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MICROPROPAGATION OF *CAMPTOTHECA ACUMINATA* DECNE (*NYSSACEAE*) – ENDANGERED ORNAMENTAL AND MEDICINAL TREE

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Abstract

Camptotheca acuminata Decne (*Nyssaceae*) (happy tree, tree of life, cancer tree) is a rapidly growing deciduous and endangered tree endemic to east Tibet and southern China (Liu *et al.*, 2002). *C. acuminata* is an ornamental tree with monopodial growth, beautiful leaves and shade- and cold- tolerant in their natural environment. Recently, its demand has grown rapidly due to secondary metabolites production like Camptothecin and its derivatives with anti cancer and antivirus activity. The aim of the present study was to develop an efficient protocol for *in vitro* micropropagation of this valuable plant. Different cultural media based on both MS (Murashige and Skoog, 1962) or DKW (Driver and Kuniyuki, 1984) formulations with different cytokinins (6-benzylaminopurine, BAP or 2-isopentenyladenine, 2iP or *meta*-topolin, mT) have been involved. The aromatic cytokinin *meta*-topolin stimulated plant growth. The optimum multiplication rate of *Camptotheca* shoots occurred on the DKW basal medium, supplemented with 2.5 μM *meta*-topolin, grown under mixed LED light. The highest number of roots per plantlets was recorded on the rooting medium with 0.3 mgl^{-1} NAA.

Keywords: *in vitro* culture, multiplication rate, *meta*-topolin, growth regulators, light emitting diodes (LEDs), Camptothecin

INTRODUCTION

Camptotheca acuminata Decne (*Nyssaceae*) (happy tree, tree of life, cancer tree) is a rapidly growing deciduous and endangered tree endemic to east Tibet and southern China (Liu *et al.*, 2002). *C. acuminata* is the only well-known species of its genus and is distributed mostly along the Yangtze River, growing in forests and on hillsides at altitudes between 250 and 1500 metres. *Camptotheca* trees exhibit monopodial growth that can reach a height of 20 to 25 m. The bark of fully-grown trees is pale-grey with deep furrows. Leaves are petiolate, alternate, pinnately veined, entire or occasionally dentate with a length of 10 to 30 cm and 6 to 15 cm in width. In their natural environment *C. acuminata* plants are shade- and cold- tolerant (Chou and Li, 1990). Recently, its demand has grown rapidly due to secondary metabolites production (Li and Wang, 2014). Camptothecin (CPT) is a monoterpenic indole alkaloid isolated from the bark and stems of *C. acuminata* and, along with its derivatives, are used to treat various cancers and have the potential to treat a wide range of viruses and parasites. Besides, CPT is the first known inhibitor of topoisomerase

(Topo I) and also inhibits retroviruses such as HIV and the equine infectious anemia virus (Priel *et al*., 1991a, b; Li *et al*., 1994; Li and Wang, 2014).

Advances in plant cell cultures and *in vitro* propagation could provide new means for cost-effective, commercial production of rare, exotic or valuable ornamental and medicinal plants with improved economic performance. The vegetative reproduction of plantlets by means of *in vitro* techniques (micropropagation) provokes interest in order to select more preferable genotypes for faster reproduction.

There are some reports describing *in vitro* methods of *C. acuminata* Decne cultivation using shoot tip, leaf and axillary bud cultures (Jain and Nessler, 1996; Liu and Li, 2001; Sankar-Thomas *et al.*, 2008). Most of them, however, are related to the *in vitro* production of secondary bioactive metabolites. The aim of the present study was to develop an efficient protocol for *in vitro* micropropagation of this valuable plant.

MATERIALS AND METHODS

Plant Material

Seeds of *Camptotheca acuminata* Decne were obtained from the Botanical Garden in Beijing, China. Explant source was 3-months-old seedlings. Explants were cut in nodal segments, washed with tap water for an hour. The surface disinfection procedure consisted of a treatment with 70% ethanol for 30 s and 2% silver nitrate solution (plus 1 drop Tween 20) for 5 min and four rinses with sterile distilled water (10 min each). Nodal segments with one axillary bud were placed in test tubes (25 × 150 mm) on 5 ml nutrient media based on both MS (Murashige and Skoog, 1962) or DKW (Driver and Kuniyuki, 1984) formulations. They were enriched with different cytokinins: 6-benzylaminopurine (BAP, 2.5 μM) or 2-isopentenyladenine (2iP, 2.5 μM) or *meta*-topolin (mT, 2.5 μM), 30.0 gl^{-1} sucrose and 6.5 gl^{-1} agar (Phytoagar, Duchefa). The pH of the media was adjusted to 5.6 before autoclaving. All aseptic shoots were subcultured every 3 weeks on the same nutrient media.

Multiplication Stage

Experiment 1

Uniformly developed shoots (10–15 mm with two leaves) were transferred on the corresponding nutrient media. Five replications, each containing 5 explants, were cultured in baby food jars on 25 ml nutrient medium for each variant. The same nutrient medium without growth regulators served as a control. In three passages of three weeks on the respective nutrient media, the number of newly formed shoots, mean number of leaves, shoot length (mm), fresh (FW) and dry mass (DW) were recorded. The experiment was repeated twice.

The cultures were maintained in the growth room at temperature of 22 ± 2 °C with 16/8 hours photoperiod supplied by cool-white fluorescent lamps (OSRAM 40 W, 60 $\mu molm^{-2}s^{-1}$ PPFD).

Experiment 2

The aim of this experiment was to study the effect of light source on the growth of *Camptotheca* shoots. Three different light sources were used:

- Cool-white fluorescent lamps (OSRAM 40 W, 68 $\mu molm^{-2}s^{-1}$ PPFD) – **LL**;
- White LED (Philips GreenPower LED research module, 70 $\mu molm^{-2}s^{-1}$ PPFD) – **LED-W**;
- Mixed LED (Red : Blue : DeepRed : White = 1 : 1 : 1 : 1, Philips GreenPower LED research module, 100 $\mu molm^{-2}s^{-1}$ PPFD) – **LED-M**.

The best nutrient media from Experiment 1 (DKW basal medium with 2.5 μMmT) was selected for the Experiment 2. The experiment was conducted twice with 30 explants for variant (Six baby food jars with 5 explants each).

Rooting Stage

Microcuttings (15 mm length) were obtained from mixed LED light from Experiment 2. They were transferred into polypropylene microboxes (600 ml) with white gas permeable filter (Sac O2, Belgium). DKW basal medium with half strength of macroelements, supplemented with IAA or IBA or NAA (0.1 mgl^{-1} or 0.3 mgl^{-1}) were used. The same nutrient medium without growth regulators served as a control. In each vessel onto 100 ml nutrient medium ten shoots were cultivated. Cultures were grown in a growth chamber at 22 ± 2 °C under 16 h photoperiod at mixed LED light described in Experiment 2 (100 $\mu molm^{-2}s^{-1}$ PPFD). Rooting was evaluated after 20 days. The experiment was conducted twice with 30 explants for each variant.

Statistical Analyses

Statistical analyses were carried out by One-Way ANOVA and the means are separated using the Duncan's multiple range test (DMRT) ($P < 0.05$).

RESULTS AND DISCUSSION

Establishment of *in Vitro* Culture

One week after initiation of culture about 70% of explants inoculated on media were not contaminated. In our previous studies with *Taxus* (Ibrahim, 2011) and *Ginkgo* (Nacheva and Ivanova, 2017) we found that silver nitrate is an effective agent for *in vitro* surface-sterilization protocol of shoot explants. The routinely used surface disinfection procedures for woody ornamental and fruit plants in the laboratory is with 5% solution of calcium hypochlorite. But this procedure had proven to be relatively unsuccessful with *Taxus baccata* and some other woody fruit and ornamental

species. For sterilization of some woody and mature plants growing in the open field known to harbor a lot of microflora and/or limited amount of explants we prefer to use silver nitrate for better results. Ten days after initiation, bud induction was noticed on some of used media supplemented with cytokinin. No development of buds and slow growth was noticed with any of the used hormone free media.

Multiplication Stage

Experiment 1

Cultures grown on the two basal media (MS and DKW) free of growth regulators did not proliferate (Tab. I, Fig. 1). An increase in height was observed, as with a DKW medium it was higher than MS, but this trend is not statistically proven. Significant multiplication was observed in both the main MS and DKW enriched cytokinins BAP and mT, with the best results being achieved in mT-enriched DKW medium. This treatment was not only with the best multiplication, but also with the highest dry plant mass and the highest number of leaves. *Meta*-topolin also significantly influenced the mean branch length – among the cytokinin-enriched nutrient media of the mT-enriched nutrient media, the greatest length of the shoots was recorded. Our research confirmed the results of Jain and Nessler (1996) that cytotokinin, such as BAP or kinetin, was required for multiplication of *C. acuminata* shoots. Similar to Liu and Li (2001), we found between 2 and 6 shoots per explant (mean 4.2 for MS and 4.1 for DKW) when applying BAP, achieving these results at a 1.7-fold lower concentration (2.5 μM). We have confirmed Liu and Lee's conclusion that low BAP remained the key to optimal shoot formation, supporting the notion that multiple shoot induction has a requirement for low levels of BA, indeed higher BA concentrations may become detrimental for *C. acuminata* multiple shoot induction (Jain and Nessler, 1996). Recently, the natural cytokinin, hydroxylated 6-benzyladenine derivative, mT was reported to induce more shoot proliferation, in respect to BAP, in different species (Aremu *et al.*, 2012) such as *Aloe* spp. (Bairu *et al.*, 2007), *Musa* spp. (Bairu *et al.*, 2008), *Pyrus* (Dimitrova *et al.*, 2016), *Corylus* (Gentile *et al.*, 2017) while it was observed to reduce shoot formation in *Citrus* (Niedz and Evens, 2010).

The application of 2iP did not lead to the expected result – no proliferation was observed on both studied basal media (MS or DKW). The length of the shoots and the mean number of leaves was equal to those of the control (DKW) or even lower than the control (MS).

Experiment 2

Light is one of the most important environmental factor affecting plant growth, morphogenesis and development. The results obtained showed differences between the light treatments. The fresh, dry mass and length of plantlets increased substantially in the cultivation at LED lights compared to control plants grown under LL (Tab. I, Fig. 2). However, there was no significant difference in the number of leaf and shoots.

Light affects the plants through their spectral quality, photon flux and photoperiod and besides as an energy source for the photosynthesis it can also act as external signal in regulating various

I: *Effect of the nutrient media on growth parameters (FW and DW, number of newly formed shoots, shoot length, mean number of leaves) of the Camptotheca (Experiment 1 and Experiment 2)*

Variant	FW mg	DW mg	Mean number of shoots	Shoot length mm	Mean number of leaves
Experiment 1					
0	81.2 ± 11.2 e	10.7 ± 1.2 g	1.1 ± 0.5 b	16.83 ± 2.13 a	11 ± 2 bc
BAP	347.3 ± 27.1 b	35.2 ± 2.1 c	4.2 ± 1.2 a	8.22 ± 1.15 c	15 ± 4 b
2iP	100.5 ± 11.3 d	9.6 ± 1.1 g	2.4 ± 1.1 ab	7.11 ± 3.12 c	9 ± 2 c
mT	228.4 ± 18.3 c	26.4 ± 2.3 e	4.3 ± 1.3 a	10.67 ± 3.33 b	26 ± 3 a
DKW					
0	160.2 ± 15.1 d	30.8 ± 2.6 d	1.1 ± 0.5 b	20.91 ± 2.5 a	12 ± 2 b
BAP	606.2 ± 28.2 a	44.2 ± 3.1 b	4.1 ± 2.1 a	10.23 ± 2.25 b	16 ± 3 b
2iP	112.1 ± 12.1 d	14.6 ± 2.1 f	1.2 ± 0.6 b	7.98 ± 2.13 c	12 ± 2 b
mT	556.5 ± 36.2 a	55.2 ± 4.7 a	5.5 ± 2.3 a	13.19 ± 3.12 b	27 ± 4 a
Experiment 2					
LL	556.5 ± 36.2 c	55.2 ± 4.7 c	5.5 ± 2.3 a	13.19 ± 3.12 c	27 ± 4 a
LED-W	703.4 ± 27.2 b	67.3 ± 5.3 b	5.6 ± 1.2 a	21.13 ± 2.71 b	26 ± 4 a
LED-M	1207.3 ± 42.2 a	109.4 ± 9.3 a	5.4 ± 1.1 a	29.41 ± 4.13 a	27 ± 3 a

1: *Effect of the nutrient media on in vitro growth of the Camptotheca shoots (Experiment 1)*

morphogenic and physiological processes. The mixed LED light (**LED-M**) applied in our experiment was significantly higher (100 $\mu molm^{-2}s^{-1}$ PPFD) compared to **LL** (68 $\mu molm^{-2}s^{-1}$ PPFD). This could be one reason for significantly higher plantlet biomass. On the other hand the use of LEDs in growth chambers provides an opportunity to control light spectrum. The application of blue, red and deep red LED light had a favorable effect on growth of *Camptotheca* plantlets compared to **LL** and **LED-W**.

Plant growth and morphogenesis is affected by both red and blue light (Rajapakse and Shahak, 2007). The photosynthetic apparatus is mostly affected by red light (Saebo *et al.*, 1995) while blue light strongly affects photomorphogenesis, chlorophyll formation and stomata opening (Dougher and Bugbee, 1998). Plant height (stem elongation) is therefore affected by different combinations of red and blue, as blue light plays an important role since it is required for a functional photosynthetic and physiological operation (Hogewoning *et al.*, 2010).

Fluorescent lamps have been the most popular in tissue culture rooms (Economou *et al.*, 1987). However, these lights have a wide range of wavelengths (350–750 nm) but appears to be unnecessary and are of low quality for promoting plant growth, and the system consumes a lot of electrical power while generating heat in a tissue culture laboratory. In recent years, light emitting diodes (LEDs) have been proposed as a potential alternative light source for in vitro plant growth and development (Gupta and Jatothu, 2013). Upon exposure to LED, in vitro-raised plants have shown significant improvements in growth and morphogenesis. In particular, red and blue lights, either alone or in combination, have a significant influence on plant growth. It has been observed that a mixture of blue and red LEDs enhanced plant growth, with increased fresh and dry weights, compared to monochromatic LEDs (Gupta and Jatothu, 2013). Similar to our results with *Camptotheca*, Li *et al.* (2010) have found increased fresh and dry weights of upland cotton when cultured under blue and red LEDs in equal proportion. Similar results with B : R (1 : 1) LEDs were also obtained from in vitro plants of Lilium (Lian *et al.*, 2002), banana (Duong *et al.*, 2003), strawberry (Nhut *et al.*, 2003), and chrysanthemum (Kim *et al.*, 2004), *Rehmannia* (Manivannan *et al.*, 2015), *Myrtus communis* (Cioc *et al.*, 2018) etc. Detailed research is needed on the impact of various LED lights for optimal growth of *Camptotheca acuminata* plantlets.

Rooting Stage

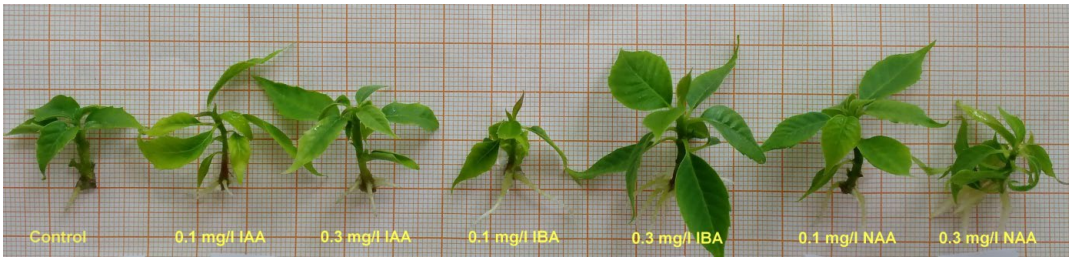
Some *C. acuminata* plantlets (about 15%) presented spontaneous rhizogenesis on the hormone free medium. In all nutrient media supplemented with auxins, a very high rooting percentage was recorded – from 90 to 98% (Tab. II, Figs. 3, 4).

In all of these variants, a significantly larger number and longer roots than the hormone free control medium were observed. Higher concentration (0.3 mgl^{-1}) of the three tested auxins (IAA, IBA, NAA) induced a greater number of roots per plant. The best result was achieved at 0.3 mgl^{-1} of NAA, which recorded 98% rooting and the highest number of roots per plant (up to 7). As is known, a greater number of roots is a prerequisite for a more successful acclimatization of plants to ex vitro conditions.

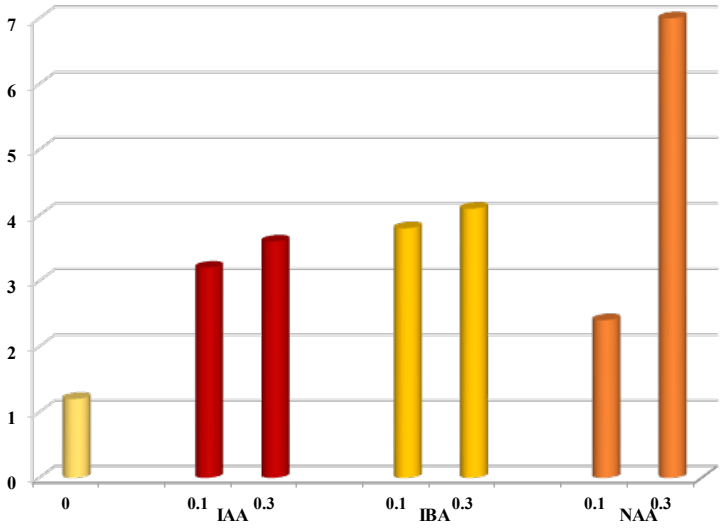
2: *Effect of the light source on in vitro growth of the Camptotheca shoots (Experiment 2)*

II: *Effect of the nutrient media on the rooting of Camptotheca acuminate plantlets*

Variant	Rooting, %	Mean root length, mm	Mean number of leaves
0 (Control)	15	1.22 ± 0.6 d	6.1 ± 1.2 b
0.1 mgl^{-1} IAA	90	7.68 ± 1.2 bc	8.5 ± 2.2 ab
0.3 mgl^{-1} IAA	93	5.76 ± 1.7 c	8.8 ± 2.1 ab
0.1 mgl^{-1} IBA	96	16.59 ± 2.3 a	8.2 ± 2.3 ab
0.3 mgl^{-1} IBA	98	14.61 ± 3.5 a	10.4 ± 2.5 a
0.1 mgl^{-1} NAA	97	9.26 ± 2.4 b	8.6 ± 2.4 ab
0.3 mgl^{-1} NAA	98	7.78 ± 2.3 bc	9.2 ± 2.2 a



3: *Effect of the auxin type and concentration on the rooting of the Camptotheca plantlets*



4: *Effect of the auxin (in mgl^{-1}) on the mean number of roots per plantlets of Camptotheca acuminata*

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. Jane and Nessler (1996) received the best results (82% of rooted plantlets) on the Gamborg's B5 medium (Gamborg *et al.*, 1968) at the lowest tested IBA concentration (1 mgl^{-1}).

According to Chen *et al.* (2004) half strength MS medium + NAA 1.0 mgl^{-1} +IBA 1.0 mgl^{-1} is the best medium for rooting induction. For roots inducing Wang *et al.* (2006) recommend 9.6 μM IBA (2 mgl^{-1}). Both *in vitro* and *ex vitro* rooting of the microcuttings are feasible with indole-3-butyric acid in the culture media, but *ex vitro* rooting lead to high plantlet survival (Liu and Li, 2001). Our results are similar to the results obtained from Chen *et al.* (2004) and Wang *et al.* (2006) but have been achieved at significantly lower auxin concentrations. Our hypothesis is that the conditions of cultivation have contributed to the better rooting of the plants. We used containers with a gas-permeable filter on the lid that provide good gas exchange with the environment but preserve the sterility. On the other hand, the application of mT we used at the multiplication step could affect the rooting. Werbrouck *et al.* (1996) and some other authors suggested a lower inhibition of rhizogenesis by mT as compared to BAP.

CONCLUSION

The optimum multiplication rate of *Camptotheca* shoots occurred on the DKW basal medium, supplemented with 2.5 μM *meta*-topolin, grown under mixed LED light. Further experiments will focus on the dose-response curve of mT and quality of LED light.

The addition of auxin in the culture medium stimulate rooting of the microcuttings. The highest number of roots per plantlets was reported on the rooting medium with 0.3 mgl^{-1} NAA.

The protocols described here efficient and could be applied in vegetative propagation, which could enhance large-scale propagation of valuable genotypes of medicinal and ornamental plant *Camptotheca acuminata* Decne.

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