

Article

Efficient In Vitro Regeneration and Camptothecin Accumulation in *Camptotheca acuminata*

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Abstract

Camptotheca acuminata produces valuable camptothecin, a potent anticancer agent. To overcome the limitations of wild resources, we developed efficient in vitro regeneration and camptothecin production systems. Key findings include: Optimal sterilization of plant material was achieved using Plant Preservative Mixture (12 min). Axillary shoot induction was most effective on MS medium with 2.5 mg/L 6-BA and 0.25 mg/L NAA, while adventitious shoot regeneration showed a preference for 1 mg/L 6-BA and 0.1 mg/L NAA. Synchronous induction reached its peak at 83.45% using MS medium with 0.5 mg/L 6-BA, 0.05 mg/L NAA, and 0.5 mg/L GA₃. Shoot elongation benefited from 5 mg/L phloroglucinol and 20 mg/L CaCl₂. For biomass production, 2/3 MS macroelements yielded maximum adventitious shoot biomass (50.52 mg). Rooting reached 100% efficiency on 1/2 MS medium containing 1 mg/L IBA, 0.5 mg/L NAA, 5 mg/L phloroglucinol, and 2 g/L AC, averaging 10.50 roots per shoot. The four-stage camptothecin enrichment system produced shoots containing 795.10 µg/g DW camptothecin—fivefold higher than natural leaves. This breakthrough establishes: (1) the first in vitro camptothecin enrichment platform; (2) a sustainable alternative to wild harvesting; (3) a novel circular production model for endangered medicinal plants. The optimized protocols address the challenges of camptothecin supply while demonstrating remarkable productivity enhancements through controlled in vitro culture systems.

Keywords: *Camptotheca acuminata*; camptothecin; in vitro regeneration; in vitro drug accumulation; tissue culture



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1. Introduction

Camptotheca acuminata, a species belonging to the Nyssaceae family and the Camptotheca genus, is a tall deciduous tree endemic to China and is classified as a second-class nationally protected wild plant. In 1966, Wall ME from the United States isolated camptothecin (CPT) from the bark of *C. acuminata*. Tumor trials demonstrated that this tryptophan-terpenoid alkaloid possessed anticancer activity [1], thereby attracting widespread attention. Given the inherent toxicity of CPT, there is potential for its development as a botanical pesticide for the control of field pests [2]. In addition to its medicinal value, *C. acuminata* also holds considerable economic value. As a fast-growing tree species, it can mature

into a forest in approximately three years. Its high-yield characteristics make large-scale cultivation of *C. acuminata* an effective means to alleviate China's timber shortage and meet the country's timber demands [3]. However, current research on the production technology of CPT and the artificial cultivation of *C. acuminata* is relatively limited both domestically and internationally, primarily focusing on the artificial synthesis and pharmaceutical development of CPT. The chemical synthesis of CPT involves complex steps and an imperfect production system. The production of CPT through cultivation methods such as suspension cells and callus tissues is relatively simpler and more efficient. Over the past few decades, researchers have established regeneration systems for *C. acuminata* using explants such as shoot tips, stem segments, embryonic axes, and leaves. They have explored the effects of factors such as explant type, plant hormones, basic media, and additives on the regeneration of *C. acuminata* and the synthesis of CPT. Nevertheless, the CPT content in the products of *C. acuminata* tissue culture is currently not satisfactory [4]. Callus tissues induced on specific culture media exhibited detectable levels of camptothecin, albeit with relatively low overall content, ranging approximately from 0.0002% to 0.0004% (*w/w*). In contrast, camptothecin production in suspension cell cultures of *C. acuminata* varied between 0.001% and 0.02% (*w/w*). Furthermore, when *Agrobacterium rhizogenes* was employed to infect *C. acuminata* and induce hairy root formation, the resulting hairy roots demonstrated camptothecin accumulation levels in the range of approximately 0.2 mg/g to 1.0 mg/g dry weight [5–11].

From the current research status and trends both domestically and internationally, CPT and its analogs are primarily obtained through isolation from *C. acuminata* [12]. The development of the anticancer applications of CPT should be based on an adequate supply of *C. acuminata* resources. However, wild *C. acuminata* resources are severely limited, hindering the industrial and large-scale comprehensive utilization of *C. acuminata* resources. The bottleneck in the artificial cultivation of *C. acuminata* lies in the dormancy and low germination rate of its seeds. Moreover, in recent years, the issue of pests and diseases affecting *C. acuminata* has become increasingly prominent [13,14]. To rationally utilize, develop, and protect *C. acuminata* resources, tissue culture technology can be employed for the rapid propagation of *C. acuminata* seedlings, thereby addressing the shortage of *C. acuminata* resources.

This study established, for the first time, a whole-plant-based in vitro regeneration system for high-yield CPT accumulation by integrating dual-pathway strategies of organogenesis-driven regeneration and metabolic flux redirection. The system achieved scalable quantitative production of medicinal organs (shoots), overcoming the yield limitations of conventional cell suspension cultures and hairy root cultures. Notably, the entire shoot system demonstrated a CPT accumulation of 795.10 ± 5.02 $\mu\text{g/g}$ dry weight (DW). By employing intact shoots as CPT accumulation carriers instead of cell/tissue fragments, this innovation resolved two critical industrialization bottlenecks: (1) the genetic instability inherent in long-term cell suspension cultures, and (2) the dependency on *Agrobacterium rhizogenes*-mediated transformation in hairy root systems. The developed production paradigm features a non-seasonal continuous harvesting system, enabling year-round raw material procurement with exceptional phenotypic consistency and biochemical uniformity.

2. Materials and Methods

2.1. Experimental Materials and Culture Conditions

The experimental samples were derived from 15-year-old mature *Camptotheca acuminata* trees, specifically young spring shoots with terminal shoots harvested in the current growing season. These explants were collected from *C. acuminata* specimens cultivated at Shanghai Chenshan Botanical Garden. To maintain favorable growth conditions, the

culture media's pH was set at 5.8. The incubation environment was kept at a constant temperature of 25 ± 2 °C. Additionally, the cultures were exposed to a light intensity of $33.6 \mu\text{mol}/\text{m}^2/\text{s}$ under a 16/8 h light/dark cycle, the light source comes from LED.

2.2. Preparation of Sterile Materials

Young stem segments of *Camptotheca acuminata* were used as the initial explants. After rinsed under running tap water for 1 h, the explants were placed in a laminar flow hood. They were then treated with 75% (v/v) ethanol for 30 s, followed by immersion in a 50% (v/v) plant preservative mixture (PPM, add 500 mL of the PPM stock solution to an equal volume of deionized water to prepare a 50% (v/v) disinfectant solution). for 10, 12, 15, 17, or 20 min, respectively. Subsequently, the explants were rinsed 3–5 times with sterile distilled water. The treated explants were inoculated onto MS medium supplemented with 1 mg/L 6-benzylaminopurine (6-BA) and 0.1 mg/L α -naphthaleneacetic acid (NAA). Inoculate 50 explants for each treatment and repeat three times. After a 2-week culture period, the contamination and growth status of the materials under each treatment were recorded. The medium was fortified with 30 g/L sucrose and solidified using 5 g/L agar. The culture medium was sterilized by autoclaving (121 °C, 0.105 MPa) for 20 min. Sucrose, agar, and other medium components were procured from Hangzhou Lin'an Bottled Scientific Experimental Supplies Department in Hangzhou, China. For plant growth regulation, all phytohormones and supplements were provided by Hangzhou Morebert Biotechnology Co., Ltd., Hangzhou, China. Additionally, the plant preservative mixture was acquired from Yesen Biotechnology Co., Ltd., located in Shanghai, China. The high-pressure steam sterilization pot is sourced from Shanghai Shen'an Medical Machinery Factory (Shanghai, China), with the model LDZF-75L-I; The vertical laminar flow purification workbench is sourced from Shanghai Shangjing Purification Equipment Co., Ltd., Shanghai, China, model CA920-3.

2.3. Efficient In Vitro Regeneration for *Camptotheca acuminata*

2.3.1. Induction of Axillary Shoots in *Camptotheca acuminata*

Sterilized *C. acuminata* explants were transferred to MS-based culture media enriched with 6-BA (1.5–3 mg/L) and NAA (0.15–0.3 mg/L). Inoculate 32 explants for each treatment and repeat three times. The inoculated cultures were incubated for 3–5 weeks, during which axillary shoot emergence time and germination frequency were monitored.

2.3.2. Induction of Adventitious Shoots in *Camptotheca acuminata*

For adventitious shoot induction, the previously generated axillary shoots were transferred to MS medium supplemented with various growth regulator combinations: 6-BA (0.5–1.5 mg/L) plus NAA (0.05–0.2 mg/L), or KT (0.1–0.5 mg/L) alone. Inoculate 32 explants for each treatment and repeat three times. After 3–5 weeks of culture, quantitative assessments included the percentage of explants producing shoots and the average shoot proliferation rate per explant.

2.3.3. Elongation of *Camptotheca acuminata* Adventitious Shoots

Adventitious shoots of *C. acuminata* induced in Section 2.3.2 were inoculated onto a synchronized elongation-inducing medium for adventitious shoots. The basal medium used was MS medium supplemented with 0.1–0.8 mg/L 6-BA, 0.01–0.08 mg/L NAA, and 0.1–0.8 mg/L GA₃. Inoculate 25 explants for each treatment and repeat three times. After 3–5 weeks of culture, synchronized adventitious shoot clusters with uniform growth and development were obtained. The number of effective shoots with a height of approximately 2 cm, the high-frequency synchronization rate, and the uniformity were statistically analyzed.

2.4. In Vitro Drug Accumulation of Camptothecin

2.4.1. Stage I Culture for In Vitro CPT Enrichment

For stage I camptothecin enrichment, adventitious shoot clusters of *C. acuminata* showing synchronous elongation (>2 cm height) were cultured on two media formulations: (1) modified MS medium containing 0.5 mg/L 6-BA, 0.05 mg/L NAA, 0.5 mg/L GA₃, and 0.005 mg/L Trp; and (2) control MS medium with identical components except Trp omission. Following a 3–5 week culture period, developed adventitious shoots were collected for subsequent camptothecin analysis.

2.4.2. Stage II Culture for In Vitro CPT Enrichment

Adventitious shoots obtained from Stage I culture of in vitro CPT enrichment were inoculated onto a Stage II culture medium for in vitro CPT enrichment. The basal medium used was MS, supplemented with 0.5 mg/L 6-BA, 0.05 mg/L NAA, 1–10 mg/L phloroglucinol (PG), and 0–40 mg/L calcium chloride (CaCl₂). Inoculate 40 explants for each treatment and repeat three times. After 4–6 weeks of culture, the height and elongation rate of the adventitious shoots were statistically analyzed.

2.4.3. Stage III Culture for In Vitro CPT Enrichment

Adventitious shoots from Stage II CPT enrichment cultures were inoculated onto MS media containing modified macroelement concentrations (ranging from 1/3× to 4/3× standard strength). All media formulations included consistent supplements: 0.5 mg/L 6-BA, 0.05 mg/L NAA, 5 mg/L PG, and 20 mg/L CaCl₂. Inoculate 20 explants for each treatment and repeat three times. Following a 4-week cultivation period, *C. acuminata* biomass production was quantitatively assessed.

2.4.4. Stage IV Culture for In Vitro CPT Enrichment

Adventitious shoots following Stage III culture for in vitro CPT enrichment were transferred to either a medium containing 1/2 MS + 0.5–1.5 mg/L IBA + 0.1–1 mg/L NAA + 5 mg/L PG + 2 g/L AC or a medium containing 1/2 MS + 0.5–2 mg/L IBA + 0.1–1 mg/L NAA + 5 mg/L PG. Inoculate 20 explants for each treatment and repeat three times. These shoots were then cultured for 4–8 weeks to induce rooting of *C. acuminata* adventitious shoots, with the aim of accumulating camptothecin in whole *C. acuminata* plants. The number of rooted adventitious shoots and the rooting rate were statistically analyzed.

2.5. Analysis of CPT Content

CPT analysis samples originated from four culture regimes: (i) Stage I control (MS + 0.5 mg/L 6-BA + 0.05 mg/L NAA + 0.5 mg/L GA₃); (ii) Stage I with Trp supplementation (additional 0.005 mg/L Trp); (iii) Stage II (MS base with 0.5 mg/L 6-BA, 0.05 mg/L NAA, 5 mg/L PG, and 20 mg/L CaCl₂); and (iv) Stage III using 2/3 MS medium with identical supplements to Stage II. The aerial parts of shootlets cultivated in 1/2 MS medium supplemented with 1 mg/L IBA, 0.5 mg/L NAA, 5 mg/L PG, and 2 g/L AC during the in vitro CPT enrichment culture of adventitious roots. The root systems of shootlets cultivated in 1/2 MS medium supplemented with 1 mg/L IBA, 0.5 mg/L NAA, 5 mg/L PG, and 2 g/L AC during the in vitro CPT enrichment culture of adventitious roots. Young leaves collected from mature *C. acuminata* trees. The CPT content in these samples was determined.

2.6. Statistical and Data Analysis

The mathematical expressions utilized for assessing various tissue culture regeneration parameters in the current study are as follows:

- Axillary shoot induction rate (%) = (Number of axillary shoots per explant)/(Initial number of explants) $\times$ 100%
- Adventitious shoot induction rate (%) = (Number of adventitious shoots per explant)/(Initial number of explants) $\times$ 100%
- High-frequency synchronization rate (%) = (Number of adventitious shoots showing high-frequency synchronous growth)/(Initial number of explants) $\times$ 100%
- Adventitious shoot elongation rate (%) = (Number of elongated shoots)/(Initial number of explants) $\times$ 100%
- Root induction rate (%) = (Number of plantlets with roots)/(Initial number of shoots) $\times$ 100%
- Data analysis involved one-way ANOVA with Duncan's test ($\alpha = 0.05$) using Excel 2020 and SPSS 27.

3. Results

3.1. Effects of PPM on the Preparation of *Camptotheca acuminata* Explants

Healthy and juvenile shoots of *C. acuminata* were harvested, and stem segments with apical shoots were selected. These segments were subjected to rinsing under running water for 1 h and then transferred to a laminar flow hood. They were treated with 75% ethanol for 30 s and subsequently immersed in 50%(v/v) PPM for a duration ranging from 10 to 20 min (Table 1). Following this, the segments were inoculated onto MS culture media supplemented with 1 mg/L 6-BA and 0.1 mg/L NAA. After 2 weeks of culture, the contamination and growth status of the materials under each treatment were statistically analyzed. The results indicated that as the PPM treatment duration increased, the sterilization rate gradually elevated, reaching 100% with a 20 min PPM treatment. However, the survival rate progressively declined from 100% to 83.54%. Prolonged PPM treatment could lead to the inactivation or growth retardation of some explants. The optimal sterilization treatment was identified as a 12 min PPM treatment, which achieved a sterilization rate of 98.5% and a survival rate of 100%.

Table 1. Effects of PPM on the sterilization efficacy of starting materials.

PPM Treatment Duration (min)	Cleanliness Rate (%)	Survival Rate (%)
10	80.00 $\pm$ 0.06 b	100 a
12	94.00 $\pm$ 0.03 a	100 a
15	96.00 $\pm$ 0.06 a	89.65 $\pm$ 0.02 b
17	98.00 $\pm$ 0.02 a	87.75 $\pm$ 0.01 b
20	100 a	86.00 $\pm$ 0.03 b

These values represent mean $\pm$ standard deviation. Duncan's various lowercase letters show significant differences between the data at the 5% level, using the multiple range test at $p < 0.05$.

3.2. Effects of PGRs on Axillary Shoot Induction

Surface-sterilized *C. acuminata* explants were cultured on MS medium containing 1.5–3 mg/L 6-BA and 0.15–0.3 mg/L NAA for 3–5 weeks. Axillary shoot emergence timing and frequency were quantitatively analyzed. As presented in Table 2, both parameters exhibited a unimodal response to phytohormone concentrations, with initial enhancement followed by decline at higher levels. Maximum shoot induction efficiency occurred at 2.5 mg/L 6-BA combined with 0.25 mg/L NAA, achieving peak performance with 95.37% shoot emergence frequency and the shortest emergence time (17.42 days) (Figure 1).

Table 2. Effects of different combinations of PGRs on axillary shoot germination.

6-BA (mg/L)	NAA (mg/L)	Adventitious Shoots Induction Rate (%)	Days for Determined Shoots to Sprout
1.5	0.15	70.37 ± 4.24 c	32.41 ± 2.34 d
2.0	0.2	84.26 ± 6.99 b	24.44 ± 3.17 b
2.5	0.25	95.37 ± 1.61 a	17.42 ± 2.44 a
3	0.3	78.70 ± 4.24 bc	29.36 ± 3.10 c

These values represent mean ± standard deviation. Duncan's various lowercase letters show significant differences between the data at the 5% level, using the multiple range test at $p < 0.05$.



Figure 1. Induction of axillary shoots in *Camptotheca acuminata*. (A) Initial explant; (B) Germinated axillary shoots 2 weeks after inoculation; (C) Axillary shoots 5 weeks after inoculation. The culture medium used was MS + 2.5 mg/L 6-BA + 0.25 mg/L NAA. Bar = 1 cm.

3.3. Effects of PGRs on Adventitious Shoots Induction

The induced axillary shoots were subsequently transferred to MS-based regeneration media formulated with either: 0.5–1.5 mg/L 6-BA + 0.05–0.2 mg/L NAA, or 0.5–1.5 mg/L 6-BA + 0.1–0.5 mg/L KT. After a 3–5 week culture period, quantitative analysis demonstrated superior performance in terms of adventitious shoot formation in NAA-containing media compared to the media supplemented with KT (Table 3). The optimal hormonal combination (1 mg/L 6-BA + 0.1 mg/L NAA) yielded remarkable regeneration efficiency, resulting in the production of 22.64 shoots per explant at a 92.75% induction frequency (Figure 2B).

Table 3. Effects of different combinations of PGRs on adventitious shoot induction in *Camptotheca acuminata*.

6-BA (mg/L)	NAA (mg/L)	KT (mg/L)	Adventitious Shoot Induction Rate (%)	Average Number of Adventitious Shoots
0.5	0.05	/	77.33 ± 3.01 bc	16.77 ± 2.51 c
1.0	0.1	/	92.75 ± 3.00 a	33.24 ± 3.32 a
1.5	0.2	/	80.87 ± 4.16 b	23.31 ± 4.46 b
0.5	/	0.1	64.67 ± 4.63 e	8.17 ± 2.71 e
1.0	/	0.3	73.33 ± 5.00 cd	15.29 ± 3.49 c
1.5	/	0.5	70.00 ± 3.46 de	13.76 ± 4.07 d

These values represent mean ± standard deviation. Duncan's various lowercase letters show significant differences between the data at the 5% level, using the multiple range test at $p < 0.05$.

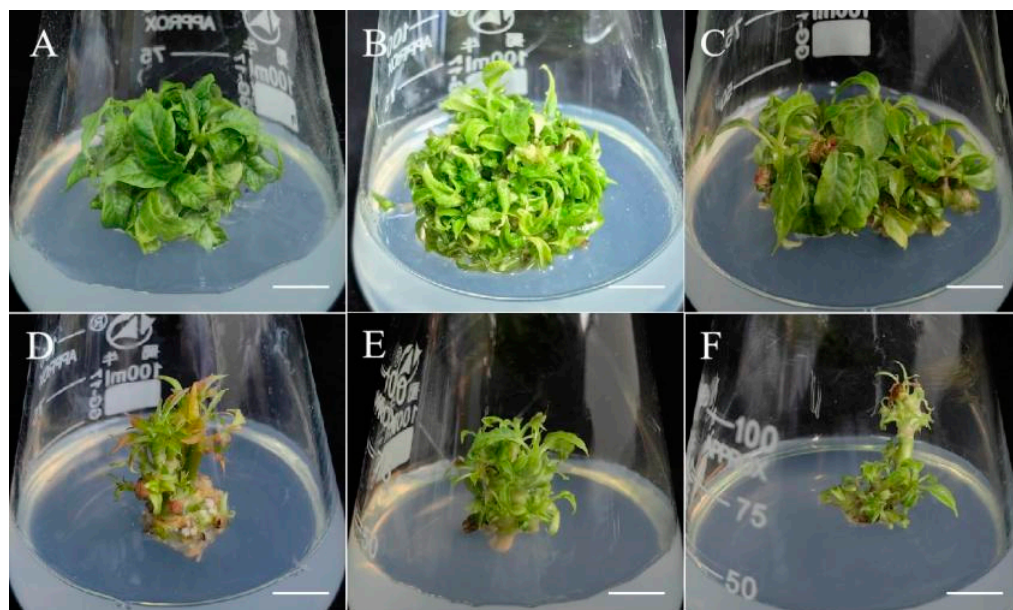


Figure 2. The effect of PGRs combinations on the induction of adventitious shoots in *Camp-totheca acuminata*. (A) 0.5 mg/L 6-BA + 0.05 mg/L NAA; (B) 1.0 mg/L 6-BA + 0.1 mg/L NAA; (C) 1.5 mg/L 6-BA + 0.2 mg/L NAA; (D) 0.5 mg/L 6-BA + 0.1 mg/L KT; (E) 1.0 mg/L 6-BA + 0.3 mg/L KT; (F) 1.5 mg/L 6-BA + 0.5 mg/L KT. Bar = 0.9 cm.

3.4. Effects of Exogenous Additive Combinations on High-Frequency Synchronous Adventitious Shoots Induction

The adventitious shoot clusters obtained from the preceding step were transferred to a culture medium for Stage I of in vitro CPT enrichment, which was composed of MS basal medium supplemented with 0.1–0.8 mg/L 6-BA, 0.01–0.08 mg/L NAA, 0.1–0.8 mg/L GA₃. After 3–5 weeks of culture, synchronously developing adventitious shoot clusters were acquired. The quantity of effective shoots with a height exceeding 1 cm, the high-frequency synchronization rate, and the uniformity of growth were statistically documented. As presented in Table 4, the treatment comprising MS basal medium supplemented with 0.5 mg/L 6-BA, 0.05 mg/L NAA, and 0.5 mg/L GA₃ demonstrated superior performance in synchronous induction, attaining peak efficiency (83.45%) among all tested combinations. Figure 3A,B depict the process of high-frequency synchronized elongation of *C. acuminata* adventitious shoots, while Figure 3C presents the case where the adventitious shoots failed to undergo synchronized elongation.

Table 4. Effects of exogenous additive combinations on high-frequency synchronous adventitious shoot induction.

Additive Concentration (mg/L)			High—Frequency Synchronization Rate (%)	Uniformity
6-BA	NAA	GA ₃		
0.1	0.01	0.1	59.33 ± 3.77 c	Low
0.3	0.03	0.3	62.67 ± 3.21 c	Low
0.5	0.05	0.5	84.33 ± 1.53 a	High
0.8	0.08	0.8	72.33 ± 2.52 b	Relatively high

These values represent mean ± standard deviation. Duncan's various lowercase letters show significant differences between the data at the 5% level, using the multiple range test at $p < 0.05$.

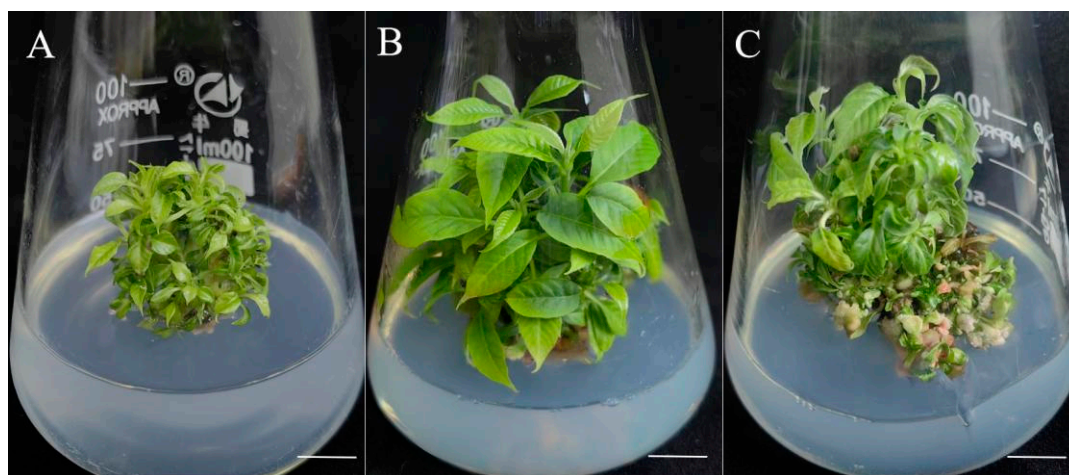


Figure 3. High-frequency synchronized elongation and non-high-frequency synchronized elongation of *Camptotheca acuminata* adventitious shoots. (A) Early stage of high-frequency synchronous elongation culture; (B) Cultivate high-frequency synchronous elongation for 4 weeks; (C) Cultivate non-high-frequency synchronous elongation for 4 weeks. Bar = 1 cm.

3.5. Effects of Exogenous Additives on the Elongation of *Camptotheca acuminata* Adventitious Shoots

Figure 3 weeks of Stage I CPT enrichment, adventitious shoots were transferred to Stage II culture medium was composed of MS basal salts supplemented with 0.5 mg/L 6-BA, 0.05 mg/L NAA, and varying concentrations of PG (1–10 mg/L) and CaCl_2 (0–40 mg/L). After a culture period of 4–6 weeks, shoot elongation parameters were quantified. The results indicated that both PG and CaCl_2 significantly enhanced shoot elongation, though the effects were concentration-dependent (Table 5). Shoot growth response exhibited a bell-shaped curve, with optimal performance observed at 5 mg/L PG combined with 20 mg/L CaCl_2 . This treatment produced the highest elongation rate (77.04%) and mean shoot length (64.16 mm), along with excellent morphological characteristics (Figure 4A). However, supraoptimal concentrations (>7 mg/L PG or >30 mg/L CaCl_2) induced symptoms of phytotoxicity, including leaf chlorosis and shoot mortality, despite retaining some growth-promoting activity (Figure 4B).

Table 5. Effects of exogenous additives on the elongation of *Camptotheca acuminata* adventitious shoots.

PG (mg/L)	CaCl_2 (mg/L)	Adventitious Shoot Elongation Rate (%)	Average Seedling Height (mm)
1	0	60.74 ± 3.39 c	48.66 ± 2.33 e
3	10	71.11 ± 3.85 b	61.05 ± 2.09 b
5	20	77.04 ± 1.28 a	64.16 ± 2.90 a
7	30	69.63 ± 1.17 b	58.62 ± 3.37 c
10	40	62.96 ± 1.76 c	57.51 ± 2.91 d

These values represent mean $\pm$ standard deviation. Duncan's various lowercase letters show significant differences between the data at the 5% level, using the multiple range test at $p < 0.05$.

3.6. Effect of Basic Media on the Biomass of *Camptotheca acuminata*

Adventitious shoots from preceding stages were cultured on MS-based media standard MS concentration (ranging from $1/3\times$ to $4/3\times$ standard MS concentration), along with consistent supplements: 0.5 mg/L 6-BA, 0.05 mg/L NAA, 5 mg/L PG, and 20 mg/L CaCl_2 . After a 4-week cultivation period, biomass accumulation was quantified (Table 6). The $2/3\times$ MS formulation demonstrated optimal performance for biomass production,

achieving a maximum biomass yield of 50.52 mg per explant. Additionally, plants grown under these conditions exhibited vigorous growth with healthy, green foliage.

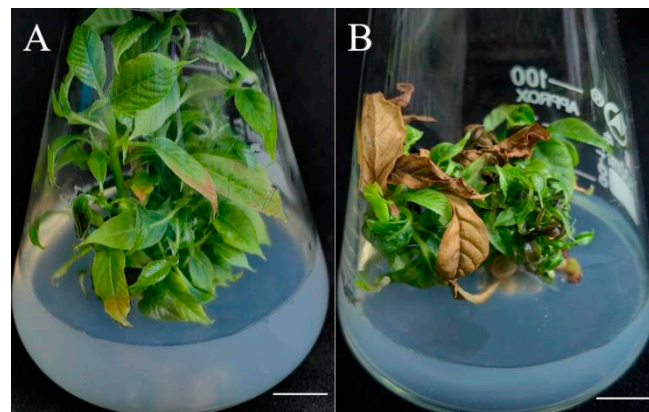


Figure 4. Elongation of *Camptotheca acuminata* adventitious shoots. (A) MS + 0.5 mg/L 6-BA + 0.05 mg/L NAA + 5 mg/L PG + 20 mg/L CaCl₂; (B) MS + 0.5 mg/L 6-BA + 0.05 mg/L NAA + 10 mg/L PG + 40 mg/L CaCl₂. Cultured for 5 weeks, bar = 1 cm.

Table 6. Effects of basic media on the biomass amplification of *Camptotheca acuminata* adventitious shoots.

Basic Medium	Starting Biomass (mg)	Biomass After 4-Week Culture (mg)	Amplification Factor	Growth Status
1/3 MS	20.10 ± 3.12	41.28 ± 1.86 d	2.11 ± 0.36 c	The plants were robust with fresh green leaves.
1/2 MS	19.65 ± 2.94	46.25 ± 2.68 b	2.41 ± 0.39 b	The plants were robust with fresh green leaves.
2/3 MS	19.35 ± 3.00	50.52 ± 1.86 a	2.67 ± 0.43 a	The plants were robust with fresh green leaves.
MS	20.28 ± 2.93	43.03 ± 1.97 c	2.17 ± 0.36 c	The leaf tips turned yellow.
4/3 MS	19.68 ± 3.03	36.37 ± 2.33 e	1.89 ± 0.32 d	The leaves turned yellow, and the plants withered.

These values represent mean ± standard deviation. Duncan's various lowercase letters show significant differences between the data at the 5% level, using the multiple range test at $p < 0.05$.

3.7. Rooting of *Camptotheca acuminata* Shootlets

The elongated adventitious shoots obtained from the preceding procedures were transferred to one of the following media for rooting induction, with the objective of accumulating CPT in whole *C. acuminata* plants: 1/2 MS medium supplemented with 0.5–1.5 mg/L IBA, 0.1–1 mg/L NAA, 5 mg/L PG, and 2 g/L AC; or 1/2 MS medium supplemented with 0.5–2 mg/L IBA, 0.1–1 mg/L NAA, and 5 mg/L PG. The cultures were incubated for 4–8 weeks. The rooting rate and the quantity of roots per shootlet were recorded for each treatment. The experimental results are presented in Table 7 and Figure 5. All treatments promoted rooting in the aseptic shootlets of *C. acuminata*. The 1/2 MS medium supplemented with 1 mg/L IBA, 0.5 mg/L NAA, 5 mg/L PG, and 2 g/L AC demonstrated superior adventitious root induction capacity, producing fully developed roots with a 100% regeneration frequency and an average of 10.50 roots per explant (Figure 5H).

Table 7. Effects of different culture formulations on the rooting of *Camptotheca acuminata* shootlets.

AC (g/L)	IBA (mg/L)	NAA (mg/L)	Rooting Rate (%)	Number of Adventitious Roots
0	0.5	0.1	78.33 ± 2.89 c	5.13 ± 1.65 d
	1	0.5	91.67 ± 3.51 b	7.57 ± 1.70 b
	1.5	1	79.00 ± 6.08 c	6.27 ± 1.82 c
2	0.5	0.1	83.66 ± 2.08 c	7.36 ± 1.89 b
	1	0.5	100.00 a	10.50 ± 1.65 a
	1.5	1	78.67 ± 3.51 c	6.31 ± 1.86 c

These values represent mean ± standard deviation. Duncan's various lowercase letters show significant differences between the data at the 5% level, using the multiple range test at $p < 0.05$.



Figure 5. Effects of different culture formulations on the rooting of *Camptotheca acuminata* shootlets. (A) Medium with activated carbon, bar = 1 cm; (B) Medium without the addition of activated charcoal, bar = 1 cm; (C) Rooted *C. acuminata* adventitious shoots, bar = 2 cm; (D) 0.5 mg/L IBA + 0.1 mg/L NAA + 5 mg/L PG, bar = 1 cm; (E) 1 mg/L IBA + 0.5 mg/L NAA + 5 mg/L PG, bar = 1 cm; (F) 1.5 mg/L IBA + 1 mg/L NAA + 5 mg/L PG, bar = 1 cm; (G) 0.5 mg/L IBA + 0.5 mg/L NAA + 5 mg/L PG + 2 g/L activated charcoal, bar = 1 cm; (H) 1 mg/L IBA + 0.5 mg/L NAA + 5 mg/L PG + 2 g/L AC, bar = 1 cm; (I) 1.5 mg/L IBA + 1.0 mg/L NAA + 5 mg/L PG + 2 g/L AC, bar = 1 cm.

3.8. Analysis of Camptothecin Content

Samples for camptothecin content analysis were collected from the following treatments:

- Treatment 1: Shootlets cultivated in MS medium supplemented with 0.5 mg/L 6-BA, 0.05 mg/L NAA, 0.5 mg/L GA₃, and 0 mg/L tryptophan during the first stage of in vitro camptothecin accumulation.
- Treatment 2: Shootlets cultivated in MS medium supplemented with 0.5 mg/L 6-BA, 0.05 mg/L NAA, 0.5 mg/L GA₃, and 0.005 mg/L tryptophan during the first stage of in vitro camptothecin accumulation.

- Treatment 3: Shootlets cultivated in MS medium supplemented with 0.5 mg/L 6-BA, 0.05 mg/L NAA, 5 mg/L PG, and 20 mg/L CaCl₂ during the second stage of in vitro camptothecin accumulation.
- Treatment 4: Shootlets cultivated in 2/3 MS medium supplemented with 0.5 mg/L 6-BA, 0.05 mg/L NAA, 5 mg/L PG, and 20 mg/L CaCl₂ during the third stage of in vitro camptothecin accumulation.
- Treatment 5: The aerial parts of shootlets cultivated in 1/2 MS medium supplemented with 1 mg/L IBA, 0.5 mg/L NAA, 5 mg/L PG, and 2 g/L AC for the in vitro enrichment of camptothecin in adventitious roots.
- Treatment 6: The root systems of shootlets cultivated in the same 1/2 MS medium formulation as in Treatment 5.
- Treatment 7: Tender leaves collected from mature adult trees.

The camptothecin content in each of these samples was subsequently determined.

The quantification of camptothecin was performed employing liquid chromatography-mass spectrometry (LC-MS). Detailed Detection Protocol:

1. Sample Preprocessing

Fresh tissue samples were lyophilized to obtain dry-weight specimens. An appropriate mass of plant material was accurately weighed and dissolved in methanol (MeOH) to prepare a sample of suitable concentration. The mixture was subjected to ultrasonic extraction for 30 min, followed by centrifugation at 15,000 rpm for 10 min. The supernatant was collected and diluted with a MeOH/H₂O (1:1, *v/v*) mixture prior to liquid chromatography-mass spectrometry (LC-MS) analysis.

2. Standard Solution Preparation

Camptothecin reference standard was precisely weighed and dissolved in dimethyl sulfoxide (DMSO) to obtain a 1 mg/mL stock solution. This stock solution was diluted with a MeOH/H₂O (1:1, *v/v*) mixture to prepare a 1 µg/mL external standard working solution (L6). Serial dilutions were performed as follows:

L5: 500 µL of L6 was mixed with 500 µL of MeOH/H₂O (1:1, *v/v*).

L4: 200 µL of L5 was mixed with 800 µL of MeOH/H₂O (1:1, *v/v*).

L3: 500 µL of L4 was mixed with 500 µL of MeOH/H₂O (1:1, *v/v*).

L2: 200 µL of L3 was mixed with 800 µL of MeOH/H₂O (1:1, *v/v*).

L1: 500 µL of L2 was mixed with 500 µL of MeOH/H₂O (1:1, *v/v*).

The resulting standard solutions (L1–L6) were used for the quantification of camptothecin in plant samples.

3. LC-MS Analysis

Instrument: XEVO-TQS WAA499 mass spectrometer (Waters Technology (Shanghai) Co., Ltd., Shanghai, China)

Chromatographic Column: ACQUITY UPLC BEH C18 (1.7 µm particle size, 2.1 mm × 50 mm) (Waters Technology (Shanghai) Co., Ltd., Shanghai, China)

Mobile Phases:

Phase A: 0.1% formic acid (FA) in water (H₂O)

Phase B: 0.1% FA in acetonitrile (ACN)

Flow Rate: 0.5 mL/min

Injection Volume: 0.5 µL

Mass Spectrometry Conditions: Electrospray ionization positive mode (ES+).

The analytical results, presented in Table 8 and Figure 6, reveal a marked upward trend in camptothecin content within *C. acuminata* aseptic seedlings following in vitro accumulation culture. Notably, the camptothecin extracted from tissue-cultured *C. acuminata* shoots exhibited no compositional discrepancies compared to that obtained from

natural plant materials, as confirmed by chromatographic analysis (Figure 6). In media supplemented with tryptophan, the mean camptothecin content in aseptic shootlets increased by 39.20 $\mu\text{g/g}$ dry weight (DW). However, at this stage, the camptothecin content in in vitro-grown shootlets was still lower than that in tender leaves from natural materials. Compared with the first stage of camptothecin accumulation, the addition of appropriate concentrations of phloroglucinol and calcium chloride during the second stage significantly promoted camptothecin accumulation, resulting in a 2.6-fold increase in camptothecin content. A moderate reduction in the nitrogen concentration in the culture medium was conducive to both biomass expansion and camptothecin accumulation in aseptic shootlets. Compared with the second stage of camptothecin accumulation, a 1.8-fold increase in camptothecin content was achieved in the third stage after reducing the nitrogen content in the medium. Adventitious shoots of *C. acuminata* also continued to accumulate camptothecin during the rooting stage. The camptothecin content in the aerial parts of shootlets reached up to 795.10 $\mu\text{g/g}$ DW, which was five times that in tender leaves from natural materials. Moreover, the camptothecin content in the adventitious roots was only marginally lower than that in tender leaves from natural materials.

Table 8. Camptothecin content.

Sample	Camptothecin Content ($\mu\text{g/g}$ DW)	Camptothecin Content (%)
Treatment 1	109.00 $\pm$ 3.77 g	0.0109 $\pm$ 0.0004 g
Treatment 2	145.20 $\pm$ 9.28 e	0.0145 $\pm$ 0.0009 e
Treatment 3	375.60 $\pm$ 8.68 c	0.0376 $\pm$ 0.0009 c
Treatment 4	677.60 $\pm$ 7.96 b	0.0677 $\pm$ 0.0008 b
Treatment 5	795.10 $\pm$ 5.02 a	0.0795 $\pm$ 0.0005 a
Treatment 6	125.00 $\pm$ 5.14 f	0.0125 $\pm$ 0.0005 f
Treatment 7	159.40 $\pm$ 5.44 d	0.0159 $\pm$ 0.0002 d

Note. Ten biological replicates were analyzed per treatment group, with data presented as mean values. Letter (a–g) denote statistically significant differences ($p < 0.05$) between groups within each measured parameter.

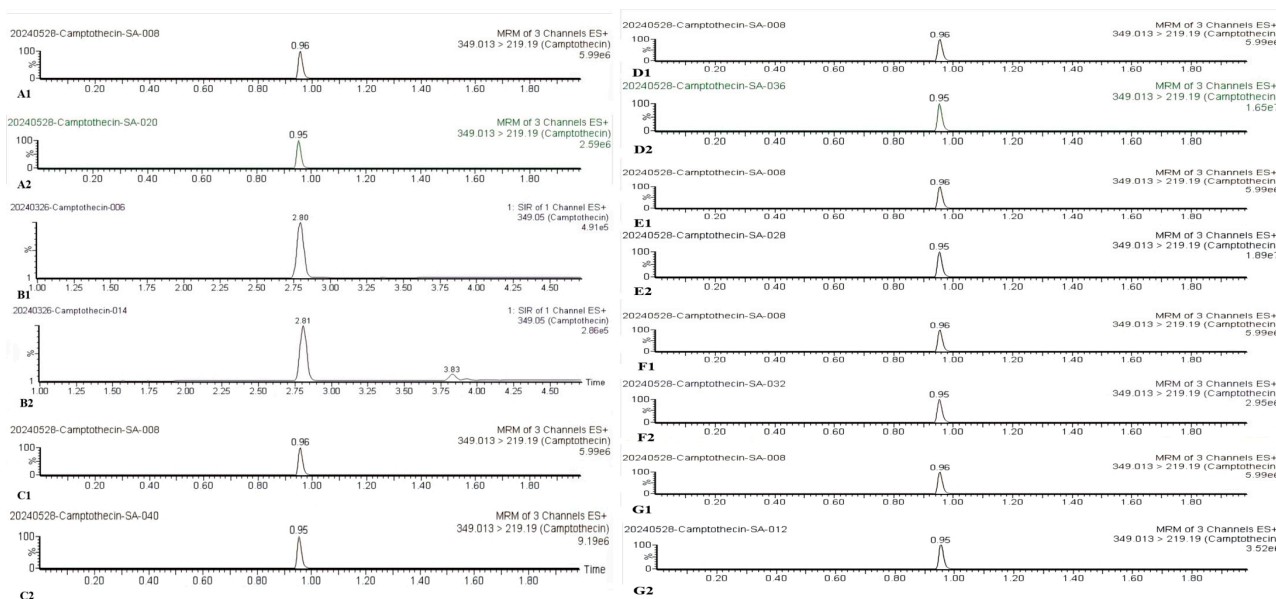


Figure 6. Test spectrum of camptothecin sample. (A1,A2) processing 1; (B1,B2) processing 2; (C1,C2) processing 3; (D1,D2) processing 4; (E1,E2) processing 5; (F1,F2) processing 6; (G1,G2) processing 7.

4. Discussion

The wild resources of *C. acuminata* in China are quite limited, and the extracted camptothecin is insufficient to meet the current demand [4]. To achieve rational utilization, development, and conservation of *C. acuminata* resources, tissue culture technology provides an effective approach for the rapid propagation of *C. acuminata* seedlings. This technology enables the accumulation of camptothecin throughout the entire plant under in vitro conditions, thereby offering a viable solution to the problems of limited resources and insufficient medicinal sources of *C. acuminata*.

During the process of plant tissue culture, PGRs represent one of the critical factors influencing morphogenesis. Research has demonstrated that signaling molecules derived from plant hormones, such as 6-benzylaminopurine (6-BA), salicylic acid (SA), indole-3-butyric acid (IBA), naphthaleneacetic acid (NAA), indole-3-acetic acid (IAA), and abscisic acid (ABA), can effectively regulate the biosynthesis of camptothecin in *C. acuminata* [15–17]. Liu revealed that exogenous SA treatment of *C. acuminata* tissue-cultured seedlings could promote the expression of relevant genes and enhance camptothecin synthesis [16]. The research conducted by Lü Y and others revealed that a low concentration of exogenous 6-BA was beneficial for the growth of *C. acuminata* tissue-cultured seedlings [18]. It promotes camptothecin synthesis by enhancing the expression of key enzyme genes. When 0.1 mg/L of 6-BA was applied to the culture medium, the camptothecin content in the callus tissue of *C. acuminata* reached 0.75 µg/mg fresh weight (FW) [18]. The study by Yang et al. showed that 6-BA can induce the transcription of RESPONSE REGULATOR 1 (*StRR1*) at wounds of potato tubers, and overexpression of *StRR1* can induce *NbPAL*, *Nb4CL*, and *NbCAD10*, thereby promoting the accumulation of phenolic acids and lignin monomers [19]. Recent studies have indicated that gibberellic acid (GA) and ABA can modulate primary and secondary metabolism in grapevines, thereby alleviating biotic and abiotic stresses [20]. In *Ophiorrhiza pumila* hairy roots, the transcription factor *OpWRKY3* responds to GA signals to regulate camptothecin biosynthesis [17]. Research by Huo et al. showed that under the stimulation of GA and methyl jasmonate (MeJA), the expression levels of *CaTDC1*, *CaSTR*, *Ca7DLGT*, and *CaG8O*, which are involved in the camptothecin synthesis pathway in *C. acuminata* suspension cells, were increased [21]. Although numerous studies have confirmed that plant hormones (including MeJA, ABA, GA, and SA) can regulate camptothecin biosynthesis, the specific mechanisms underlying this regulation remain unclear.

The biosynthesis pathway of camptothecin can be divided into the upstream and downstream pathways. Currently, the specific synthesis processes in the downstream pathway remain unclear. The upstream biosynthesis of camptothecin involves three main pathways: the shikimate pathway, the mevalonate (MVA) pathway, and the methylerythritol phosphate (MEP) pathway. During the synthesis of camptothecin, strictosidine synthase (STR), tryptophan synthase (TSB), and tryptophan decarboxylase (TDC) in the shikimate pathway are key enzymes. Specifically, TDC catalyzes the conversion of tryptophan into tryptamine, which is essential for forming the indole ring in camptothecin and its derivatives. Tryptamine is derived from tryptophan, which is synthesized from serine and subsequently decarboxylated, thereby providing the necessary precursor amino acid for camptothecin synthesis [22,23]. Some studies have demonstrated that adding a certain amount of the camptothecin precursor, tryptophan, to the B5 medium can effectively increase the camptothecin content in cultured cells of *C. acuminata*, with the camptothecin content reaching 0.115 g/kg FW [24]. Our findings demonstrate that *C. acuminata* adventitious shoots cultured in medium containing 0.005 mg/L tryptophan accumulated significantly higher camptothecin levels than non-supplemented controls. These findings are supported by previous experimental evidence.

Calcium (Ca), an essential micronutrient among the 17 vital elements for plant physiology, serves critical biological functions. In response to abiotic stresses, calcium mitigates the detrimental effects of low temperature, high temperature, drought, high salinity, and pests and diseases by activating protective enzyme systems and improving photosynthesis [25]. Calcium facilitates the conversion of carbohydrates and the absorption and metabolism of other nutrients, thus promoting plant growth and development. Both calcium deficiency and excess can adversely affect plant growth [26]. Prolonged calcium deficiency or excess can inhibit plant growth [27]. Calcium, while essential for basic plant physiology, also exerts considerable influence on secondary metabolite accumulation. Research has established that optimal calcium ion concentrations enhance flavonoid biosynthesis across multiple medicinal species, including *Glycyrrhiza uralensis* (licorice) seedlings [28], *Silybum marianum* (milk thistle) cell cultures [29], *Atractylodes lancea rhizomes* [30], and *G. uralensis* [31]. It can also increase the alkaloid content in medicinal plants including *Corydalis yanhusuo* W. T. Wang (corydalis), *Sophora tonkinensis* Gagnep. (sophora root), *Fritillaria thunbergii* Miq. (Zhejiang fritillary bulb), and *Pinellia ternata* (Thunb.) Ten. ex Breitenb. (pinellia tuber) [32–35]. Calcium chloride (25 mmol/L) functioned as an effective elicitor, boosting camptothecin biosynthesis in *N. nimmoniana calli* [36]. During Stage II of in vitro camptothecin accumulation, culture media were supplemented with optimized CaCl₂ concentrations. It was observed that the camptothecin content in *C. acuminata* test-tube seedlings significantly increased after the addition of CaCl₂, the observed effects are congruent with previous reports.

Previous studies have demonstrated that phenolic compounds can react with indole-3-acetic acid (IAA) in the presence of polyphenol oxidase to form an “IAA-phenolic acid complex”. This complex can serve as an auxiliary factor for rooting and promotes the initiation of adventitious roots [37,38]. Additionally, phenolic substances can influence the rooting rate of cuttings by affecting the content or activity of certain enzymes, such as polyphenol oxidase (PPO) and peroxidase (POD) [39,40]. Research conducted by Balakrishnamurthy et al. and Hatmanr revealed that high concentrations of phenolic compounds accumulated in plant shoots can facilitate the formation of rooting-promoting substances [41,42]. Phloroglucinol supplementation was initiated during Stage II of in vitro camptothecin accumulation, with concentration optimized through preliminary trials. It was observed that phloroglucinol, in interaction with auxins, promoted the initiation of adventitious roots in *C. acuminata*.

Nitrogen (N) is one of the most essential mineral nutrients required by plants in large quantities, playing a pivotal role in promoting biomass accumulation [43,44]. The critical role of nitrogen in regulating the production and deposition of secondary metabolites in plants has been well-documented in multiple investigations. Alkaloids are basic organic compounds found in nature, distributed across approximately 20% of plant species. Most alkaloids are derived from amino acids or adenosine monophosphate [45]. Among the most clinically representative plant alkaloids are CPT, taxol, berberine, L-ephedrine, morphine (a pain-relieving drug), and codeine (an opium alkaloid) [46,47]. Research has indicated that appropriately increasing the application rates of nitrogen [48–52], phosphorus [53,54], or potassium can promote the growth of tissue-cultured hairy roots and the accumulation of their secondary metabolites, alkaloids [55]. Evidence indicates that the interactive effects of N, P and K nutrients significantly enhance biomass production and alkaloid content in medicinal plants [56]. Camptothecin is an alkaloid derived from amino acids. Nitrogen is a crucial element for amino acid synthesis and also constitutes part of the molecular structure of camptothecin [57]. Research on *C. acuminata* reveals that leaf camptothecin levels exhibit a biphasic response to nitrogen fertilization, rising initially before declining at higher concentrations. Low nitrogen levels are conducive to promoting the synthesis

and accumulation of camptothecin [58–60]. Pan et al. conducted a study on the cultivation of *C. acuminata* suspension cells and found that the maximum camptothecin yield, reaching 0.36 mg/g, was achieved when the total nitrogen content in the culture medium was 40 mM [61]. During the final accumulation phase, 2/3-strength MS medium supported optimal shoot growth, as evidenced by biomass measurements. Quantitative analysis revealed maximal camptothecin content in axenic shoots grown in 1/2-strength MS medium, aligning with established literature.

With the ever-increasing incidence of cancer, the demand for camptothecin-based anticancer drugs has been rising annually, and the clinical supply has emerged as a critical issue that demands urgent resolution. Leveraging modern biotechnology to enhance CPT production holds immense application prospects. In this study, through tissue culture techniques, we achieved, for the first time, whole-plant enrichment of CPT under in vitro conditions. The produced camptothecin exhibits no compositional differences from natural CPT, and there are no toxic or harmful by-products. Moreover, this production method is not constrained by seasonal limitations, enabling the year-round provision of *C. acuminata* germplasm and CPT drug sources. Subsequently, we will further optimize the established system, conduct molecular mechanistic studies on key regulatory genes involved in camptothecin biosynthesis, perform a comprehensive life cycle cost analysis, and proceed with pilot-scale production to advance toward industrial-scale manufacturing.

5. Conclusions

Taken together, this study presents an efficient in vitro regeneration and CPT accumulation framework utilizing *C. acuminata* stem explants (Figure 7). The system we developed facilitates the quantitative production of CPT-rich adventitious shoots of *C. acuminata*, promoting vigorous development of its medicinal organs and shortening the growth cycle. Moreover, the harvesting of raw materials is not constrained by seasonal factors, allowing for the continuous supply of CPT-enriched pharmaceutical base materials with stable properties and consistent quality to the market, thereby addressing the issue of CPT raw material sources. Optimal axillary shoot germination in *C. acuminata* necessitates supplementation with elevated concentrations of 6-BA and NAA. The induction efficiency of adventitious shoots is significantly enhanced with judicious reduction in 6-BA and NAA levels. An adequate amount of GA₃ can promote the high-frequency synchronized emergence of adventitious shoots in *C. acuminata*. The inclusion of activated charcoal in the culture medium simplifies to induce rooting of adventitious shoots. Appropriate concentrations of tryptophan, PG, and CaCl₂ can facilitate the accumulation of CPT in axenic seedlings of *C. acuminata*, and a similar promoting effect can be achieved by appropriately decreasing the nitrogen concentration in the culture medium. After undergoing in vitro CPT enrichment from Stage I to Stage IV, the CPT content in the aerial parts of the adventitious shoots of *C. acuminata* reached up to 795.10 µg/g DW, which is five times the CPT content in the tender leaves of natural plant materials.

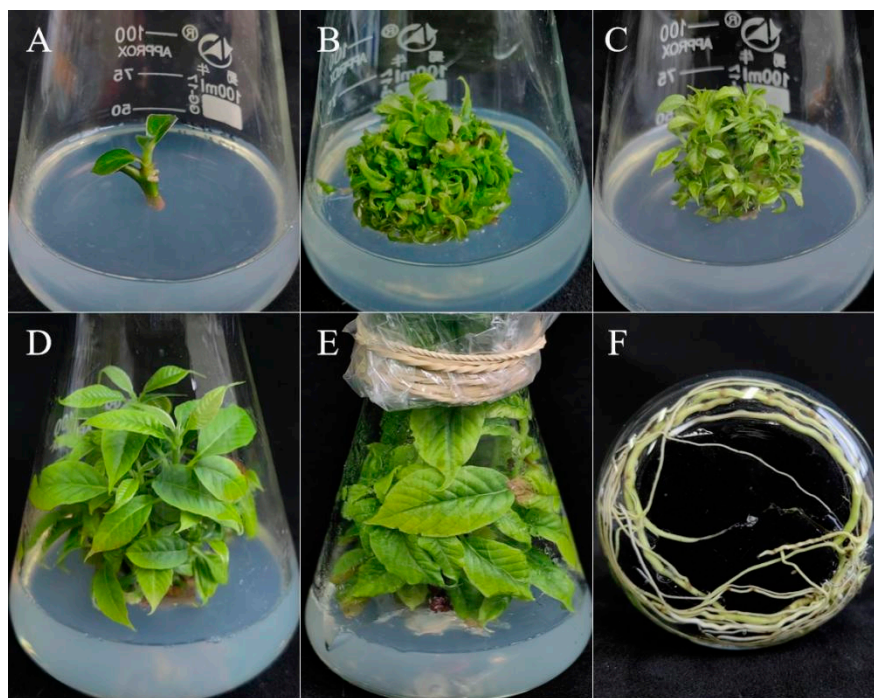


Figure 7. In vitro camptothecin accumulation system in *Camptotheca acuminata*. (A) Germination of axillary shoots; (B) Induction of adventitious shoots; (C) High-frequency synchronized occurrence of adventitious shoots and Stage I of in vitro CPT accumulation; (D) Elongation of adventitious shoots and Stage II of in vitro CPT accumulation; (E) Biomass expansion of *C. acuminata* and Stage III of in vitro CPT accumulation; (F) Induction of adventitious roots and in vitro CPT accumulation in adventitious roots.

Author Contributions: Responsible for experimental execution, data analysis, and drafting the initial manuscript, H.Z.; collaborated on data analysis, K.Z. and Y.X.; oversaw manuscript review and finalization of the publication-ready version, W.Z. (Wenqing Zhang) and W.Z. (Weizhong Zhang); led experimental design, manuscript revision, and approval of the final publication-ready draft, M.Z. All authors have read and agreed to the published version of the manuscript.

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