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IN VITRO PROPAGATION OF NANJING LINDEN (*TILIA MIQUELIANA* MAXIM.)

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Abstract

Tilia miqueliana Maxim. is one of the most important tree species used in landscape architecture, especially in larger cities of China. Because of the high demand for this species, the development of an efficient micropropagation system can help to ensure an adequate supply of plants needed for city landscaping and reforestation. Seedlings were induced from seeds cultured on woody plant medium (WPM) without glycine, but supplemented with 8.66 μM Giberellic acid. Nodal segments with axillry buds were cultured on establishment WPM with 2.28 μM zeatin and 0.2 μM IBA. The resulting elongated axillary shoots were cut into nodal segments and transferred to shoot multiplication medium. The highest mean number of shoots (2.5) and nodes (4.5) was induced on WPM with 2.22 μM BA, 2.28 μM zeatin, and 0.1 μM IBA; while highest rooting (91.7%) was achieved on half-strength Murashige and Skoog medium with 14.76 μM IBA.

Key words: axillary bud, multiplication, nodal segment

INTRODUCTION

Nanjing linden (*Tilia miqueliana* Maxim., Tiliaceae) is a native tree species of Jiangsu province, China. It is highly valued as an ornamental tree and is nectar source for bees. Propagation of linden is conducted primarily *via* cuttings and seeds. However, these methods suffer from disadvantages such as poor rooting or low germination rates (Rose 1919, Spaeth 1932, Yang et al. 2011). Also, it has been demonstrated that rooting of cuttings vary between 8.9% and 43% and depends from the age of stock plants, the season of cutting collection, and the type of cuttings (Yang et al. 2010). In contrast, micropropagation is regarded as an effective method of obtaining seedlings of linden. Chalupa (1984, 1990) established a shoot proliferation method by using nodal segments from 1-year old seedlings and plant regeneration by somatic embryogenesis from cultured immature embryos of *T. cordata*. To our knowledge, no study has investigated the tissue culture of *T. miqueliana*. To satisfy an ever increasing horticultural demand for this species it was therefore considered useful to develop an effective *in vitro* multiplication and propagation protocol.

Nodal segments that already have a developed meristem are suitable for micropropagation, as these are more easily manipulated, and additionally have a high proliferation rate while retaining clonal fidelity (Vijaya and Padmaja 1999). It has proven difficult to establish a tissue culture protocol for *T. miqueliana* using mature plant material, obtained directly from the field. This seems to be mostly attributable to high level of contamination (Lu et al. 2004). It has been reported that embryo culture is considered to be an efficient approach for eliminating dormancy and is a convenient initial source for establishment of shoot cultures (Ucler and Mollamehmetoglu 2001). However, seed-surface disinfection procedures can have a negative effect on the viability of seedlings (Obeidy and Smith 1990, Renukdas et al. 2010).

The objective of our research was to study the effect of plant growth regulators for axillary shoot induction, multiplication, and rooting *in vitro*.

MATERIALS AND METHODS

Plant material

Mature fruits were collected from one 20-year-old tree in mid-November. Following extraction, the seeds were washed in sterile water for 20 min and then disinfected by 500 mg l^{-1} carbendazim solution for 2 h (Sigma Chemical Co., St. Louis, MO, USA). After

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that they were washed three times with sterile water, and then placed into 10% (v/v) liquid anionic detergent (Amway, China), and agitated on a rotary shaker for 20 min at 100 rpm. The seeds were washed for 60 min under running water and disinfected by immersion in 75% (v/v) ethanol for 40 s. Following this pre-treatment, the seeds were then subjected to one of the following treatments: 1) 5.65% (v/v) sodium hypochlorite (NaClO) for 12 min; 2) 0.1% (w/v) mercuric chloride ($HgCl_2$) for 12 min; 3) 5.65% (v/v) NaClO for 6 min and subsequent treatment with 0.1% (w/v) $HgCl_2$ for 6 min; or 4) 2.83% (v/v) NaClO for 6 min, and subsequent treatment with 0.2% (w/v) $HgCl_2$ for 6 min. Finally, the seeds were rinsed in sterile deionized water for 5 min, with this washing step being repeated 4 times. Disinfected seeds were then transferred to a glass bottle (230 ml, 5 × 12 cm) containing 30 ml woody plants medium (WPM) without glycine (Lloyd and McCown 1980, McCown and Lloyd 1981) but supplemented with 8.66 μM Gibberellic acid (GA_3) (Sigma) (WPM1). The contamination rate was calculated after ten days.

Breaking of seed dormancy

The following treatments were used for the breaking of seed dormancy,

- Stored at 4°C for 2 days, and then cultured on WPM1 after disinfection.
- Placed in 40°C water for 2 h and then stored at 4°C for 2 days, followed by culture on WPM1 after disinfection.
- Cultured on WPM without glycine and plant growth regulators (WPM0) after incubation in 1.44 μM GA_3 for 3 days at 4°C.
- Cultured on WPM1 immediately after the thick peel was removed.

Germination of seeds was scored after 4 and 8 weeks of culture.

Culture establishment and multiplication of the axillary shoots

After culturing on WPM with 8.66 μM GA_3 for 8 weeks (Fig. 1A), nodal explants were isolated from the epycotils (Fig. 1B). Each nodal segment bearing a single axillary bud was cultured on WPM establishment medium, supplemented with 0.05, 0.91, 2.28, and 4.56 μM (6-[4-Hydrooxy-3-methil-but-2-enylamino] purine (ZT, zeatin, Sigma) or 0.04, 0.89, 2.22, 4.44 μM N^6-benzyladenine (BA, Sigma), and 0.20 μM indole-3-butyric acid (IBA, Sigma). Data were recordered after 4 weeks.

After 4 weeks of culture, elongated shoots (longer than 6 cm) were divided into nodal segments, each bearing a single axillary bud. Segments were then cultured on multiplication medium consisting of WPM supplemented with ZT (0.05, 0.45, 2.28, and 4.56 μM) or BA (0.04, 0.44, 2.22, and 4.44 μM) and 0.10 μM IBA, or supplemented with combination of 2.22 μM BA, 2.28 μM ZT, and 0.10 μM IBA, or with combination of 4.44 μM BA, 4.56 μM ZT and 0.98 μM IBA. Data were recordered after 4 weeks.

Rooting of the shoots

The rooting of elongated shoots (4-6 cm in length) was studied on half-strength Murashige and Skoog (1962) medium (MS) that contained IBA at various concentrations (0, 2.46, 4.92, 9.84, 14.76, or 24.60 μM). The result were evaluated after 4 weeks.

Culture conditions

All variants of the medium, were supplemented with 30 g l^{-1} sucrose (Ucler and Mollamehmetoglu 2001), while the rooting medium contained 20 g l^{-1} sucrose. The variants of the medium were solididified with 7.5 g l^{-1} agar (Sanland Chemical Co. Ltd., USA) and pH was adjusted to 5.8 before autoclaving at 121°C and 151 kPa for 15 min. All cultures were maintained at 25 ± 1°C with 14 h light at a photosynthetic photon flux density of 50 μmol m^{-2} s^{-1}.

Statistical analysis

Each treatment comprised either 100 seeds or 20 explants, and was repeated three times. Data about the germination rate of the seeds, response of explants, mean number of shoots, and mean number of nodes were analyzed using SPSS 13.0 for Windows (SPSS, Chicago, IL, USA). The mean differences were investigated at $p \leq 0.05$ level, according to one-way ANOVA and LSD's multiple range rest. For paired-comparisons between treatments, post-hoc analysis and independent-samples *t* test were used.

RESULTS AND DISCUSSION

Disinfection of seeds

After disinfection treatments, the contamination of seeds varied between 4.3% and 96.3% (Table 1). The combination of sodium hypochlorite and $HgCl_2$ was significantly better than sodium hypochlorite or $HgCl_2$ alone ($df = 3$, $F = 229.40$, $p < 0.001$). The effect of the combination of sodium hypochlorite and $HgCl_2$ was larger using high concentration sodium hypochlorite and low concentration $HgCl_2$ than using low concentration of sodium hypochlorite and high concentration of $HgCl_2$ ($df = 4$, $t = 12.59$, $p < 0.001$), which agrees with the results of He et al. (2011).

Breaking of seed dormancy

The breaking of seed dormancy was important factor for germination of the seeds. A long dormancy period in *Tilia* seeds had been previously reported (Dirr and

Table 1. Contamination rate of Nanjing linden seeds after disinfection treatments.

Treatment	Explants with bacterial contamination (%)
Sodium hypochlorite (5.65%) 12 min	96.3 ± 2.7 c
$HgCl_2$ (0.1%) 12 min	91.3 ± 1.2 c
Sodium hypochlorite (5.65%) 6 min, and $HgCl_2$ (0.1%) 6 min	4.3 ± 2.4 a
Sodium hypochlorite (2.83%) 6 min, and $HgCl_2$ (0.2%) 6 min	63.7 ± 4.1 b

Means ± SE followed by the same letters were not significantly different at $p \leq 0.05$, according to LSD's multiple range test.

Heuser 1987, Suszka et al. 1996, Baskin and Baskin 2001, Yang et al. 2011). The germination rates of seeds treated with the four treatments to break dormancy showed considerable variation (Table 2). The germination rate of seeds consecutively subjected to hot and cold treatment (incubated at 40°C water for 2 h and then stored at 4°C for 2 days) was not significantly different from the cold-treated seeds (stored in a refrigerator at 4°C for 2 days) after 4 weeks ($df = 4$, $t = 0.791$, $p = 0.437$) and 8 weeks of culture ($df = 4$, $t = 1.306$, $p = 0.262$). Seeds that were incubated in 1.44 µM GA_3 for 3 days and cultured on basal medium without GA_3 showed the lowest germination. However, the germination rate of completely untreated seeds that were directly cultured on WPM1 was in both cases higher than all other three treatments ($df = 4$, $t = 11.663$, $p < 0.001$; $df = 4$, $t = 32.870$, $p < 0.001$). During germination the seeds started to develop roots after 4 weeks and a shoot was observable after 5 weeks.

Culture establishment

Overall ZT performed better than BA with regard to induce axillary shoots formation on the explants (66-98% vs. 50-73%, respectively), with significant difference for ZT concentrations above 2.28 µM (Table 3). This was in accordance with results for *Acacia senegal* (Simon et al. 1993) and *Betula pendula* (Iliev et al. 2010). The medium supplemented with 2.28 µM ZT and 0.2 µM IBA yielded the best results (Table 3, Fig. 1C,D) were the explants demonstrated the highest induction response (98%), faster growth resulting in a mean length of 6.8 cm, and 4.3 nodes.

Shoot multiplication and elongation

Shoot multiplication is an important factor for tissue culture, determining the suitability of the protocol in question for mass propagation (Quraishi et al. 1996). Zeatin was one of the most effectively used cytocinins for shoot multiplication of woody species *in vitro* (Reed and Abdelnour-Esquivel 1991, Nour and Thorpe 1993, Al-Juboory et al. 1998, Lucchesini and Mensuali-Sodi 2004, Sghir et al. 2005). In the present study, some axillary buds of nodal segments elongated after 2 weeks of culture. After 4 weeks of culture, axillary buds developed into shoots and the mean number of shoots was significantly higher on medium containing low concentration of ZT and BA, in comparison to the medium containing higher concentration of cytokinins and the one containing only one of these cytokinins ($df = 9$, $F = 4.471$, $p = 0.003$, Table 4). In addition, the observed number of nodes correlated in the same way. The shoots grown on medium containing 2.28 µM ZT and 2.22 µM BA had a significantly higher mean number of nodes in comparison with shoots grown on the other medium ($df = 9$, $F = 3.524$, $p = 0.009$, Table 4). The highest mean number of shoots (2.5), as well as the highest mean number of nodes (4.5), were obtained on medium with 2.28 µM ZT and 2.22 µM BA combined with 0.1 µM IBA (Table 4).

We concluded that combining ZT and BA was a successful strategy for enhancing shoot multiplication, as long as certain concentrations were not exceeded. Similar results have been obtained for *Tilia platyphyllos* by Chalupa (2003), who reported longer shoots using MS medium supplemented with combination of (1-Phenyl-3-(1,2,3,-thiadiazol-5-yl)urea (thidiazuron, TDZ) or BA, in combination with IBA at low concentrations.

Rooting of shoots

Table 2. Germination rate of Nanjing linden seeds after application of different dormancy breaking treatments.

Treatment	Germinated seeds (%)	
	After 4 weeks	After 8 weeks
Stored at 4°C for 2 days without hot-treatment	28.3 ± 0.3 b	45.0 ± 0.6 b
Incubated in 40°C water for 2 h	30.0 ± 2.1 bc	49.7 ± 3.5 b
Incubated in 500 mg l^{-1} GA_3 for 3 days	10.3 ± 0.9 a	16.3 ± 1.2 a
Cultured on modified WPM with 1.44 µM GA_3	33.3 ± 1.8 c	65.3 ± 0.9 c

Means ± SE followed by the same letters were not significantly different at $p \leq 0.05$, according to LSD's multiple range test.

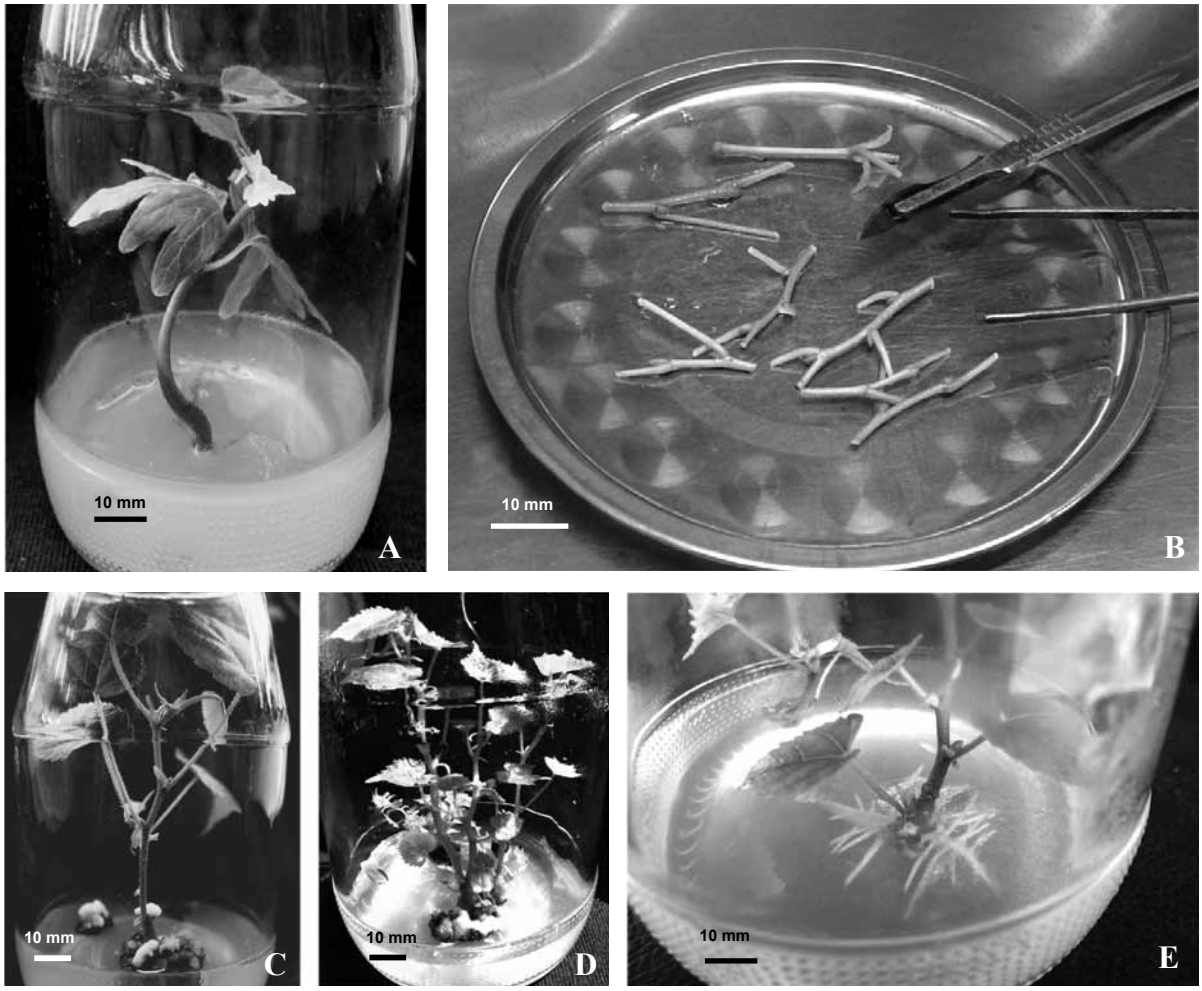

Fig. 1. Micropropagation of Nanjing linden (*T. miqueliana*). A) Seedling developed from disinfected seed, B) Nodal explants used for culture, C) Elongated shoots on medium supplemented with 2.28 µM ZT and 0.2 µM IBA, D) Multiplication of axillary shoots, E) Roots induced on rooting medium.

Low content of nutrients and low concentration of auxin in the medium (IBA, indole-3-acetic acid or α-naphthaleneacetic acid) has been widely used as the basic rooting medium for *Olea europaea* (Rugini et al. 1984), *Tilia platyphyllos* (Chalupa 2003), *Phellodendron amurense* (Azad et al. 2005), *Vaccinum angustifolium* (Brissette et al. 1990), and *Fraxinus nigra* (Beasley and Pijut 2013). Earlier studies on *in vitro*

Table 3. Effects of ZT and BA on axillary shoots initiation in Nanjing linden.

Plant growth regulators (µM)		Explants response (%)	Length of shoots (cm)	No. of nodes
Cytokinin	IBA			
BA 0.04	0.20	50.0 ± 5.0 a	4.3 ± 0.2 a	2.4 ± 0.2 a
BA 0.89	0.20	73.3 ± 1.7 b	4.5 ± 0.2 a	2.8 ± 0.1 ab
BA 2.22	0.20	70.0 ± 2.9 b	5.1 ± 0.2 ab	2.1 ± 0.2 a
BA 4.44	0.20	68.3 ± 4.4 b	4.3 ± 0.1 a	2.8 ± 0.1 ab
ZT 0.05	0.20	66.7 ± 8.8 b	5.5 ± 0.2 bc	3.3 ± 0.2 bc
ZT 0.91	0.20	80.0 ± 2.9 bc	5.8 ± 0.2 bc	3.6 ± 0.1 c
ZT 2.28	0.20	98.3 ± 1.7 d	6.8 ± 0.1 d	4.3 ± 0.2 d
ZT 4.56	0.20	91.7 ± 6.0 cd	6.0 ± 0.3 c	3.8 ± 0.4 cd

Means ± SE followed by the same letters were not significantly different at $p \leq 0.05$, according to LSD's multiple range test.

Table 4. Effects of ZT and BA on shoots multiplication in Nanjing linden.

Plant growth regulators (μM)			Explants response (%)	No. of shoots	No. of nodes
BA	ZT	IBA			
0.04	0.00	0.10	80.0 ± 5.0 a	1.0 ± 0.00 a	4.4 ± 0.3 b
0.44	0.00	0.10	83.3 ± 4.4 ab	1.0 ± 0.00 a	3.8 ± 0.2 ab
2.22	0.00	0.10	88.3 ± 4.4 ab	1.5 ± 0.3 ab	4.0 ± 0.4 ab
4.44	0.00	0.10	76.7 ± 8.8 a	2.0 ± 0.2 ab	4.8 ± 0.1 b
0.00	0.05	0.10	78.3 ± 7.3 a	1.1 ± 0.1 a	3.0 ± 0.2 a
0.00	0.45	0.10	85.0 ± 5.8 ab	1.9 ± 0.2 ab	4.3 ± 0.5 ab
0.00	2.28	0.10	90.0 ± 5.8 ab	1.5 ± 0.3 ab	3.4 ± 0.1 ab
0.00	4.56	0.10	80.0 ± 0.0 a	2.0 ± 0.5 ab	3.6 ± 0.3 ab
2.22	2.28	0.10	98.3 ± 1.7 b	2.5 ± 0.3 b	4.5 ± 0.2 b
4.44	4.56	0.98	91.7 ± 8.3 ab	1.1 ± 0.0 a	3.8 ± 0.4 ab

Means ± SE followed by the same letters were not significantly different at $p \leq 0.05$, according to LSD's multiple range test.

rooting of linden species indicated that half-strength basal medium had a better effect on rooting success than full-strength basal medium (Chalupa 1984, Liu et al. 2009). Root induction was observed on rooting medium after 4 weeks of culture (Fig. 1E). The correlation of IBA concentrations and rooting of *T. miqueliana* shoots was significant ($R^2 = 0.954$, $p < 0.001$). Over 91.7% rooting was observed at 14.76 μM IBA, and 30.7%, 50%, 70.3%, and 88% rooting were observed at 2.46, 4.92, 9.84, or 24.6 μM IBA, while no rooted shoot was found on the auxin free medium (Fig. 2). The rooting effect of IBA was better on medium containing 14.76 μM and 24.6 μM than on medium containing 2.46 μM ($df = 4$, $t = 32.25$, $p < 0.001$, $df = 4$, $t = 34.40$, $p < 0.001$). The better rooting at high auxin concentrations contrast with the results obtained for *T. cordata*, where a high rate of rooting was induced at much lower concentrations (Chalupa 1984).

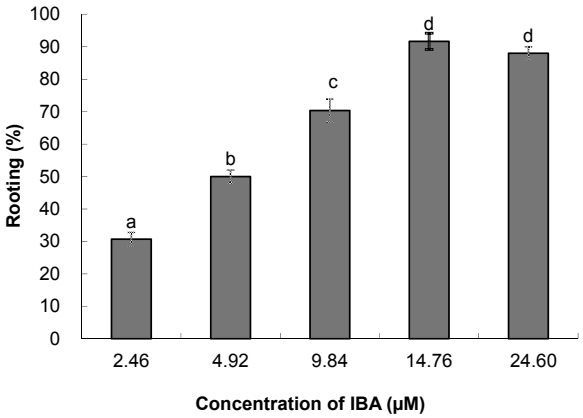

Fig. 2. Effects of IBA on the rooting rate of shoots. Treatments with the same letters were not significantly different at $p \leq 0.05$, according to LSD's multiple range test.

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