Original Paper

Development of an efficient regeneration system for the precious and fast-growing timber tree *Toona ciliata*

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Abstract   *Toona ciliata* (Chinese mahogany) is an important timber species and secondary protected plant due to excessive exploitation in China. Here we developed a robust and efficient regeneration system for adventitious shoot induction using hypocotyl explants of *T. ciliata*. To facilitate plant growth, different regulators were added to Murashige–Skoog (MS) medium (0.5 mg/l 6-BA, 1.0 mg/l KT and 0.1 mg/l IBA). A regeneration frequency of 58.67% with four shoots per explant was achieved by horizontal setting of hypocotyls on MS medium and following a 20-day seeding period. MS medium supplemented with 0.3 mg/l 6-BA and 0.2 mg/l NAA was optimal for shoot multiplication and elongation, with a multiplication coefficient of 3.06. A rooting frequency of 93.33% was achieved using the half-strength MS containing 0.1 mg/l NAA. After acclimatization, plantlets were transplanted to sterilized nutrient soil containing a 2 : 1 ratio of vermiculate with 90% survival frequency. Thus, the regeneration system developed in this study would be useful for genetic transformation and other biotechnology endeavours in *T. ciliata*.

Key words: hypocotyl explant, plant regeneration, precious tree, *Toona ciliata*.

Introduction

Toona ciliata belongs to the Meliaceae family and is a precious timber species in China, *T. ciliata* is primarily distributed in south China (Chen et al. 2014; Feng et al. 2015), and is also sporadically distributed along the east coast of India, Laos, Myanmar, Pakistan and the east coast of Australia (Heinrich and Banks 2005; Li et al. 2017a). Referred to Chinese mahogany, *T. ciliata* has straight trunks and produces red wood with a desirable grain (Heinrich and Banks 2005; Li et al. 2012; Liang et al. 2011). It has high economic value in wood industry. The impacts of environmental changes, low population renewal rates and over-harvesting of *T. ciliata* leading to it being an endangered species and listed as a level II national key protected wild plant in the *China Plant Red Data Book* (Li et al. 2015). Recently, increased attention has been paid to research and use of this tree species.

A broad-leaf tree species that with high hardness, high density and beautiful material appearance and texture can be called precious broad-leaved tree (Jiang 2013). They have special process properties which suitable to produce high-end furniture, high-grade instruments or high value-added terminal high quality arts and crafts

products. Therefore, precious broadleaf trees such as *T. ciliata* have become scarce resources on the market (Luo et al. 2010). *T. ciliata* is the focus of development and utilisation programs designed to enhance the planting of fast-growing timber species in southern China (Li et al. 2017b; Zou 1994). *T. ciliata* also has applications in landscaping and potential medicinal uses. Thus, all of these uses of *T. ciliata* have led to its depletion (Chen et al. 2009; Cheng and Cui 2010; Chowdhury et al. 2003; Zhou et al. 2015).

The sustainability of *T. ciliata* is challenged owing to its growth characteristics and natural environmental impacts. Due to straight and tall adult tree, artificial seed harvesting is more difficult and the viability of the seeds decline in short time (Liu et al. 2014a; Zhao et al. 2005). The long breeding cycle and labour intensiveness of *T. ciliata* are disadvantageous for its conventional breeding. Our previous studies also found that *T. ciliata* is readily damaged by *Hypsipyla robusta*, which can cause death of *T. ciliata*. Furthermore, *T. ciliata* is subjected to freezing injury in some regions, which slows growth and unable to perform the characteristics of the rapid growth. All these influences negatively affect the species growth and practical demand in a large scale. Plant genetic

Figure 1.   Plant regeneration from hypocotyl explants of *T. ciliata* and acclimatization. A: Hypocotyl segments were cut from aseptic seedling. a: the upper of hypocotyl; b: the middle of hypocotyl; c: the lower of hypocoty. B: Shoots induction from hypocotyl in *T. ciliata* on MS media containing 0.5 mg/l 6-BA, 1 mg/l KT and 0.1 mg/l IBA. a: the explant that was cultured for 7 days, callus inducted; b: the explant that was cultured for 10 days, callus inducted continuously; c: the explant that was cultured for 20 days, shoots elongated from the calli; d: the explant that was cultured for 35 days, shoots elongated continuously; C: Shoot multiplication and elongation on MS medium containing 0.3 mg/l 6-BA and 0.2 mg/l NAA after 30 days of growth; D: Roots formed in half-strength MS with 0.1 mg/l NAA, 1.5% sucrose (w/v) and 0.5% agar (w/v). E: Acclimatized plantlet after 20-day transplantation. F: Acclimatized plantlet after two month; G: Acclimatized plantlet after four month.

engineering can provide an effective way for genetic improvement (Raza et al. 2017). The establishment of genetic transformation system that is a key technique for genetic engineering can be used for plant conservation (Anjusha and Gangaprasad 2016; Chauhan 2016). But few studies have focused on the development of tissue culture methods for *T. ciliata*. The stems of mature trees or seedlings have been used as explants; however, low regeneration frequencies and difficulty in disinfecting

have limited the methodological development (Angeloni et al. 1992; Chen et al. 2014; Liu et al. 2014a; Mroginski et al. 2003).

As an explant, hypocotyls has the advantages of short cycles, simple culture procedures and adequate repeatability (Shahzad et al. 2014). Hypocotyls are embryogenic organs derived from zygotic embryos with high embryonic activity that undergo division and differentiation via the parenchymal cells of the vascular-

forming layer, and hypocotyls have high vitality and the ability to differentiate and regenerate (Liao et al. 2015). Notably, hypocotyls from *Vernicia fordii* (Mu et al. 2016), *Cyphomandra betacea* (Kahia et al. 2015), *Fagopyrum esculentum* (Hou et al. 2015), *Tectona grandis* (Tambarussi et al. 2017), nectarines (Yue et al. 2007) and *Eucalyptus dunnii* (Lu et al. 2016) are used routinely to produce regenerated plants. Besides, hypocotyls are excellent for genetic transformation as a result of their high rates of differentiation, convenience of selection, lack of seasonal restrictions and a high rate of infection with *Agrobacterium* (Huang 2006; Liao et al. 2015; Liu et al. 2017).

In this study, we developed an efficient and stable shoot regeneration system for *T. ciliata* using hypocotyls as explants. Such an approach provided the necessary technical support to achieve rapid propagation, which allowed identification, isolation and improvement of the desirable traits of *T. ciliata*.

Materials and methods

Preparation of culture medium and conditions
Murashige and Skoog (MS) medium (Murashige and Skoog 1962) containing 3% sucrose (w/v) and 0.5% agar (w/v) was used as the base medium in all studies, except the rooting studies, in which 1.5% (w/v) sucrose was used instead. All media were adjusted to a pH of 5.8 prior to the addition of agar and were autoclaved at 121°C for 20 min. Cultures were maintained at 25 ± 2°C under white fluorescent light (30 μmol m^{-2}s^{-1} photosynthetic photon flux) and a 12/12-h light/dark cycle.

Seed samples and sterilization
Seeds of *T. ciliata* were collected from healthy mature trees in Pupiao, Yunnan, China (99°06′E, 25°04′N; altitude of 1,513 m; annual average temperature of 14°C) and stored at 4°C in the South China Agricultural University in Guangzhou.

After the wings were removed, the seeds were submerged in sterile water for 3–5 h at 45°C initial temperature. Seeds lacking wings were sterilized in 75% ethanol 60 s and then washed in sterile water for 60 s. Following washing, seeds were sterilized in 0.1% HgCl$_2$ and 10% NaClO for different periods of time. The treatments with 0.1% HgCl$_2$ were 5, 10 and 15 min, while the treatments with 10% NaClO were 10, 15 and 20 min. Seeds were then washed five times for 4 min each with sterilized water.

Sterilized seeds were inoculated on MS medium without growth regulators. Following incubation, 10 seeds from each treatment were placed in a single flask (100 seeds total) and grown for 10 days. Then, the contamination and germination frequencies were assessed.

Adventitious shoot induction
Hypocotyls were cut once the aseptic seedlings had grown to 2–3 cm (Figure 1A) and were inoculated on MS medium supplemented with 0.1 mg/l IBA; 0.3, 0.5 or 1 mg/l 6-BA; and

0.5, 1 or 1.5 mg/l KT. The medium without growth regulators was used as control. The optimal medium composition was determined based on the callus growth and shoot induction of hypocotyls.

Hypocotyls were inoculated from 15-, 20- and 25-day-old aseptic seedlings (in optimal medium, as described above), and shoot induction was assessed based on seedling age.

To assess shoot induction based on growth position, hypocotyls were divided into three 1-cm sections: one near the cotyledonary node (the upper) (Figure 1Aa), one in the central section (the middle) (Figure 1Ab) and one near the radicle (the lower) (Figure 1Ac). Sections were inoculated vertically stand on the medium, or placed horizontally, and shoot induction was assessed according to the position and the inoculation method.

Each assessment usually had three replicates, with 10 culture flasks containing five explants each. Shoot regeneration was assessed after 35 days of culture.

Shoot multiplication and elongation
Multiple shoots were placed in MS media supplemented with 0.1, 0.3 or 0.5 mg/l 6-BA and 0.1, 0.2 or 0.3 mg/l NAA. Shoot multiplication and elongation were assessed following growth under light. Each treatment usually had three replicates, with 10 culture flasks containing three explants each. Multiplication frequencies and seedling heights were assessed after 30 days of growth.

Rooting and field acclimatization
Four half-strength MS media containing 0, 0.1, 0.2 or 0.3 mg/l NAA were assessed to optimise the rooting medium for growth. Shoots 3–4 cm in length were grown in each medium, and the rooting frequency was calculated following growth under light for 20 days. Each treatment usually had three replicates, with five culture flasks containing three shoots each.

Culture flasks containing healthy plantlets that were adequately adapted to the culture environment were opened for 1 day, and the plantlets were washed to remove the medium and placed in water for 1 h. Plantlets were then transferred to plastic cups containing sterilized nutrient soil and vermiculite at a 2:1 ratio (Zhang et al. 2017). Cups were covered with plastic film for 3 days and watered to maintain moisture. Survival frequencies were evaluated after 20 days.

Statistical analysis
Analysis of variance (ANOVA) to compare mean values was performed using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). The means were also compared using Duncan's multiple range tests. A *p*-value of ≤0.05 was considered the statistically significant level.

The contamination frequency (%) was calculated using the following formula: number of contaminated seeds/total number of seeds ×100%. The germination frequency (%) was calculated as the number of germinated seeds/total number of seeds ×100%. The shoot regeneration frequency (%) was calculated

as the number of hypocotyl explants producing shoots/total number of hypocotyl explants ×100%. The multiplication frequency (%) was calculated as the number of new multiplied shoots/total number of shoots ×100%. The rooting frequency (%) was calculated as the number of shoots with roots/total number of shoots ×100%.

Results and discussion

Establishment of sterile seeds

The large quantity of aseptic seedlings provided sufficient experimental materials to establish the hypocotyl regeneration system. Therefore, seed sterilization was an important step in this study. The germination frequency was higher when sterilization was performed using 0.1% $HgCl_2$ for 5 min or 10% NaClO in all three treatment time (Table 1). Following 3 days of culture, the radicle developed in seeds sterilized using 10% NaClO, while 6 days of culture were required for radicle development in seeds sterilized using 0.1% $HgCl_2$.

Contamination was the lowest among seeds sterilized using 0.1% $HgCl_2$ for 15 min or 10% NaClO for 20 min, compared with all other treatments (Table 1). These data indicated that contamination was reduced with increasing 0.1% $HgCl_2$ treatment time; however, 0.1% $HgCl_2$ affected the germination frequency. This finding was consistent with studies in processing tomato (Tang et al. 2016) and Tung Tree (*Vernicia fordii*) (Mu et al. 2016).

The optimal sterilization treatment was 75% ethanol for 60 s, followed by 10% NaClO for 20 min; 75% ethanol treatment resulted in the lowest contamination without affecting germination. Additionally, cotyledons grew from seeds cultured for 7 days.

Effects of plant growth regulators on shoot induction

Plant growth regulators are important for callus formation and adventitious shoot induction. Hormone levels in plants change continuously in vitro, and exogenous hormones directly impact the endogenous levels (Baskaran et al. 2015; Liu et al. 2014b). The formation of callus and adventitious shoots is influenced by the interactions between endogenous and exogenous plant growth regulators (Guo et al. 2016). Multiple studies have shown that 6-BA influences adventitious shoot differentiation more than do other cytokinins (Al Khateeb et al. 2013; Lee and Pijut 2017; Zhang et al. 2017), but only in the condition of rational configuration of cytokinin and auxin, there is a high frequency induction in explants.

The hypocotyls from aseptic *T. ciliata* seedlings were used as explants and incubated on MS medium supplemented with different combinations of plant growth regulators. When the concentration of IBA was kept at 0.1 mg/l, the shoot regeneration frequency initially increased with higher 6-BA and KT

Table 1.   Effect of different sterilization time on the seed sterilization.

Sterilization agent	Sterilization time (min)	Contamination frequency (%)	Germination frequency (%)
10% NaClO	10	34.67±3.21b	77.67±2.52b
	15	28.00±2.65c	88.33±2.08a
	20	6.33±2.52e	91.67±1.53a
0.1% $HgCl_2$	5	31.00±4.58bc	79.00±3.61b
	10	53.00±4.36a	60.00±3.00c
	15	18.00±1.73d	49.33±5.01d

Values are mean±standard error. The different letters behind data mean significantly different from each other at $p \leq 0.05$ level, according to Duncan's multiple range test. All the treamnets were after sterilized in 75% ethanol 60 s and then washed in sterile water for 60 s.

Table 2.   Effect of different concentrations of cytokinin and auxin on shoot induction in *T. ciliata* on MS after 35 days of culture.

No.	Plant growth regulators and concentration			Shoot regeneration frequency (%)	Number of shoots per explant
	6-BA (mg/l)	KT (mg/l)	IBA (mg/l)		
T1	0	0	0	0f	0c
T2	0.3	0.5	0.1	19.33±5.03e	2.00±0b
T3	0.3	1	0.1	26.67±4.16de	2.67±0.58ab
T4	0.3	1.5	0.1	42.00±4.00bc	2.67±1.15ab
T5	0.5	0.5	0.1	34.67±7.02cd	2.33±0.58b
T6	0.5	1	0.1	58.67±5.03a	4.00±1.00a
T7	0.5	1.5	0.1	48.67±4.08b	2.33±0.58b
T8	1	0.5	0.1	21.33±9.24e	2.67±1.15ab
T9	1	1	0.1	42.00±7.21bc	2.67±0.58ab
T10	1	1.5	0.1	24.67±2.31e	2.67±1.15ab

Values are mean±standard error in triplicate, each with 50 explants. The different letters behind data mean significantly different from each other at $p \leq 0.05$ level, according to Duncan's multiple range test.

concentrations (6-BA was in 0.5 mg/l and KT was in 1 mg/l), followed by a decrease with a further increase in concentration (6-BA was in 1 mg/l and KT was in 1.5 mg/l) (Table 2). Thus, effects of the two cytokinins were concentration-dependent.

Among the 10 medium compositions containing with different concentrations of 6-BA and KT, calli were observed at the wounds of hypocotyls after 7 days of culture on MS containing 0.5 mg/l 6-BA, 1 mg/l KT and 0.1 mg/l IBA (optimal composition). Callus growth continued gradually following the initial growth. After 20 days of culture, shoots elongated from the calli (Figure 1B). The shoot regeneration frequency reached 58.67%, while the number of shoots per explant reached 4.0 (Table 2).

Table 3. Effect of aseptic seedling age and position of hypocotyl on adventitious shoot induction from hypocotyl of *T. ciliata*.

Effect factors		Shoot regeneration frequency (%)	Number of shoots per explant
Seedling age	15-day-old	28.37±7.39b	3.33±0.58b
	20-day-old	43.82±6.64a	4.67±0.58a
	25-day-old	21.49±3.49b	2.67±0.58b
Position	Upper	45.19±2.57	3.67±1.54
	Middle	43.70±10.02	3.33±0.58
	Lower	40.74±20.12	3.33±0.58

Values are mean±standard error in triplicate, each with 50 explants. All the culture media contained 0.5 mg/l 6-BA, 1 mg/l KT and 0.1 mg/l IBA. The different letters behind data mean significantly different from each other at $p \leq 0.05$ level, according to Duncan's multiple range test.

Optimization of culture conditions

Except plant growth regulator, the differentiation of adventitious shoots from hypocotyls was influenced by seedling age, explant genotype and inoculation method.

Seedling age is important factor for the inducting of adventitious shoots in *T. ciliata*. Significant differences in regeneration capacity of hypocotyls from 15-, 20- and 25-day-old seedlings were observed (Table 3). The reason for that is seedling age determined the physiological state of the explants, and the young seedlings used in this study reflected the strong differentiation and regeneration capabilities of the parenchymal cells in incision (Gaur and Srivastava 2017; Zhu et al. 2005). Differences in physiological state also existed in the same explant comprised of seedlings of different ages (Compton et al. 1993). Additionally, the highest shoot regeneration frequency was 43.82%, observed in 20-day-old seedlings, in which the number of shoots per explant was 4.67 (Table 3). These data suggest that regeneration of explants younger than 20 days old was less than ideal, and that after 20 days of growth, the regeneration ability decreased with seedling age.

Time difference in the onset of differentiation was based on the positioning of hypocotyls; however, this finding may have been due to different auxin contents in the sections themselves (Lin et al. 2016), which led to differences in the meristematic cells. Statistical analyses revealed that the regeneration frequency of adventitious shoots did not differ among the positions of hypocotyls after 35 days of culture. The shoot regeneration frequency ranged from 40.74 to 45.19%, and the number of shoots

Table 4. Effects of different concentrations of 6-BA and NAA on the adventitious shoots multiplication and elongation.

Plant growth regulators		Multiplication coefficient	Shoot height (cm)
6-BA (mg/l)	NAA (mg/l)		
0.1	0.1	1.36±0.13c	2.31±0.86bc
0.1	0.2	2.06±0.85bc	3.04±0.31abc
0.1	0.3	1.67±0.33bc	2.93±0.80abc
0.3	0.1	2.00±0.33bc	3.15±0.51ab
0.3	0.2	3.06±0.59a	3.87±0.53a
0.3	0.3	1.78±0.63bc	3.24±0.39ab
0.5	0.1	2.28±0.25b	1.92±0.22c
0.5	0.2	1.28±0.25c	2.70±1.46abc
0.5	0.3	1.50±0.17bc	2.86±030abc

Values are mean±standard error in triplicate, each with 30 explants. The different letters behind data mean significantly different from each other at $p \leq 0.05$ level, according to Duncan's multiple range test.

Table 5. Effect of NAA on root induction and growth of adventitious shoots.

No.	NAA (mg/l)	Rooting rate (%)	The number of roots	Growth status of roots
TCR1	0	67.22±7.52b	10.33±0.58ab	Thin roots without callus at the base
TCR2	0.1	93.33±11.55a	12.33±2.08a	Strong roots with few callus at the base
TCR3	0.2	80.56±17.35ab	8.33±0.58b	Strong roots with a lot of calli at the base
TCR4	0.3	85.00±13.23ab	8.67±1.53b	Short roots with many calli at the base

Values are mean±standard error in triplicate, each with 30 explants. The different letters behind data mean significantly different from each other at $p \leq 0.05$ level, according to Duncan's multiple range test.

per explant was 3.33 to 3.67 (Table 3). Those data indicated that each section induced adventitious shoots under appropriate culture conditions. This finding is consistent with a previous study on *Brassica napus* L. (Shi and Zhou 1998), but inconsistent with a study on *Platanus acerifol* (Liu and Bao 2009) and *Solanum melongena* (Sharma and Rajam 1995; Zhang et al. 2014). In *P. Acerifol*, the cotyledon section readily induced adventitious shoots, while polarity phenomenon easy shown near the radicle in S. *melongena*.

The placement of explants influenced the induction of shoots. Significant increases in differentiation were observed when the explants horizontally placed on the medium. Hypocotyls which placed vertically stand on the medium exhibited poor differentiation, with calli observed only in the wounds that were exposed to the culture medium, the shoot regeneration frequency was just 3.82%. Additionally, calli were brown until explant death. However, horizontal explants expanded rapidly, exhibited transparent calli containing green buds and produced adventitious shoots, the shoot regeneration frequency was 41.64%, and the number of shoots per explant was 3.33.

Effects of plant growth regulators on shoot multiplication and elongation

To achieve normal growth following shoot induction, healthy adventitious shoots were transferred to medium optimized for multiplication and elongation. Compared with the adventitious shoots induction culture medium, this medium had reduced 6-BA but added NAA instead of IBA to promotes cell division and elongation (Campanoni and Nick 2005). The result is shown in Table 4. Assessments of the nine different culture media combinations for multiplication and elongation revealed that increases in NAA concentrations initially increased the multiplication frequency when 6-BA was kept in the same concentration, but then decreased with further as the concentration increased. Shoot heights of 3.87 cm were achieved in plants cultured in MS medium containing 0.3 mg/l 6-BA and 0.2 mg/l NAA, and this height was significantly greater than that of seedlings grown on other media (Figure 1C). Furthermore, a multiplication coefficient of 3.06 was observed in these adventitious shoots. Thus, this medium composition was most suitable for shoot multiplication and elongation.

Rooting culture

Shoots were cut to lengths of 3–4 cm and transferred to half-strength MS medium containing different concentrations of NAA that played a mainly role in rooting (Wei et al. 2015). After 7 days of culture, adventitious roots were formed. Rooting frequencies and the number of roots were determined after 20 days of culture. As shown in the Table 5, no significant differences in rooting frequencies were observed based on the culture medium used. But many calli were induced on the medium supplemented with more than 0.3 mg/l NAA.

Treatment with TCR2 generated the highest rooting frequency and average root number, were 93.33% and 12.33, respectively. TCR2 was comprised of half-strength MS with 0.1 mg/l NAA, 1.5% sucrose (w/v) and 0.5% agar (w/v) (Figure 1D). Additionally, roots grown in TCR2 were sufficiently strong for acclimatization and transplantation.

NAA is routinely used in rooting studies, and therefore, we only added it to our rooting medium. Also, as was found with *Syzygium alternifolium* (Khan et al. 1999), *Vernicia fordii* (Lin et al. 2016), *Crassocephalum crepidioides* (Opabode et al. 2017) and *Couroupita guianensis* aubl. (Shekhawat and Manokari 2016), the sucrose and nutrient contents (used half-strength MS) were decreased to increase rooting.

Acclimatization and transplantation

Washed plantlets were transplanted to sterilized nutrient soil containing vermiculite at a 2 : 1 ratio. Using this mixture, the 20-day survival frequency was greater than 90%. After 20 days of growth, the root systems were developed, and the leaves were dispersed with new leaves growing (Figure 1E). Plant heights were greater than 30 cm in two months after transplantation, then grew healthy (Figure 1F, G).

Conclusions

T. ciliata is an important and precious tree species in China and abroad. Since this species is exposed to pests and cold temperatures, production is often impeded. Genetic engineering has been actively promoted to increase its production; however, this has not been systematically investigated, and the transgenic technologies for *T. ciliata* are lacking.

In this study, we optimised transplantation methods and culture conditions for adventitious shoot induction, multiplication and elongation, and rooting using hypocotyl explants. We investigated multiple factors that affected regeneration. The regeneration system established in this study could provide experimental and technical references for future genetic improvements, conservation and production of *T. ciliata*.

Conflict of interest statement

The authors declare no conflict of interest.

Author Contribution Statement

CX designed the experiments. LP and SY performed them and analyzed the data. LP wrote the manuscript. ZW, MW, LJ and LJ carried out the shoot multiplication and elongation and field acclimatization experiment. All authors read and approved the final manuscript.

Statement

The researchers only collected the seeds from *Toona ciliata* and didn't harm the tree body. The collected behavior through the consent and supervision of the local protection agency and the forestry bureau. In this study, we get the help from Baoshan forestry bureau. Hereby declare.

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