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Full Length Article

High-efficiency Regeneration of Seedlings from Hypocotyl Explants of Tung Tree (Vernicia fordii)

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Abstract

Tung tree (*Vernicia fordii*) is one of the most important woody oil plants and provides the sole source of tung oil widely used in the industry. An efficient and reproducible *in vitro* regeneration method is required for genetic transformation in tung tree. In this study, we reported an effective and reproducible regeneration method for tung tree using hypocotyl explants. The optimum condition for callus induction was observed in woody plant medium (WPM) supplemented with 5.0 mg L^{-1} 6-benzyladenine (BA), 1.0 mg L^{-1} kinetin (KT) and 0.1 mg L^{-1} α-naphthaleneacetic acid (NAA), with an induction rate of 100%. High frequency of shoot initiation was achieved in WPM supplemented with 1.0 mg L^{-1} 6-BA, 0.05 mg L^{-1} NAA and 2.0 mg L^{-1} gibberellic acid (GA_3), with an induction rate of 82.46%. Efficient shoot proliferation was obtained in WPM supplemented with 2.0 mg L^{-1} KT, 0.05 mg L^{-1} indole-3-acetic acid (IAA), and 2.0 mg L^{-1} GA_3, with a multiplication index of 6.8. Shoot elongation and strengthening was noted in WPM supplemented with 1.0 mg L^{-1} NAA and 2.0 mg L^{-1} GA_3. High rooting rate of 97.1% was achieved in 1/2 Murashige and Skoog (1962) (MS) medium supplemented with 0.1 mg L^{-1} indolebutyric acid (IBA). The well-rooted *in vitro* regenerated plantlets were successfully transferred to mixed substrates of peat moss: perlite: loess (1:1:1) and their survival rate reached 91.67% under natural environment. The efficient and reproducible *in vitro* regeneration method is established from hypocotyl explants, which could be applied for agrobacterium-mediated genetics transformation in tung tree. © 2016 Friends Science Publishers

Keywords: Adventitious shoot; Callus; Hypocotyl; Plant regeneration; Tung tree
Abbreviations: WPM, Woody plant medium; MS, Murashige and Skoog; 6-BA, 6-Benzylaminopurine; GA_3, Gibberellin acid; IAA, indole-3-acetic acid; IBA, indolebutyric acid; KT, Kinetin; NAA, α-naphthaleneacetic acid

Introduction

Tung tree (*Vernicia fordii*), one member of Euphorbiaceae family, is an important woody oil tree species native to China. Together with Oil-tea tree (*Camellia oleifera*), walnut (*Juglans regia*) and Chinese tallow tree (*Sapium sebiferu*m), they are called the four major woody oil trees in China (Tan *et al*., 2011). Tung oil extracted from tung seeds possesses excellent characteristics such as quick drying, excellent adhesion, light specific gravity, good gloss, acid and alkali resistance, heat resistance, frost crack resistance and waterproof. It is considered as the top-quality plant drying oil in the world, and can be used for producing high quality paint and printing pink, and for synthesizing new composite materials with a wide range of applications (Tan *et al*., 2011).

Tung blight (*Fusariumoxy sporum* f. sp. aleuritidis) is known as a devastating disease in the distribution areas of tung tree, which hinders the steady development of tung oil industry (Fang and He, 1998). Disease-resistant tung tree plants may be obtained via genetic transformation method, which has bright prospects in tung tree genetic improvement. Genetic transformation system of tung tree has not been established yet, although tung tree regeneration by *in vitro* leaves via indirect organogenesis means has been reported (Tan *et al*., 2013). To our knowledge, the tender leaves of tung tree are easily damaged by agrobacterium and susceptible to antibiotics such as kanamycin and hygromycin, which may result in lower regeneration activity. An efficient method for plant regeneration is a basic prerequisite for the molecular genetic improvement. Both leaves and hypocotyls contain a lot of protoplasts that have the potential for regenerating into intact plants (Grzebelus *et al*., 2012). Hypocotyls are derived from zygotic embryo and accordingly possess high embryogenic activity and regeneration potential. Hypocotyl explants have been used to achieve regenerated plants in several species including *Capsicum* (Gunay and Rao, 1978), *Peganum harmala* (Ehsanpour and Saadat, 2002), and *Cucumis sativus* (Andr´ysková *et al*., 2009). In comparison with leaves and

cotyledons, the hypocotyl explants were found to have higher differentiation capacity for *in vitro* plant regeneration in *Arctium lappa* L. (He *et al*., 2006), *Brassica oleracea* L. var. Capitata (Munshi *et al*., 2007), and *Lepidium campestre* (Ivarson *et al*., 2013), *Solanum melongena* L. (Muthusamy *et al*., 2014). To date, agrobacterium-mediated genetic transformation method has been successfully established using hypocotyl explants in *Poncirus trifoliate* Raf. (Kaneyoshi *et al*., 1994), *Antirrhinum majus* L. (Cui *et al*., 2003) and *Solanum lycopersicum* L. (Sivankalyani *et al*., 2014). For these species, leaf explants also could be used for agrobacterium infection producing transgenic plants but with low transformation efficiency since leaf segments are easily damaged by agrobacterium and susceptible to antibiotics. By contrast, the hypocotyls have a strong resistance to antibiotics, which is good for introduction of foreign genes and regeneration of transformed plants. We have established a procedure for plant regeneration from tung tree leaves, but seedlings regenerated from leaf explants developed abnormally and only 40% of the regenerated plants survived later (Tan *et al*., 2013). Therefore, it was necessary to develop a more efficient protocol for regenerating plants for transgenic studies in the future. The objective of this study was to establish an efficient protocol for regenerating plants from tung tree hypocotyls.

Materials and Methods

Plant Materials

Mature seeds were collected from 4-years old 'Putaotong' planted at Central South University of Forestry and Technology Germplasm Repository (CSUFTGR) in Qingping Town, Yongshun County, Hunan Province (110°29'E, 28°32'N with altitude at 530-600 m and annual average temperature of 16°C). After air dried seed coats were removed, the remaining seeds were washed thoroughly with tap water for 5 - 6 times, soaked in tap water for 24 h, sterilized in 0.1% $HgCl_2$ for 3 min, and finally rinsed three times with sterile distilled water. Embryos were isolated and cultured on 1/2MS medium supplemented with 0.5 mg L^{-1} IAA to produce aseptic seedlings (Li *et al*., 2012).

Callus Induction

Hypocotyls of 30-days-old seedlings derived from isolated tung tree embryos were cut into segments of approximately 5 mm in length. The hypocotyl segments were inoculated into the WPM medium supplemented with 6-BA (3.0, 5.0, 7.0, 10.0 mg L^{-1}), NAA (0.05, 0.1, 0.5 mg L^{-1}), and KT (1.0 mg L^{-1}) and incubated in the dark for 3 days and then exposed to light for 25 days. The culture without hormones was included as a control. Four hypocotyl explants were cultured per flask and three replicates with 40 explants per treatment were conducted.

Adventitious Shoot Induction

After callus induction for 30 days, the hypocotyl explants were transferred onto WPM medium supplemented with 6-BA (0.5, 1.0, 2.0 mg L^{-1}), NAA (0, 0.01, 0.05, 0.1 mg L^{-1}) and GA_3 (2.0 mg L^{-1}) under light for formation of adventitious shoots. The culture without hormones was included as a control. Four hypocotyl explants were cultured per flask and three replicates with 40 explants per treatment were conducted.

Multiple Shoot Formation

Adventitious shoots with approximately 1 cm in length were excised after 40 days in culture and inoculated into multiplication media i.e., WPM supplemented with KT (1.0, 2.0, 3.0 mg L^{-1}), IAA (0, 0.05, 0.1, 0.2 mg L^{-1}), and GA_3 (2.0 mg L^{-1}) under light for multiple shoot formation. The culture without hormones was included as a control. Two explants were cultured per flask and three replicates with 40 explants per treatment were conducted.

Shoot Elongation and Strengthening

The multiple shoots were cut apart and placed in flasks containing WPM medium supplemented with NAA (0.5, 1.0, 2.0 mg L^{-1}) and GA_3 (0.5, 1.0, 2.0, 3.0 mg L^{-1}) under light for shoot elongation and strengthening culture. The culture without hormones was included as a control. Two explants were cultured per flask and three replicates with 40 explants per treatment were conducted.

Rooting Culture

The healthy and uniform shoots with about 2.0 cm in length were inoculated into 1/4MS, 1/2MS and MS media supplemented with IBA (0, 0.1, 0.3, 0.5 mg L^{-1}) and NAA (0, 0.1, 0.3, 0.5 mg L^{-1}), respectively, for rooting culture. The rooting culture without hormones was included as a control. The shoots were first cultured in the dark for 5 days and then exposed to light. One explant was cultured per flask and three replicates with 30 explants per treatment were conducted.

Acclimatization and Transplantation of Plantlets

For acclimatization, plantlets with well-developed root systems (about 5 cm long) were transferred to a climate chamber with humidity at 60% - 70% for 2 - 3 days. Afterwards, the plantlets were transferred to pots in mixed substrates of peat moss: perlite: loess = 1:1:1 after cleaning the roots to fully remove the media. The pots covered with plastic film were placed in greenhouse. The plastic films were removed one week later and then pray water to keep the microenvironment humidity above 80.0%. Plant survival rate was calculated after 30 days.

Cultivation Conditions

WPM was used as the basal medium for tung tree regeneration with exception of rooting culture using 1/2MS medium, supplemented with 3% sucrose and 0.7% agar and pH was adjusted to 5.8 prior to autoclaving at 121°C for 20 min. All cultures were maintained at 26±1°C under a 14 h photoperiod with 50-60 $\mu molm^{-2} s^{-1}$ irradiance provided by cool white fluorescent light.

Experimental Design and Data Analysis

Callus induction rate was calculated using the formula: (number of hypocotyl explants producing callus/total number of hypocotyl explants) × 100%. Adventitious shoot induction rate was calculated using the formula: (number of explants differentiating into adventitious shoots/total number of explants) × 100%. Rooting percentage was calculated as (number of shoots with rooting/total number of shoots inoculated) × 100%. Tests were conducted in randomized block design with three replicates as described by Cavusoglu *et al*. (2011). The data were expressed mean standard error and analyzed by the Duncan's multiple range test at P = 0.05 to determine significant differences. The statistical analyses were carried out with SPSS17.0 software package.

Results

Callus Induction from Hypocotyl Explants in Tung Tree

Hypocotyl segments derived from tung tree aseptic seedlings were inoculated into the WMP medium with various hormonal combinations. After 3 days cultured in the dark, no obvious change was observed on the explants. When exposed to light (Fig. 1A), callus was observed at wounds of hypocotyl explants within around 30 days (Fig. 1B). Callus could be successfully induced from all of the hypocotyl explants under each hormonal combination (Table 1). When 6-BA concentration of 5.0 mg L^{-1} combined with NAA of 0.1 mg L^{-1} were applied, the callus induction rate reached 100% and the calli were of high quality and easy to develop into adventitious shoots. When higher concentration of 6-BA was increased, the callus induction rate was still high but the calli were of low quality and not easy to develop into adventitious shoots. Therefore, the optimum medium for callus induction from tung tree hypocotyls was WPM containing 5.0 mg L^{-1} 6-BA, 1.0 mg L^{-1} KT, and 0.1 mg L^{-1} NAA, which resulted in an induction rate of 100%.

Adventitious Shoot Induction from Callus Tung Tree

The hypocotyl segments with calli were inoculated onto WPM medium supplemented with various 6-BA, NNA and GA_3 concentrations for adventitious shoot induction (Fig. 1C-E). Adventitious shoots could be produced from

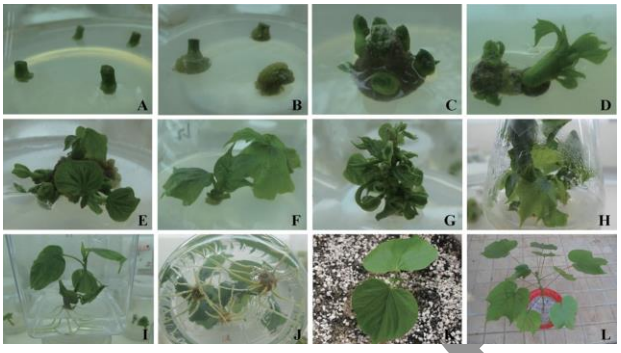

Fig. 1: Plant regeneration from hypocotyl explants of tung tree

A: Inoculation of hypocotyl segments; B: Calli induced from hypocotyls: C, D, and E: Adventitious shoots differentiated from callus; F and G: Multiplication of adventitious shoots; H: Shoot elongation and strengthening; I and J: Rooted plants; K: Regenerated plantlets transplant after acclimatization; L: 90 days after transplant

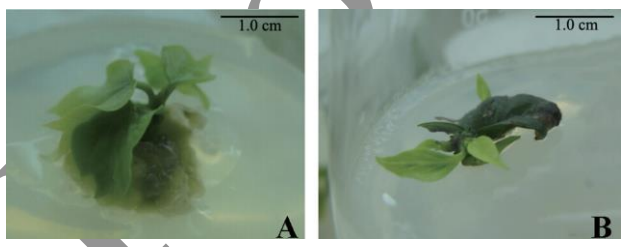

Fig. 2: Comparison of shoots produced from hypocotyls and leaves

A: Shoots derived from hypocotyls; B: Shoots derived from leaves

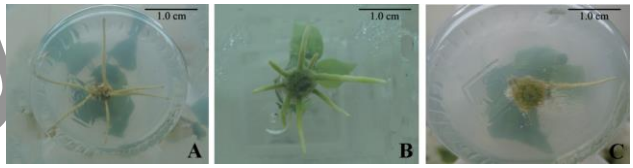

Fig. 3: Effects of different concentrations of IBA on rooting culture of tung tree

A: 0.1 mg L^{-1} IBA produces healthy roots without callus at base; B: 0.3 mg L^{-1} IBA leads to development of callus at base; C: 0.5 mg L^{-1} IBA leads to decrease of root numbers

the callus under all hormonal combinations but with different shoot growth vigour. The adventitious shoot induction rate first increased and then decreased as 6-BA concentration increased under the condition of constant NAA concentration (Table 2). When 6-BA concentration was constant, the shoot induction rate also first increased and then decreased as NAA concentration increased. When 6-BA of 1.0 mg L^{-1} combined with NAA of 0.05 mg L^{-1} were applied, adventitious shoot induction rate reached at 82.46%. The produced adventitious shoots were green with good growth vigour and easy for subculture multiplication. Therefore, the optimum medium for adventitious shoot induction from tung tree hypocotyl callus was WPM+1.0 mg L^{-1} 6-BA+0.05 mg L^{-1} NAA+2.0 mg L^{-1} GA_3.

Table 1: Effects of different hormonal combinations on callus induction from tung tree hypocotyls

No.	Plant growth regulators			Callus induction rate (%)	Description of callus growth
	6-BA (mg L^{-1})	NAA (mg L^{-1})	KT (mg L^{-1})		
A_1	0	0	0	0^{f}	No callus was produced
A_2	3.0	0.05	1.0	68.23 ± 2.14^{d}	The callus was small and not easy to develop into adventitious shoots
A_3	3.0	0.1	1.0	86.12 ± 1.87^{c}	The callus was small and greenish white
A_4	3.0	0.5	1.0	100 ± 0^{a}	A large number of compact calli were induced but not easy to develop into adventitious shoots
A_5	5.0	0.05	1.0	86.05 ± 3.01^{c}	The callus was loose and pale surface
A_6	5.0	0.1	1.0	100 ± 0^{a}	The callus was easy to develop into adventitious shoots
A_7	5.0	0.5	1.0	100 ± 0^{a}	A large number of calli were induced but not easy to develop into adventitious shoots
A_8	7.0	0.05	1.0	92.33 ± 1.98^{b}	The callus was loose and dark brown
A_9	7.0	0.1	1.0	100 ± 0^{a}	The callus was not easy to develop into adventitious shoots
A_{10}	7.0	0.5	1.0	100 ± 0^{a}	The callus was compact and not easy to develop into adventitious shoots
A_{11}	10.0	0.05	1.0	100 ± 0^{a}	The callus was pale with loose surface and not easy to develop into adventitious shoots
A_{12}	10.0	0.1	1.0	100 ± 0^{a}	The callus was yellowish and grows rapidly
A_{13}	10.0	0.5	1.0	100 ± 0^{a}	The callus was dark green, compact, and not easy to develop into adventitious shoots

Table 2: Effects of different hormonal combinations on adventitious shoot induction from tung tree hypocotyl callus

No.	Plant growth regulators			Adventitious induction rate	Shoot Description of shoot growth
	6-BA (mg L^{-1})	NAA (mg L^{-1})	GA_3 (mg L^{-1})		
B1	0	0	0	0^{f}	No adventitious shoots were produced
B_2	0.5	0	2.0	$66.32\%\pm2.24^{c}$	The adventitious shoots were short with slow growth
B_3	0.5	0.01	2.0	$76.15\%\pm2.20^{b}$	The adventitious shoots were short with slow growth
B_4	0.5	0.05	2.0	$80.47\%\pm1.86^{a}$	The adventitious shoots were strong with a number of leaves
B_5	0.5	0.1	2.0	$63.24\%\pm3.32^{c}$	The shoot leaves were broad without clear apical buds
B_6	1.0	0	2.0	$74.23\%\pm2.85^{b}$	The adventitious shoots were short with few leaves
B_7	1.0	0.01	2.0	$80.46\%\pm2.34^{a}$	The adventitious shoots were strong with a number of leaves
B_8	1.0	0.05	2.0	$82.46\%\pm1.97^{a}$	The adventitious shoots were green and strong with rapid growth
B_9	1.0	0.1	2.0	$67.15\%\pm2.05^{c}$	The adventitious shoots were yellowish with a number of leaves
B_{10}	2.0	0	2.0	$42.36\%\pm3.17^{e}$	A number of multiple shoots were produced and not easy for subculture multiplication
B_{11}	2.0	0.01	2.0	$56.32\%\pm2.66^{d}$	The adventitious shoots were green and strong
B_{12}	2.0	0.05	2.0	$53.54\%\pm2.38^{d}$	The shoot leaves were small without clear apical buds
B_{13}	2.0	0.1	2.0	$33.69\%\pm3.55^{e}$	Low quality shoots were produced with calli

Multiple Shoot Formation in Tung Tree

High shoot multiplication can result in high frequency and efficiency of plant reproduction, which is also an important indicator estimating plantlets output in industrialized seedling production. The WPM medium supplemented with only KT could produce multiple shoots with the multiplication index ranging from 1.6 to 2.6, whereas the multiple shoots were not strong enough (Table 3). When IAA was applied, the shoot multiplication index was increased by 161.54% with a significant difference (Fig. 1F and G). This result indicated that KT dominated the multiplication of adventitious shoots derived from tung tree hypocotyls. The medium most suitable for adventitious shoots multiplication of tung tree was WPM+2.0 mg L^{-1} KT+0.05 mg L^{-1} IAA+2.0 mg L^{-1} GA_3, with a multiplication index of 6.8.

Shoot Elongation and Strengthening Culture in Tung Tree

After 30 days in culture, the multiple shoots were cut apart and inoculated for elongation and strengthening culture. The diameters and lengths of shoots were measured after 30 days in culture (Fig. 1H). When GA_3 concentration was constant, the diameter and length of shoots first increased and then decreased as 6-BA concentration increased (Table 4). The diameter and length of shoots reached the maximum value at 0.46 cm and 5.3 cm, respectively, in the WPM medium supplemented with NAA of 1.0 mg L^{-1} and GA_3 of 2.0 mg L^{-1}. Under such a culture condition, the shoots were dark green and showed high growth vigour, which was good for subsequent rooting culture.

Rooting Culture in Tung Tree

The shoots of appropriately 2 cm in length were inoculated into 1/4MS, 1/2MS, and MS media supplemented with different concentrations of IBA and NAA for rooting culture. Adventitious roots were developed within 7 days (Fig. 1I and J). As shown in Table 5, in the treatment of E1, E5 and E12, no roots were produced without IBA or NAA in the media. Therefore, either IBA or NAA was required for root formation from tung tree shoots. Moreover, different types and concentrations of auxin and different types of basal medium resulted in different effects on rooting rate and root growth. The 1/2MS medium was better than 1/4MS and MS media for rooting culture. IBA was better than NAA for root formation and the latter also led to serious callus at the base of shoots with low rooting rate,

Table 3: Effects of different hormonal combinations on adventitious shoot multiplication of tung tree

No.	Plant growth regulators			Multiplication index
	KT (mg L^{-1})	IAA (mg L^{-1})	GA_3 (mg L^{-1})	
C_1	0	0	0	0^{f}
C_2	1.0	0	2.0	1.6 ± 0.3^{d}
C_3	1.0	0.05	2.0	4.2 ± 0.2^{b}
C_4	1.0	0.1	2.0	3.7 ± 0.2^{b}
C_5	1.0	0.2	2.0	2.5 ± 0.4^{c}
C_6	2.0	0	2.0	2.6 ± 0.3^{c}
C_7	2.0	0.05	2.0	6.8 ± 0.3^{a}
C_8	2.0	0.1	2.0	3.9 ± 0.1^{b}
C_9	2.0	0.2	2.0	3.6 ± 0.2^{b}
C_{10}	3.0	0	2.0	2.0 ± 0.3^{c}
C_{11}	3.0	0.05	2.0	2.6 ± 0.4^{c}
C_{12}	3.0	0.1	2.0	2.3 ± 0.3^{c}
C_{13}	3.0	0.2	2.0	1.5 ± 0.1^{e}

Table 4: Effects of different hormonal combinations on shoot elongation and strengthening of tung tree

No.	Plant growth regulators		Diameter of shoots (cm)	Length of shoots (cm)
	NAA (mg L^{-1})	GA_3 (mg L^{-1})		
D_1	0	0	0.12 ± 0.01^{f}	1.5 ± 0.1^{d}
D_2	0.5	0.5	0.15 ± 0.01^{ef}	2.2 ± 0.1^{c}
D_3	0.5	1.0	0.17 ± 0.01^{e}	2.9 ± 0.2^{c}
D_4	0.5	2.0	0.23 ± 0.03^{d}	3.5 ± 0.1^{b}
D_5	0.5	3.0	0.19 ± 0.02^{de}	1.8 ± 0.3^{d}
D_6	1.0	0.5	0.20 ± 0.01^{d}	3.3 ± 0.2^{b}
D_7	1.0	1.0	0.36 ± 0.03^{b}	5.2 ± 0.4^{a}
D_8	1.0	2.0	0.46 ± 0.02^{a}	5.3 ± 0.3^{a}
D_9	1.0	3.0	0.33 ± 0.02^{b}	3.2 ± 0.2^{b}
D_{10}	2.0	0.5	0.14 ± 0.01^{f}	2.2 ± 0.3^{c}
D_{11}	2.0	1.0	0.20 ± 0.02^{d}	3.7 ± 0.2^{b}
D_{12}	2.0	2.0	0.28 ± 0.02^{c}	3.6 ± 0.3^{b}
D_{13}	2.0	3.0	0.13 ± 0.01^{f}	1.9 ± 0.2^{d}

and the produced roots were not healthy enough for acclimatization and transplantation. The optimum media for tung tree rooting was 1/2MS +0.1 mg L^{-1} IBA, with a rooting rate of 97.12%.

Acclimatization and Transplants

The plantlets were put in climate chamber with humidity at 70% - 80% for acclimatization for 2 - 3 days. Then the plantlets were taken out, cleaned the media by tap water and then transplanted into mixed substrates of peat moss: perlite: loess = 1:1:1 (Fig. 1K). In total, 60 plantlets were transplanted and 55 survived and grew well after 30 days with a survival rate of 91.67%. The survived plants, generally, could reach 45 cm in height within 3 months (Fig. 1L). All the regenerated plantlets were transferred to soil and can be used for conservation and plantation purposes.

Discussion

Establishment of a highly efficient regeneration system for tung tree will provide a convenient tool for tung tree tissue culture and transformation, thereby facilitating the transformation of foreign genes into tung tree. The endogenous hormones contained in different organs of the same plant, and in different parts of the same organ are different (Nagalakshmi *et al*., 2014). Therefore in different organs of a plant and in different parts of the same organ the regeneration capacity is different. In the process of adventitious shoot induction, it is found that the callus growth and adventitious shoots induction are different between the top and base of hypocotyls segments, mainly due to different auxin content in different parts of hypocotyls. Auxin is mainly synthesized at apical meristem and transported to the base and other parts. Therefore, a conclusion could be drawn that different auxin content in different parts leads to different potential of adventitious shoot induction. In our previous study, we used the hormonal combinations developed for tung tree regeneration from leaves (Tan *et al*., 2013) to produce the regenerated plantlets using hypocotyl explants but failed, which further confirms hypocotyls and leaves contain different content of endogenous hormones. For plant *in vitro* culture, healthy and strong adventitious shoot culture is the key step for successful plant regeneration. The adventitious shoots derived from hypocotyls in this study show better growth vigour and are stronger (Fig. 2A) than those from leaves (Fig. 2B), indicating hypocotyls might bear more potential of plant regeneration than leaves. Therefore, hypocotyls could be considered as better materials for establishment of genetic transformation system in tung tree.

In the process of subculture multiplication, we found that adventitious shoots were not easy to develop into multiple shoots when using MS medium. Instead, the WPM medium supplemented with GA_3 had a dramatic effect on multiple shoot formation from tung tree adventitious shoots, which is consistent with the effect of GA_3 on multiple shoot formation of seedless *Siraitia grosvenorii* (Chen, 2013). According to Chen (2013), the multiplication index could increase to 16.4 when GA_3 was added into MS medium. In the process of shoot elongation and strengthening culture, increasing gibberellins and reducing cytokinins would be helpful for shoot growth, which is in accordance with results obtained from other plant species including *Acca sellowiana* (Berg) Burret (Cangahuala-Inocente *et al*., 2007), *Capsicum annuum* L. (Kumar *et al*., 2012), and *Mytilaria laosensis* Lecomte (Qiu *et al*., 2013). Culture of strong and healthy shoots is the key step for plant rooting. In this study, we found that 1/2MS medium worked well for tung tree rooting culture. According to the study of Luo and Xu (1987) on aspermous watermelon rooting experiment, low concentration of inorganic salt was good for plant rooting and root growth. In this study however, we found that rooting rate was higher by using 1/2MS medium than that by using MS medium and 1/4MS medium, which is consistent with results of rooting study on *Celastrus angulatus* (Ma *et al*., 2004). Ma *et al*. (2004) also found that 1/2MS medium was better for root formation of *C. angulatus* than MS medium and 1/4MS medium.

Table 5: Effects of different hormonal combinations on rooting from tung tree shoots

No.	Medium	Plant growth regulators		Rooting rate (%)	Number of roots	Average length of roots (cm)	Description of roots growth
		IBA (mg L^{-1})	NAA (mg L^{-1})				
E_1	1/4 MS	0	0	0^c	0^d	0^c	No roots were produced
E_2	1/4 MS	0.1	0	83.12 ± 1.45^a	6.1 ± 0.6^a	3.4 ± 0.3^a	White roots were produced without callus at base
E_3	1/4 MS	0.3	0	80.71 ± 1.40^a	5.8 ± 0.5^a	3.3 ± 0.2^a	White roots were produced with few calli
E_4	1/4 MS	0.5	0	60.05 ± 1.73^b	4.4 ± 0.6^b	2.7 ± 0.3^b	Few roots were produced with a lot of calli at base
E_5	1/2 MS	0	0	0^c	0^d	0^c	No roots were produced
E_6	1/2 MS	0.1	0	97.12 ± 1.26^a	10.2 ± 0.9^a	3.6 ± 0.3^a	White roots were produced without callus at base
E_7	1/2 MS	0.3	0	93.34 ± 1.38^a	9.5 ± 0.7^a	3.9 ± 0.2^a	White roots were produced with few calli
E_8	1/2 MS	0.5	0	79.95 ± 2.37^b	7.1 ± 0.8^b	3.1 ± 0.3^b	The produced roots were greenish with a lot of calli at base
E_9	1/2 MS	0	0.1	70.23 ± 2.58^b	7.2 ± 0.6^b	2.7 ± 0.1^b	The produced roots were thin without callus at base
E_{10}	1/2 MS	0	0.3	68.76 ± 1.97^b	6.5 ± 0.6^b	3.2 ± 0.1^b	The produced roots were greenish with few calli at base
E_{11}	1/2 MS	0	0.5	66.35 ± 3.56^b	4.3 ± 0.4^c	4.0 ± 0.2^a	The produced roots were greenish and thick with a lot of calli at base
E_{12}	MS	0	0	0^c	0^d	0^c	No roots were produced
E_{13}	MS	0.1	0	78.56 ± 1.34^a	5.6 ± 0.7^a	2.9 ± 0.2^a	The produced roots were thin without callus at base
E_{14}	MS	0.3	0	67.34 ± 1.21^a	4.7 ± 0.6^a	3.0 ± 0.1^a	The produced roots were thin with few calli at base
E_{15}	MS	0.5	0	56.08 ± 1.87^b	3.5 ± 0.7^b	2.5 ± 0.2^b	The produced roots were short and thick with a lot of calli at base

These findings indicate that too low or too high concentration of inorganic salt may prevent the rooting of shoots, and that different plant species have different response to inorganic salt. In addition, IBA was found to be better than NAA for rooting culture (Table 5). Appropriate concentration (0.1 mg L^{-1}) of IBA yielded strong roots without callus at base (Fig. 3A), whereas higher concentration (0.3 mg L^{-1}) of IBA led to formation of calli at base of roots (Fig. 3B). When IBA was increased to 0.5 mg L^{-1}, the rooting rate tended to decrease and a lot of calli were generated (Fig. 3C), which is not good for plant acclimatization and transplantation.

Conclusion

This study successfully develops an efficient regeneration system from hypocotyls of tung tree, which mainly includes callus induction, adventitious shoot differentiation, multiple shoot formation, shoot elongation and strengthening, and highly efficient rooting. This regeneration system could be applied for industrialized seedling cultivation and genetic transformation of tung tree.

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