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Clonal propagation of *Camptotheca acuminata* through shoot bud culture

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Abstract

The chinese tree *Camptotheca acuminata* produces the anti-cancer and anti-retroviral drug camptothecin. Methods were developed for the clonal propagation of this important medicinal plant through shoot bud culture. Shoot buds were excised from 25 to 30 day old seedlings, presoaked for 48 h in three different liquid media containing either BA (2.22–17.4 μM), kinetin (2.32–18.58 μM), or thidiazuron (0.1–10 μM) and were subsequently cultured on semi-solid medium of the same composition. Multiple shoots only developed from the 6-benzyladenine presoaked explants with the maximum number of shoots initiated from buds presoaked in and grown on B5 medium containing 17.4 μM 6-benzyladenine. Individual shoots were removed from clusters and rooted on B5 supplemented with indole-3-butyric acid (4.9–19.6 μM). The lowest concentration of indole-3-butyric acid (4.9 μM) gave the highest percentage of rooting (82%) and the shortest root initiation period (18 d). Over 90% of the *in vitro* rooted plantlets survived transfer to soil.

Abbreviations: BA – 6-benzyladenine; B5 – Gamborg's B5 medium (Gamborg *et al.*, 1968); CPT – camptothecin; 2,4-D – 2,4-dichlorophenoxyacetic acid; IBA – indole-3-butyric acid; kinetin – 6-furfurylaminopurine; LS – Linsmaier & Skoog medium (Linsmaier & Skoog, 1965); MS – Murashige & Skoog (Murashige & Skoog, 1962); NAA – 1-naphthaleneacetic acid; PGR – plant growth regulator; TDZ – thidiazuron; WPM – woody plant medium (Lloyd & McCown, 1981)

Introduction

Camptotheca acuminata Decaisne (Nyssaceae) is a species of tree that is native to China and produces CPT, an anticancer indole alkaloid that was first identified by Wall *et al.* (1966). The basis of CPT's antitumor activity is its ability to inhibit DNA topoisomerase I (Kjeldsen *et al.* 1990; 1992), an enzyme involved in relaxing supercoiled DNA.

CPT inhibits the replication of the human immunodeficiency virus (Priel *et al.* 1991a) and another retrovirus, equine infectious anemia virus (Priel *et al.* 1991b) in cultured cells. CPT also inhibits the induction of diseases in mice by two additional retroviruses, Moloney murine leukemia viruses and Friend spleen focus-forming virus (Priel *et al.* 1993). The anti-retroviral activity of CPT may be related to its recently demonstrated ability to inhibit Tat-mediated transcription from the viral promoter (Li *et al.* 1993; 1994). Because of the potential clinical uses of CPT, it is important to investigate methods of increasing CPT yield so that the availability of this compound does not become limiting, as recently occurred with taxol, another plant-derived anticancer drug (Cragg *et al.* 1993).

C. acuminata trees are commonly raised from seed (Perdue *et al.* 1970), but little is known about their clonal propagation, and to our knowledge, there are no reports on the propagation of this species by cuttings or grafting. Hence, there may be an opportunity for applying tissue culture approaches in the rapid clonal propagation of this species and in the *in vitro* conservation of elite genotypes. The objective of this study was to develop methods for the clonal propagation of juvenile *C. acuminata* through *in vitro* shoot proliferation.

Materials and methods

The leathery pericarp covering each *C. acuminata* seed was removed followed by washing in 5% Triton X-100 for 3 min. The seeds were repeatedly rinsed with sterile water, until free of foam. They were immersed in 70% ethanol for 1 min and transferred to 1% NaOCl (without any wetting agent) for 3 min. After 8–10 rinses with sterile water the seeds were transferred to PGR-free half-strength MS (Murashige & Skoog 1962) solidified with 0.35% Phytagel (Sigma Chemical, St. Louis, MO) and grown in continuous darkness for 1 week at 25 °C. Seedlings were transferred to a growth chamber with a 16-h photoperiod provided by cool white fluorescent light (40 μmol m^{-2} s^{-1}). Shoot tip explants (6–8 mm) were excised from 25–30 day old seedlings.

Three different basal media, LS (Linsmaier & Skoog, 1965); B5 (Gamborg *et al.*, 1968) and WPM (Lloyd & McCown, 1981) were used to standardize the basic nutrient requirements for *C. acuminata* shoot tip cultures. The media were supplemented with cytokinins to examine their effects on growth and development. Each medium contained 4% sucrose, and was adjusted to pH 5.8 prior to the addition of 0.35% Phytagel and autoclaving (at 121 °C and 103 kPa for 20 min, liquid cycle). All cultures were incubated under a 16-h photoperiod (cool white fluorescent lamps, 40 μmol m^{-2} s^{-1}) at 25 ±2 °C, throughout the study unless otherwise indicated.

To induce multiple shoots, 6–8 mm shoot tips were excised and soaked in different basal media containing 2.2, 4.4, 8.9, 17.7, or 35.5 μM BA or 2.3, 4.7, 9.3 or 18.6 μM kinetin or 0.1, 1, 2.5, 5, or 10 μM TDZ for 24, 48, or 72 h in dark on a low speed (125 rpm) orbital shaker. After presoaking, the explants were cultured on semi-solid medium containing the same kind and concentration of cytokinin. Six to eight explants were placed in petri dishes (100×15 mm) containing 30 ml of media and sealed with parafilm. Forty replicates were used for each treatment and the entire experiment was performed in duplicate. After six weeks in culture the number of buds giving rise to multiple shoots was recorded for each treatment. Ten shoots per treatment were chosen at random from these cultures and the number of shoots per explant recorded.

To induce rooting, individual shoots (15–20 mm) were excised and cultured on B5basal medium supplemented with 4.9, 9.8, 14.7 or 19.6 μM IBA. Four individual shoots were placed in Magenta boxes (110 mm, Sigma Chemical, St. Louis, MO) containing 65 ml of media and closures were sealed with parafilm. All cultures were maintained under the same conditions described above. Twenty-four shoots were used for each treatment and the experiment was performed in duplicate. Visual observations of the cultures were taken weekly and the effect of the treatment was quantified on the basis of the root initiation period, percentage of shoots with roots and average number of roots per shoot after 6 weeks.

Thirty well-rooted plantlets (80–90 mm long) from the 4.9 μM IBA treatment were removed from Magenta boxes, washed in running tap water, and transferred to 15 cm top diameter pots containing a mixture of 4 potting soil: 2 vermiculite: 1 perlite (by volume). A 250 ml glass beaker was inverted over each plant to maintain high relative humidity following transplantation. The rooted shoots were maintained in a growth chamber under 16-h photoperiod (cool white fluorescent lights, 60 μmol m^{-2} s^{-1}) at 25 ±2 °C. The beakers were removed after one week and the survival of the plantlets was recorded 3 weeks later.

Table 1. Response of *Camptotheca acuminata* freshly excised shoot buds to different basal media and cytokinins[1]

Cytokinin (μM)	% Explants with shoot growth[2]		
	B5	LS	WPM
BA (2.2)	82 [0]	15 [67]	4 [85]
BA (4.4)	86 [0]	16 [68]	7 [79]
Kinetin (2.3)	70 [0]	6 [81]	0 [67]
Kinetin (4.7)	76 [0]	11 [71]	0 [76]

[1]Untreated shoot buds were placed directly on cytokinin supplemented basal media solidified with Phytagel without presoaking and examined after 5 weeks.
[2]30 replicates/treatment. Numbers in brackets are the percent of shoots that developed callus at the explant base.

Results and discussion

Three basal media (B5, LS and WPM) were examined for their ability to support the growth of freshly excised shoot buds of *C. acuminata*. Preliminary experiments indicated that exogenous cytokinin was required for the growth of *C. acuminata* shoot buds *in vitro* (data not shown). Each of the media tested was therefore supplemented with BA or kinetin. The best shoot growth was observed in buds cultured on cytokinin supplemented

Table 2. Response of *Camptotheca acuminata* shoot buds to a 48 h presoaking and growth on different basal media and cytokinins[1]

Cytokinin (μM)	% Explants producing multiple shoots[2]		
	B5	LS	WPM
BA (2.2)	0	0	0^C
BA (4.4)	0	0	0^C
BA (8.9)	85 [6.5±1.1]	67.5 [4.8±0.7]	52.5 [2.9±0.7]
BA (17.7)	100 [14.2±2.4]	47.5 [9.8±1.5]	20 [2.4±0.5]
BA (35.5)	45 [4.4±1.1]	15 [3.9±0.7]	0
Kinetin (2.3)	0	0	0
Kinetin (4.7)	0	0	0
Kinetin (9.3)	0	0	0
Kinetin (18.6)	0	0	0
TDZ (0.1)	0	0	0^C
TDZ (1.0)	0^C	0^C	0^N
TDZ (2.5)	0^C	0^C	0^N
TDZ (5.0)	0^N	0^N	0^N
TDZ (10.0)	0^N	0^N	0^N

[1] After soaking, the explants were grown for 6 weeks on the same cytokinin and basal media solidified with Phytagel.
[2] Average of 2 experiments with 40 replicates/treatment. Numbers in brackets are the mean number of shoots formed from ten buds for each treatment. ± = standard deviation.
0^C Loose callus formed at the base of the explant.
0^N Nodular callus formed at the base of the explant.

B5 medium (Table 1). Buds grown on B5 medium produced single, dark-green, fast-growing shoots, whereas buds cultured on LS and WPM media produced single slow-growing shoots with callus at the base of the explants. These results suggest that the higher ratio of NO_3^-/NH_4^+ in B5 compared with LS and WPM may favor more vigorous growth of cultured *C. acuminata* shoot buds, although other differences in the media cannot be excluded as contributing to this response.

As noted above, excised buds transferred directly to semi-solid culture media without pretreatment produced only one shoot per explant. Presoaking of freshly excised buds in cytokinin prior to placement on semi-solid media was therefore examined as a possible method for inducing multiple shoot development in *C. acuminata*. Cytokinin presoaking has been shown to be effective for shoot multiplication in sesame (George *et al.*, 1987) and mulberry (Jain & Datta, 1992). Preliminary tests indicated that the presoaking period and concentration of cytokinin affected the frequency of shoot bud induction in *C. acuminata*. A minimum of 48 hours presoaking was found to be necessary to induce multiple shoots, whereas a longer presoaking period seemed toxic (data not shown).

The morphogenetic response of cultured shoot buds presoaked in cytokinin for 48 h is shown in Table 2. The best response for multiple shoot formation was observed when shoot tips were presoaked in B5 medium containing 17.74 μM BA for 48 h and then cultured on the same semi-solid medium. Following this treatment, each bud gave rise to 11–18 shoots (Fig. 1).

Presoaking of freshly excised buds with BA was an effective approach for inducing multiple shoots in *C. acuminata* regardless of the basal medium used, although shoot proliferation was more rapid on B5 medium (28–30 d) compared to LS medium (32–34 d) and WPM (37–40 d). The multiple shoots grown on B5 medium also appeared greener and healthier than on the other basal media.

Among the cytokinins tested, only BA effectively induced multiple shoots. Kinetin soaked buds produced only single shoots and TDZ treatment had a negative effect by inhibiting shoot growth and promoting callus formation at the base of the explant. These

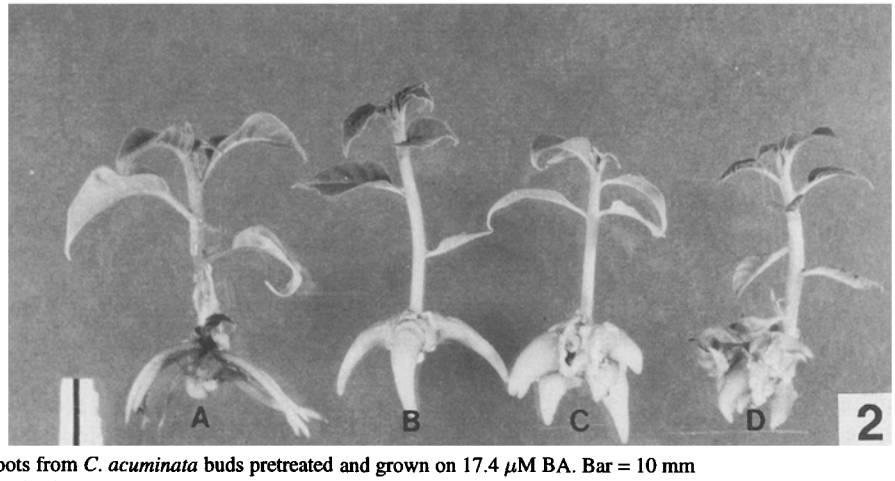

Fig. 1. Multiple shoots from *C. acuminata* buds pretreated and grown on 17.4 μM BA. Bar = 10 mm
Fig.2 Root formation in *C. acuminata* shoots in response to IBA treatment. *A* - 4.9 μM IBA; *B* - 9.8 μM IBA; *C* - 14.7 μM IBA; *D* - 19.6 μM IBA. Bar = 20 mm

results were somewhat unexpected because TDZ is known for its ability to stimulate shoot proliferation in a number of woody species (Huetteman & Preece, 1993).

To induce rooting, individual shoots were excised from the clusters and transferred to B5 medium supplemented with IBA (Table 3). Normal roots were formed in the presence of low levels of IBA (4.9 μM), whereas higher levels of IBA produced callus at the base of the shoot and formed thick, stunted roots (Fig. 2).

Although some plant species do not survive transfer from tissue culture medium to soil, transplantion of rooted *C. acuminata* was readily achieved by maintaining high humidity around the plantlets during their first week in pots. Under these conditions over 90% (28/30) of the plantlets survived and resumed normal growth.

Clonal selection and propagation of tree species by tissue and organ culture techniques has considerable potential for large scale multiplication of economically important plants. The present study provides methods for the mass propagation of a medicinally important tree *Camptotheca acuminata* through shoot bud culture. Genetically identical juvenile stocks generated by

Table 3. Rooting response of excised *Camptotheca acuminata* shoots to IBA treatment.

IBA Conc. (μM)	Time of root intiation (days)	Percent of shoots with roots[a]	Number of roots/shoot[b]
4.9	18	82	7±0.7
9.8	22	61	5±0.5
14.7	28	34	4±1.0
19.6	31	26	3±0.7

[a] Each treatment included 24 shoots with two replicates.
[b] ± standard deviation.

shoot bud multiplication can provide uniform starting materials for physiological and cellular studies aimed at increasing CPT yield.

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