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BRIEF COMMUNICATION

Establishment of *Camptotheca acuminata* regeneration from leaf explants

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Abstract

Plantlet regeneration through shoot formation from young leaf explant-derived callus of *Camptotheca acuminata* is described. Calli were obtained by placing leaf explants on Woody plant medium (WPM) supplemented with various concentrations of 6-benzyladenine (BA) and naphthaleneacetic acid (NAA) or 2,4-dichlorophenoxyacetic acid (2,4-D). Callus induction was observed in all media evaluated. On the shoot induction medium, the callus induced on the WPM medium containing 19.8 μM BA and 5.8 μM NAA was the most effective, providing high shoot regeneration frequency (70.3 %) as well as the highest number of shoots (11.2 shoots explant^{-1}). The good rooting percentage and root quality (98 %, 5.9 roots shoot^{-1}) were achieved on WPM medium supplemented with 9.6 μM indole-3-butyric acid (IBA). 96 % of the *in vitro* rooted plantlets with well developed shoots and roots survived transfer to soil.

Additional key words: callus, *ex vitro* transfer, phytohormones, rooting, shoot regeneration.

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Camptotheca acuminata Decaisne (*Nyssaceae*) is an endangered tree species in south China. Camptothecin (CPT) was isolated and identified from *C. acuminata* by Wall *et al*. (1966) and demonstrated to kill cancer cells (Wall 1998). However, the content of CPT in raw material is very low (Zu *et al*. 2003), so it is highly desirable to develop clonal lines with high CPT-synthesis capabilities for planting (Liu and Li 2001). Conventional breeding methods cannot solve this problem in short time because the life cycle of this tree is long. However, the improvement CPT production by *Agrobacterium*-mediated genetic transformation will be very attractive which may facilitate the rapid transfer of valuable genes to the genome of well-established *C. acuminata* cultivars. Adventitious regeneration is the key step for the successful application of this gene transformation system (Pérez-Tornero *et al*. 2000). Though *in vitro* shoot regeneration of *C. acuminata* from shoot buds and axillary buds have been established before (Jain and Nessler 1996, Liu and Li 2001), but these materials are not suitable for *Agrobacterium*-mediated genetic transformation. While using leaves as explants is an effective and easy protocol in *Agrobacterium*-mediated transformation system (Horsch *et al*. 1985). To our knowledge, there are no reports of regeneration using leaf tissue as an explant source for *C. acuminata*. In this study, we attempted to develop an efficient adventitious regeneration protocol from leaf explants as a necessary first step in development of a genetic transformation of *C. acuminata* for alkaloids production.

Young and not fully expanded leaves of *C. acuminata* were collected from plants grown in the greenhouse at the Northeast Forestry University, China. The leaves were sterilized with 70 % ethanol for 30 s, 5 % (v/v) $NaClO_3$ for 5 min, and subsequently washed three times with sterile water. For callus induction, leaf sections of 1 × 1 cm were placed on Woody plant medium (WPM) supplemented with different combinations of 6-benzyladenine (BA, 9.9 - 29.7 μM) with naphthaleneacetic acid (NAA, 2.9 - 11.6 μM) or 2,4-dichlorophenoxyacetic acid (2,4-D, 2.0 - 4.0 μM), containing 30 g dm^{-3} sucrose, 8 g dm^{-3} agar. After 4 weeks of culture, the percentage of explants producing callus was recorded. For shoot regeneration, the calli were transferred to shoot inducing medium, WPM medium supplemented with 9.9 μM BA, 30 g dm^{-3} sucrose and 8 g dm^{-3} agar. The callus

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Abbreviations: BA - 6-benzyladenine; IBA - indole-3-butyric acid; NAA - naphthaleneacetic acid; 2,4-D - 2,4-dichlorophenoxyacetic acid; WPM - Woody plant medium; CPT - camptothecin.

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regeneration frequency and the number of shoots per callus was recorded every 3 d, respectively. For root induction, isolated shoots were transferred to WPM medium supplemented with IBA (2.4 µM, 4.8 µM, 9.6 µM) or NAA (2.9 µM, 5.8 µM, 11.6 µM), respectively, containing 20 g dm^{-3} sucrose, 8 g dm^{-3} agar. The frequency of rooting and average root number per shoot were recorded. The pH of all the media was adjusted to 5.8 prior to autoclaving (at 121 °C, for 20 min). All cultures were incubated at 25 ± 2 °C, under a 16-h photoperiod with irradiance of 40 µmol m^{-2} s^{-1} provided by white fluorescent tubes. Four weeks after culture on rooting medium, root length was measured by taking out the rooted plantlets carefully from the media and plantlets with vigorously growing roots were transferred to soil and acclimated in the greenhouse. Each treatment consisted of 20 explants and was done in triplicate. Means and standard errors were used for statistical analysis.

The leaf explant began to enlarge after 7 d of culture on the callus induction medium. In the following two weeks, explants gradually formed callus at the cutting surface. Callus was induced in all media tested (Table 1). In general, different auxin have similar effects, however, some tissue do show specific qualitative differences (Minocha 1987, Haliloglu 2006). In the present study, 2,4-D was more effective for callus induction than NAA, which enhanced callus induction and growth. This was agreement with Wu's opinion, who reported that 2,4-D is usually more efficient than other phytohormones in the induction of callus from plant explants (Wu *et al*. 2003). In the media containing 2,4-D, the callus appeared within 14 d and callus induction frequencies were 88.5 to 97.1 %. Whereas the explants on the media containing NAA produced callus after 18 d of culture and callus induction frequencies were 70.3 % to 91.6 %. Furthermore, we noticed that the calli induced on the media containing BA and NAA were green and compact (Fig. 1*A*). while the calli induced on the media supplemented with BA and 2,4-D were white and friable (Fig. 1*B*). After 4 weeks of culture on the callus induction medium, the calli were transferred to shoot induction medium for shoot regeneration. Shooting efficiency from the calli induced by BA and 2,4-D was very low or to zero, the better results were got from the calli originally induced on the media containing BA and NAA (Table 1). In all the treatments, the highest shooting frequency, 70.3 % as well as the highest number of shoot formation, 11.2 shoots $explant^{-1}$ was achieved on the callus induced on the medium containing 19.8 µM BA and 5.8 µM NAA. The callus induced by this medium began to produce multiple shoot primordia after two weeks of culture on shoot regeneration medium (Fig. 1*C*), which developed into adventitious shoots in the following two weeks (Fig. 1*D*).

Auxins are involved in the process of adventitious root formation, but these phytohormones affect *in vitro* rooting of various species differently (Pawlicki and Welander 1995, Abdulaziz and Bahrany 2002, Agrawal and Sardar 2006). In this study, the auxins IBA and NAA produced different effects (Table 2). The best rooting quality and percentage were achieved on the medium supplemented with 9.6 µM IBA. Root frequency reached 98 % with the higher number of roots, 5.9 roots per shoot (Fig. 1*E*). In the medium containing IBA, average rooting number increased from 2.7 to 5.9 with the increasing of IBA concentration from 2.4 µM to 9.6 µM. But further increase of IBA (15.4 µM) inhibited rooting and root growth. In the medium containing NAA, the average root number decreased from 5.4 to 3.5 when NAA concentration increased from 2.9 µM to 11.6 µM. Moreover, higher concentration of NAA (> 5.8 µM) induced thick and stunted roots. After four weeks

Table 1. The effect of phytohormones on callus induction and shoot regeneration from leaf explants of *C. acuminata*. The number of shoots per callus was recorded after 4 weeks of culture on shoot induction medium. Means ± SE, n = 60.

2,4-D [µM]	NAA [µM]	BA [µM]	Explants producing callus [%]	Callus producing shoots [%]	Number of shoots [$callus^{-1}$]
0	2.9	9.9	70.3 ± 0.53	10.1 ± 0.97	1.2 ± 0.88
0	2.9	19.8	81.2 ± 0.89	57.4 ± 0.86	8.2 ± 0.40
0	2.9	29.7	83.5 ± 0.23	39.2 ± 1.23	7.8 ± 1.80
0	5.8	9.9	83.5 ± 0.31	22.4 ± 0.37	8.3 ± 0.56
0	5.8	19.8	87.8 ± 0.25	70.3 ± 0.48	11.2 ± 0.30
0	5.8	29.7	90.2 ± 0.53	60.7 ± 0.73	7.6 ± 0.26
0	11.6	9.9	85.7 ± 0.93	17.8 ± 0.35	2.2 ± 0.55
0	11.6	19.8	91.6 ± 0.34	49.4 ± 1.80	6.2 ± 0.53
0	11.6	29.7	89.3 ± 0.57	43.3 ± 1.30	4.9 ± 2.00
2.0	0	9.9	95.4 ± 0.32	0.3 ± 0.21	0.2 ± 0.13
2.0	0	19.8	97.1 ± 0.29	0.2 ± 0.15	0.1 ± 0.01
2.0	0	29.7	95.6 ± 0.21	0	0
4.0	0	9.9	88.5 ± 0.17	0	0
4.0	0	19.8	95.4 ± 0.23	0	0
4.0	0	29.7	96.2 ± 0.16	0	0

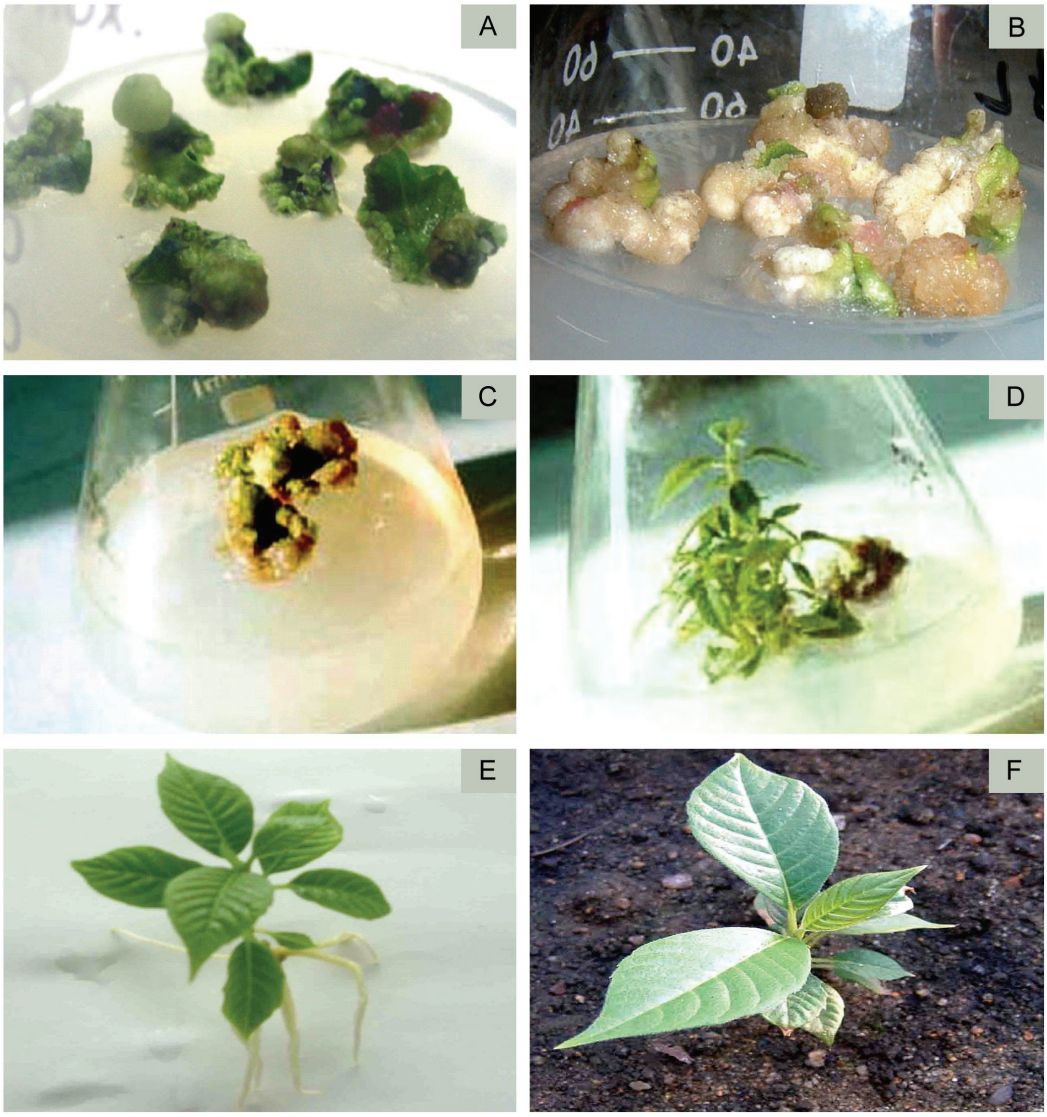

Fig. 1. *A* - Callus induction on leaf explants on the medium containing 19.8 µM BA and 5.8 µM NAA after 4 weeks of culture; *B* - Callus induction on leaf explants on the medium containing 19.8 µM BA and 4.0 µM 2,4-D after 4 weeks of culture; *C* - Multiple shoot primordia on callus after 2 weeks of culture on the shoot regeneration medium; *D* - Shoots regeneration from leaf-derived callus after 4 weeks of culture on the shoot regeneration medium; *E* - Rooted shoot of *C. acuminata* on the medium containing 9.8 µM IBA; *F* - The plantlet of *C. acuminata* growing in the soil.

Table 2. The effect of IBA and NAA on shoot rooting of *C. acuminata*. Rooting efficiency was calculated by the number of shoots giving roots divided by the total number of shoots per treatment. Root length was measured after four weeks culture on rooting medium. Data represent percentages or means ± SE. Each treatment consisting of 60 replicates (explants).

Auxin	[µM]	Rooting time [d]	Root number [shoot^{-1}]	Rooting efficiency [%]	Root length [cm]
IBA	2.4	>15	2.7 ± 0.62	95 ± 0.39	1.2 ± 0.23
	4.8	>15	3.4 ± 0.36	98 ± 0.67	1.6 ± 0.62
	9.6	>15	5.9 ± 0.53	98 ± 0.82	1.9 ± 0.49
	15.4	>17	5.0 ± 0.37	93 ± 0.32	1.0 ± 0.43
NAA	2.9	<12	5.4 ± 0.72	85 ± 0.79	0.9 ± 0.57
	5.8	<12	4.2 ± 1.04	88 ± 0.38	0.5 ± 0.32
	11.6	<10	3.5 ± 0.44	98 ± 0.45	0.4 ± 0.28

of culture on the rooting medium, the plantlets with normal shoots and roots were transferred to the soil in the greenhouse. During the first two weeks in the greenhouse, air relative humidity > 80 % was maintained. Under these conditions over 96 % of the plantlets survived and resumed normal growth (Fig. 1*F*).

In conclusion, we have established a successful *in vitro* system of *C. acuminata* for young leaf explants derived callus. These general and reproducible protocols are vital importance for the *Agrobacterium*-mediated transformation of *C. acuminata*, which is the development of a reliable transformation protocol.

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