^{-1} at pH 5.8. All experiments were performed in 250 ml shake flasks, each containing 100 ml medium on a rotary shaker with 120 rpm at (25 ± 1) °C under darkness and the inoculum was about 8 mg dry wt ml^{-1}. The nitrogen supply in the medium was modified by altering the molar ratio of NO_3^-/NH_4^+ or the total amount of initial nitrogen in standard MS media. All experiments were independently repeated at least three times.

Biomass and camptothecin analysis

Determinations (triplicate) of dry weight and camptothecin content were carried out at the time of the maximum production (after 20 days of culture) unless particularly stated. The biomass was measured on dry weight (DW) basis after the cells had been dried to constant weight and the determination of camptothecin in the samples of the cells was performed by HPLC as described (Pan *et al*. 2004).

Results

Effect of NO_3^-/NH_4^+ ratio on cell growth and camptothecin content

Biomass and camptothecin accumulation of suspension culture cells in cell line HP-22 are presented in Figure 1. Cell dry weight was improved in a medium with a higher NO_3^-/NH_4^+ ratio. Nitrate as the sole nitrogen source (i.e. 60 mM NO_3^- without NH_4^+) gave the highest cell dry weight. For secondary metabolism, a high ratio of NH_4^+/NO_3^- was favorable to camptothecin accumunation and the optimum NH_4^+/NO_3^- for camptothecin biosynthesis was 5:1. No extracellular camptothecin was detected.

Effect of initial total nitrogen amount on biomass and camptothecin content

Cell growth was inhibited at low or high initial nitrate concentrations and the most favorable concentration of nitrate for maximum biomass (dry cell wt = 33.5 g l^{-1}) was 70 mM (Table 1). Results in Table 1 also show that camptothecin

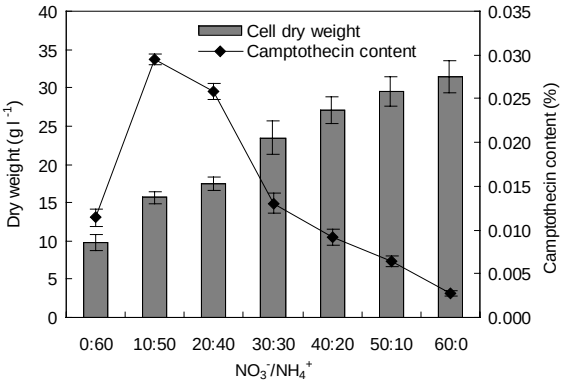

Fig. 1. Effect of NO_3^-/NH_4^+ ratio on the growth and camptothecin content in cell suspension cultures of cell line HP-22. Cell suspension culture was grown in modified MS liquid medium by altering the ratio of NO_3^-/NH_4^+ at 60 mM total nitrogen. Biomass refers to the dry weight of the cells and camptothecin content refers to the percentage weight of dry cells. Data are expressed as mean ± standard deviation of three replicates.

content and yield reached the highest (0.033% dry wt and 6.3 mg l^{-1}, respectively) with the total nitrogen 40 mM ($NH_4^+/NO_3^- = 5:1$). In addition, camptothecin accumulation in the cells cultivated in the medium with nitrate as the sole nitrogen source (growth medium) was far below than that

Table 1. Effect of initial medium nitrogen concentration on the growth rate and camptothecin biosynthesis in cell suspension cultures of cell line HP-22.

Total nitrogen concetration (mM)	Cell growth (g dry wt l^{-1})	Camptothecin Content ($\times 10^{-3}$ % dry wt)	Camptothecin yield (mg dry wt l^{-1})
50[a]	27.6 ± 1.2	1.8 ± 0.1	0.5 ± 0.1
60[a]	31.1 ± 1.6	2.7 ± 0.4	0.8 ± 0.2
70[a]	33.5 ± 1.8	5.7 ± 0.5	1.9 ± 0.1
80[a]	30.6 ± 1.2	8.4 ± 0.6	2.6 ± 0.1
30[b]	20.4 ± 1.5	25.7 ± 1.1	5.2 ± 0.2
40[b]	19.4 ± 0.9	32.8 ± 1.8	6.3 ± 0.4
50[b]	17.3 ± 1.4	29.3 ± 1.7	5.1 ± 0.4
60[b]	15.6 ± 1	27.7 ± 1	4.3 ± 0.2
70[b]	12 ± 0.7	20.9 ± 1	2.5 ± 0.2

Cell suspension cultures were maintained in modified MS liquid medium by altering initial nitrogen concentration. Data are expressed as mean ± standard deviation of three replicates.
[a]Nitrate as the sole nitrogen source in MS liquid medium and other components were not changed.
[b]Nitrogen at the ratio of NH_4^+:NO_3^- = 5:1 as the nitrogen source in MS liquid medium and other components were not changed

in those cultured in the medium with nitrogen nutrient at a NH_4^+/NO_3^- ratio of 5:1 (production medium) (Table 1). Likewise, cell growth in production medium was inferior to that in growth medium (Table 1). Therefore, to optimize culture efficiency, cell growth and secondary metabolism should be separated by using a two-stage culture.

Improve cell growth and camptothecin yield by two-stage culture

In our two-stage suspension cultures, during the first 18 days, cells were cultivated in growth medium, in which 70 mM nitrate replaced the nitrogen source in standard MS medium. The growth medium was then removed and replaced by the same volume of fresh production medium (i.e. initial nitrogen concentration of 40 mM at a NH_4^+/NO_3^- ratio of 5:1 replacing the nitrogen in standard MS medium) for secondary metabolite biosynthesis. Cells were harvested every 4 days. As expected (Table 2), the cell dry weight, camptothecin content, and camptothecin yield in suspension cells cultured increased by 30%, 280% and 340%, respectively when compared with those of control, reaching up to 35.6 g l^{-1}, 0.036%, and 12.8 mg l^{-1}, respectively.

Discussion

Nitrate was favorable to cell growth while more ammonium was optimal for camptothecin accumulation in cell suspension cultures of *C. acuminata*. Our results are similar to that of cell suspension cultures of *Catharanthus roseus* to produce steroidal alkaloids (Panda *et al.* 1992), of *Taxus yunnanensis* to produce taxol (Chen *et al.* 2003) and that of hairy root cultures of *Atropa belladonna* to produce tropane alkaloids (Bensaddek *et al.* 2001). Ammonium is very diffusive and easily accumulates into the tissues, becoming toxic if not immediately metabolized (Bensaddek *et al.* 2001). Another consequence of the accumulation of ammonium could be a repression of nitrate assimilation (Crawford 1995), which would result in medium acidification. Ammonium ions, however, would probably be rapidly assimilated into glutamate and then into glutamine which is involved in camptothecin biosynthesis (Li 2002, Yamazaki *et al.* 2004). Interestingly, Li (2001) reported that camptothecin concentrations of *C. acuminata* hydroponic seedlings increased after nitrogen deficiency although a surplus of nitrogen significantly improved *C. acuminata* seedling biomass and he hypothesized that the nitrogen deficiency increased camptothecin concentration by possibly creating an environmental stress. However, in our study, no extracellular camptothecin was detected, therefore, it seemed unlikely that the increasing ratio of ammonium to nitrate enhanced camptothecin yields as a stress factor.

Table 2. Improved camptothecin yield in suspension cells of cell line HP-22 by two-stage culture.

Culture method	Biomass (g dry wt l^{-1})	Camptothecin content (mg g^{-1} dry wt)	Camptothecin yield (mg l^{-1})
Control	27.4 ± 1.7 (20)[a]	0.09 ± 0.01 (20)	2.5 ± 0.3 (20)
Two-stage culture	35.6 ± 1.2 (24)	0.36 ± 0.01 (28)	12.8 ± 0.3 (28)

Data are expressed as mean ± standard deviation of three replicates.
[a]The day it reached the maximum.

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