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Efficient Rooting and Root Development after Transfer of Regenerated Plantlets of *Camptotheca acuminata*

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Abstract

For establishing an efficient *in vitro* rooting protocol of *Camptotheca acuminata* and improving the survival rate and acclimatization of the regenerated plantlets, the effects of auxins, sucrose concentration and different basal medium on *in vitro* rooting and root development of regenerated shoots after transfer were investigated in this study. Auxin played an important role in *in vitro* rooting and rooting ability was affected by auxin type and the concentration. IBA (indolebutyric acid) 0.5mg/l was the most effective, providing high shoot regeneration frequency (98 %) as well as the highest number of roots (5.9 roots per shoot). Sucrose concentration from 10g/l to 30g/l was positively correlated with the rooting percentage and root number, however, higher sucrose concentration (40g/l) negatively inhibited rooting. WPM or 1/2MS medium produced better results than MS medium. Optimum rooting was attained on WPM or 1/2MS medium supplemented with IBA 0.5mg/l, sucrose 30g/l and agar 6g/l. 96% of the *in vitro* rooted plantlets with well developed shoots and roots survived after transfer. Root development of regenerated plantlets was much worse than that of seedlings after transfer. The ratio between root tips and leaf area in regenerated plantlets were significant lower than that of seedlings.

Key words: *Camptotheca acuminata*, *in vitro* rooting, root development, *ex vitro* acclimatization

Introduction

Camptotheca acuminata Decaisne (Nyssaceae) is an endangered tree species native to south China (Wang *et al.* 2006). It is a well known natural source of the monoterpene-indole alkaloid camptothecin (CPT), one of the most promising anti-tumoural compounds, which was first isolated and structurally described in 1966 (Wall *et al.* 1966). *C. acuminata* has gained considerable attention because of its increasing economic value and use as a medicinal plant. But *C. acuminata* is an endangered species and the extraction of CPT will consume a lot of resources. Moreover, the content of CPT in raw material is very low (Zu *et al.* 2003). In a provenance study, *C. acuminata* seedlings from different seed sources in China and USA displayed significant differences in CPT concentrations. It is highly desirable to develop clonal lines with high CPT-synthesis capabilities for CPT extraction (Liu and Adams 1998, Liu and Li 2001). *In vitro* micropropagation offers a low cost, highly efficient technique for propagating at rates much higher than those obtained with other methods of propagation (Rout *et al.* 2000). Developing a micropropagation protocol for *C. acuminata* will be very useful because once the desirable clonal lines are identified, they can be mass propagated for planting.

Adventitious root formation is the crucial aspect in the clonal propagation of different species, because it directly affected survival rate after transfer. There is marked variation in the rooting potential of different plant species, and systematic trials are often needed to

define the conditions required for root induction (Rout *et al.* 2000). There are so many factors affected adventitious root regeneration such as carbohydrate, auxin, basal medium and so on (Nathalie and Margareta 1995). For *C. acuminata*, systematic study for *in vitro* rooting has not been reported, although some other research in tissue culture has been done for this species (Jain and Nessler 1996, Liu and Li 2001, Weidenfeld *et al.* 1997, Li and Liu 2003, Wang *et al.* 2005).

Root development such as root tip, root projection area and leaf/root ratio during *ex vitro* acclimatization was very important for water and mineral nutrient absorption, even may affect gas exchange. Learning root development during *ex vitro* acclimatization for rooted plantlets of *C. acuminata* will help us to adopt some measures for improving transplanted survival rate and accelerating acclimatization.

To develop an efficient protocol of *in vitro* rooting for *C. acuminata*, in the present study, we investigated: 1) the effects of auxins, sucrose concentration and basal medium on *in vitro* rooting of *C. acuminata*; 2) root development of rooted regenerated plantlets after transferred to soil.

Materials and Methods

Plant material

The shoots for rooting in this study were obtained from 2-month-old micro-shoots from 8 clones. The micro-shoots were sub-cultured every 5 weeks on multiplication medium, B5 medium supplemented with 0.5 mg /l of BA, 20 g/l sucrose and 6 g/l agar, pH 5.8.

The cultures were grown at 25 ± 2 °C, under a 16-h photoperiod of $40 \mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance provided by white fluorescent tubes.

Basal medium and phytohormone

To test the effect of auxin type and concentration on rooting of regenerated plantlets, WPM medium was used as basal medium supplemented with IBA (0.1mg/l, 0.2mg/l, 0.5mg/l, 0.8mg/l) and NAA (0.1mg/l, 0.2mg/l, 0.5mg/l) differently, sucrose 30g/l and agar 6g/l.

To investigate the effect of sucrose concentration on rooting of regenerated plantlets, WPM medium was used as basal medium supplemented with sucrose (0g/l, 20g/l, 30g/l, 40g/l), IBA 0.5mg/l and agar 6g/l.

To study the effect of the basal medium on rooting of regenerated plantlets, MS, 1/2 MS, and WPM medium was used supplemented with IBA 0.5mg/l, sucrose 30g/l and agar 6g/l.

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 of $40 \mu\text{mol m}^{-2} \text{s}^{-1}$ irradiance provided by white fluorescent tubes.

Transfer of rooted plantlets

After cultured on rooting medium for three weeks, the plantlets with normal roots were transferred to rectangular box containing a mixture of sterilized sand and soil (1:1,v:v). At first two weeks after transfer, the plantlets were covered with clear plastic film to maintain high humidity. Thereafter, shade cloth was removed. Survival of the rooted plantlets was recorded at 4 week.

To study root development of regenerated plantlets, one month-old seedlings of *C. acuminata* were used as control. When rooted regenerated plantlets were transferred, one-month-old seedlings were also transferred to the same substrates. After four weeks transfer, root development of regenerated plantlet and seedling was investigated. Root development was indexed by the ratio of total root tip number and total leaf area. Leaf area was measured by a LI 3000 (LiCor, Lincoln, NB, USA).

Statistical analysis

Each treatment factor consisted of 20 replicates and the experiment was repeated three times. A completely randomized design was used in all experiments and data were statistically analyzed and the means were compared using Duncan's Multiple Range Test (DMRT) significance determined at 5% level.

Results and Discussion

Effect of auxin types and concentration on *in vitro* rooting

In the present study, we found that the addition of auxins to the medium produced the more evident effects (Table 1). On the medium without auxin, the rooting percentage was very low, only 35% with 1.9 roots per shoot. When auxins were added, rooting percentage reached more than 85% with more roots per shoot. The best rooting quality and percentage were achieved on the medium supplemented with 0.5mg/l IBA. Root frequency reached 98 % with the higher number of roots, 5.9 roots per shoot (Fig. 1). We also found that IBA and NAA produced different effect on *in vitro* rooting of *C. acuminata*. The similar observation was also found in other researches (Al-Juboory *et al.* 1998, Nathalie and Margareta 1995, Abdulaziz and Bahrany 2002, Prakash *et al.* 1999, Rani and Grover 1999, Fracro and Echeverrigaray, 2001). On the medium containing IBA, average rooting number increased from 2.7 to 5.9 with the increasing of IBA concentration from 0.1 mg/l to 0.5mg/l. Further increase IBA (0.8mg/l) had no obvious effect on rooting efficiency and root number per shoot, but rooting time was longer (>20days) and root growth was inhibited. When NAA was used, NAA performed better at low concentration (0.1mg/l). Negative effects were observed with the addition of higher NAA (>0.2mg/l) to the medium, the average root number decreased and the roots were thick and stunted. These results are not in agreement with those found in a previous study by Watad *et al.* (1992) who observed that, NAA was more effective than IBA in promoting root formation in some *Grevillea* species. But in *G. rosmarinifolia*, the effects of IBA and NAA on *in vitro* rooting were similar, even

Table 1. Effect of auxins on rooting of regenerated plantlets of *C. acuminata*.

Auxin (mg/l)	Rooting time (day)	Rooting efficiency (%)	Root number per shoot
0	>15	35±1.9a	1.9±0.78a
IBA(0.1)	>15	95±3.3c	2.7±0.62a
IBA(0.2)	>15	98±1.7c	3.4±0.36b
IBA(0.5)	>15	98±0.8c	5.9±0.53c
IBA(0.8)	>20	96±3.5c	5.8±0.49c
NAA(0.1)	<12	85±2.3b	5.4±0.72c
NAA(0.2)	<12	88±1.4b	4.2±1.04b
NAA(0.5)	<10	98±0.9c	3.5±0.44b

For rooting, the regenerated shoots were cultured on WPM medium supplemented with 30g/l sucrose in all the above treatment. The values represent the mean (\pm S.E.) of three independent experiments. Rooting efficiency was calculated by the rooted regenerated shoots divided by the total regenerated shoots for rooting. Means within a column followed by the same letter are not significantly different according to Duncan's Multiple Range Test(DMRT) at 5% level.

if at different concentrations (Leonardi *et al.* 2001). From the above, we can conclude that the effects of IBA and NAA maybe depend on plant species.

Effect of sucrose concentration on *in vitro* rooting

The adventitious root regeneration is an energy consuming process which requires a source of carbohydrates (Haissig 1982). The optimal sucrose concentration for adventitious root regeneration depends on plant species (Cheng *et al.* 1992, Hyndman *et al.* 1986, Conner and Fallon 1993). The present study also proved that adventitious root formation is strongly dependent on sucrose supply. On the medium lacking sucrose, the plantlets remained green in the culture period and no adventitious roots produced. Sucrose concentration was positively correlated with the percentage of rooting and root number per rooted shoot. The rooting percentage, root number increased with the rising concentration of sucrose from 10g/l to 30 g/l. Higher concentrations of sucrose inhibited root formation (Table 2). Similar observations have been made in *Syzygium alternifolium* (Sha vallikiian *et al.* 1999). There have been some studies on the effect of carbohydrates on adventitious organ formation (Lazzeri *et al.* 1988, Calamar and de Klerk 2002, Weber *et al.* 1998, Borisjuk *et al.* 1998). Calamar and de Klerk (2002) observed interaction between auxin and sucrose *in vitro* rooting of apple, for all three auxins tested, the optimal concentration for rooting increased with the sucrose concentration. For *C. acuminata*, higher sucrose concentration inhibited root formation might reflect a genuine regulatory role of sucrose in adventitious regeneration.

Table 2. Effect of sucrose concentration on rooting of regenerated plantlets of *C. acuminata*.

Sucrose (mg/l)	Rooting efficiency (%)	Root number
0	0±0a	0a
10	78±2.9b	2.8±0.54b
20	91±1.2c	4.9±0.34cd
30	95±2.3d	5.4±0.65d
40	81±1.5b	4.1±0.83c

For rooting, the regenerated shoots were cultured on WPM medium supplemented with 0.5mg/l IBA. The values represent the mean (±S.E.) of three independent experiments. Rooting efficiency was calculated by the rooted regenerated shoots divided by the total regenerated shoots for rooting. Means within a column followed by the same letter are not significantly different according to Duncan's Multiple Range Test (DMRT) at 5% level.

Effect of basal medium on *in vitro* rooting

Basal medium has an important influence on root induction and root quality (Garland and Stoltz 1981, Zimmerman and Broome 1981). In several tissue culture of wood species, higher ion strength inhibited adventitious root induction and growth (McCown and Sellmer 1987, Manzanera and Parados 1990, Purohit *et al.* 1994, Taojing *et al.* 1999). For *C. acuminata*, we also found that MS medium inhibited root formation. On the MS medium, root percentage was only 65% with 2.8 roots per shoot. 1/2MS and WPM medium resulted in a satisfactory rooting and the rooted shoots were green and healthy (Table 3). The difference in rooting ability between MS and 1/2MS and WPM may be caused by MS medium containing higher ion concentration especially nitrogen level than WPM and 1/2 MS medium.

Root development after transfer

The rooted regenerated plantlets with normal root development and growth vigorously were transferred to soil. We found that remaining higher humidity (RH>80%) and shading were very important for improving survival rate during the first two weeks after transfer. Or else, the plantlets wilted and died quickly. In the present study, the survival rate reached 96% and rooted plantlets remained vigorously growth after transfer (Fig. 2).

After transferred for 30 days, root development was studied. The results showed that root tip number of regenerated plantlets was less and roots of regenerated plantlets usually had no root hair (Fig. 3). When root development was expressed by ratio of root tip number

Table 3. Effect of basal medium on rooting of regenerated plantlets of *C. acuminata*.

Medium	Rooting efficiency (%)	Root number
MS	65±1.7a	2.9±0.69a
1/2MS	95±1.3b	4.3±1.2ab
WPM	93±2.7b	4.8±0.83b

For rooting, 20g/l sucrose and 0.5mg/l IBA were supplemented in different media. The values represent the mean (±S.E.) of three independent experiments. Rooting efficiency was calculated by the rooted regenerated shoots divided by the total regenerated shoots for rooting. Means within a column followed by the same letter are not significantly different according to Duncan's Multiple Range Test (DMRT) at 5% level.

and total leaf area, the ratio of regenerated plantlets obviously lower than seedlings. This lower ratio may affect water absorption and development of plantlets because root tips are the most active part for water absorption (Lambers *et al.* 1998). Hence during adventitious root induction, we should improve root

development of regenerated plantlets, especially increase adventitious root number induction for improving survival rate and accelerating acclimatization for rooted plantlets of *C. acuminata*.



Fig. 1. Rooted plantlets (about 2 cm in height) developed from regenerated shoots of *C. acuminata*. They were cultured on rooting medium for three weeks.



Fig. 2. Rooted regenerated plantlets (About 3 cm in height) of *C. acuminata* survived in the soil after four weeks transfer.

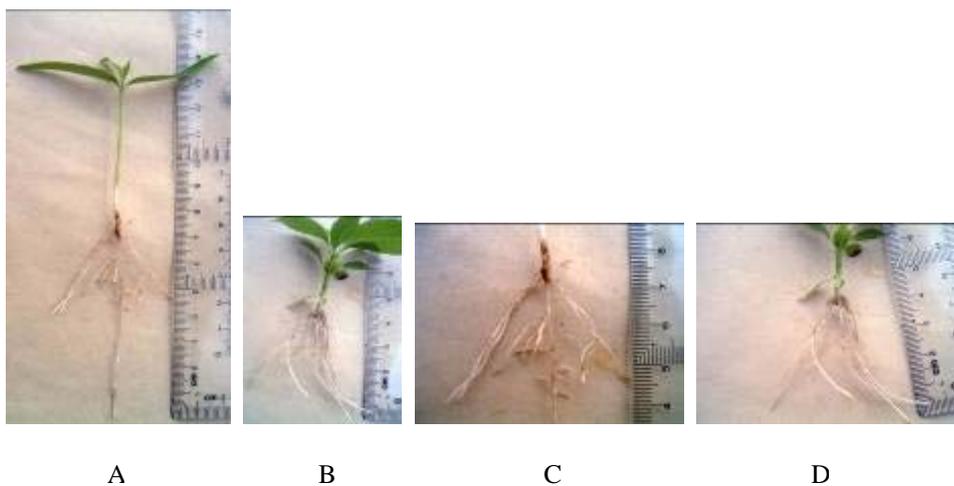


Fig. 3. Differences in root system between seedlings and regenerated plantlets of *C. acuminata*. A. Seedling individuals with taproot, about 7 cm in length; B. regenerated plantlets without taproot, about 4cm in length. C. More root tips in seedlings, with root hair; D. Less root tips in regenerated plantlets, without root hair.

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