Review of JPOP 765

* Report all plant growth regulators in microMolar (μM)

IN VITRO PROPAGATION OF NANJING LINDEN (*Tilia miqueliana* Maxim)

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Short running title: *In vitro* propagation of *Tilia miqueliana* Maxim

Abstract: *Tilia miqueliana* Maxim is one of the most important tree species used in landscape architecture, especially in larger cities? in China? Because ~~In the light~~ 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 reforestation and city landscaping. ~~In this study~~ several types of treatments and methods for micropropagation were compared to establish an optimized protocol for the propagation of *T. miqueliana* through *in vitro* axillary shoot induction. Axillary buds were obtained from seedlings grown in vitro ~~in vitro~~ from seeds cultured on ~~WPM~~. These buds ~~explants~~ were then cultured on initiation medium ~~WPM~~, and the resulting elongated shoots were cut into nodal segments and transferred to shoot multiplication medium. ~~Overall~~ best results were obtained using the following protocol: highest average number of nodes on explants (~~11.25~~ 11.3 per explant) was induced on ~~medium~~ ~~WPM~~; highest rooting success (~~91.67~~ 91.7%) was achieved after first elongating nodal segments on ~~WPM~~ and then transferring them to medium ~~MS~~.

Key words: Nanjing linden, *Tilia miqueliana*, in vitro propagation, multiplication

Half-strength Murashige and Skoog medium with 14.76μM IBA

woody plant medium (WPM) without glycine, but supplemented with 8.66μM gibberellic acid

WPM with 2.28μM zeatin and 0.2μM indole-3-butyric acid (IBA)

WPM with 2.22μM benzyladenine, 2.28μM zeatin, and 0.1μM IBA

WPM without glycine and plant growth regulators

*Use the word disinfestation or disinfested "s" instead of disinfected or disinfectation. ✓

Nanjing linden (*Tilia miqueliana* Maxim), a member of the ~~family~~ Tiliaceae (linden), is a native tree species of Jiangsu province, China. The linden is highly prized as an ornamental tree, especially when abundant foliage and good shading is desired. ~~Furthermore~~ In addition, it can be a very important nectar source for bees, and hence is welcomed by beekeepers. Propagation of linden is ~~done~~ conducted primarily via cuttings and seeding. However, these methods ~~can~~ suffer from disadvantages such as poor establishment rates or low germination rates (Spaeth 1932, Rose 1919, Yang et al. 2011). ~~At present,~~ micropropagation is regarded as an effective method of obtaining seedlings of linden. ~~However, in previous studies, only Vladimir~~ Chalupa (1984, 1990) investigated the tissue culture of small-leaved linden (*T. cordata*). ~~In 1984, he established a~~ shoot proliferation method was established by using nodal stem segments from ~~one~~ year old seedlings as explants. In 1990, ~~he established the~~ plant regeneration by somatic embryogenesis protocol was established from cultured immature embryos. ~~However,~~ To our knowledge, no study has ~~previously~~ investigated the tissue culture of Nanjing linden (*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 for Nanjing linden.

Nodal segments that already have a developed meristem are suitable for micropropagation, as ~~they~~ 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 Nanjing linden (*T. miqueliana*) using ~~adult~~ mature plant material, obtained directly ~~in~~ from the field. This seems to be mostly attributable to high levels of contamination and vitrification (Lu et al. 2004). With regards to the selection of nodal segments, we found that the axillary buds of seedlings germinated from fertile seed were the best method to obtain explants.

49 However, it ~~had~~ has been reported in other studies that rigorous seed-surface ~~sterilization~~ disinfestation
50 procedures can have a negative effect on the viability of axillary buds of seedlings
51 (Obeidy and Smith 1990, ~~Nilima et al.~~ Renukdas et al. 2010). In the present study, we present an
52 efficient disin~~fection~~festation method to minimize negative effects on seedlings ~~while still~~ and produce
53 ~~providing good surface sterilization~~ plant materials.

* Report all plant growth regulators in microMolar (μM)

54 MATERIALS AND METHODS

55 *Plant material*

56 Mature fruits of Nanjing linden were collected from one 20-year-old tree in
57 mid-November. The seeds were then extracted by ~~fixing~~ placing the fruits in a vise and
58 removing the peel with a knife. Following ~~the~~ extraction, the seeds were washed in
59 sterile water for 20 min and then sterilized by incubation in carbendazim solution for
60 2 h (~~500 mg l^{-1}~~ 500 mg l^{-1}, Sigma Chemical Co., St. Louis, MO, USA). After this sterilization the
61 seeds were immediately washed three times with water, and ~~to remove any remaining~~
62 ~~carbendazim were then put~~ placed into 10% (v/v) liquid anionic detergent (Amway, China),
63 and ~~shaken~~ agitated on a rotary shaker for 20 min. ~~Afterwards, to remove the detergent,~~ the
64 seeds were then placed into a conical flask and washed for 60 min under running
65 water. ~~Finally,~~ the seeds were then disinfected by immersing on ~~them~~ for 40 s in 75% (v/v)
66 ethanol. Following this pre-treatment, the seeds were then subjected to one of the
67 following ~~sterilization~~ treatments: 1) 5.65% (m/v) sodium hypochlorite for 12 min; 2)
68 0.1% (m/v) mercuric chloride ($HgCl_2$) for 12 min; 3) 5.65% (m/v) sodium
69 hypochlorite for 6 min, and subsequent treatment with 0.1% (m/v) mercuric chloride
70 ($HgCl_2$) for 6 min; OR 4) 2.83% (m/v) sodium hypochlorite for 6 min, and subsequent
71 treatment with 0.2% (m/v) mercuric chloride ($HgCl_2$) for 6 min. After treatment with
72 surface disinfectants, the seeds were ~~washed by swirling them~~ rinsed in sterile deionized

should this be v/v ?

water for 5 min; ~~this washing step was~~ repeated four times. Each of the disinfected seeds ~~was~~ were then transferred to a glass bottle (230 ml, 5×12 cm) containing 30 ml ~~WPM1~~. One hundred seeds per treatment with three replicates were evaluated. After ten days, the seeds without visible bacterial or fungal growth on the surface were scored, and the contamination rate was then calculated as the ratio of seeds with visible growth to the total number of seeds.

~~Medium WPM1 was~~ woody plants medium (WPM) without glycine (Lloyd and McCown 1980; McCown and Lloyd 1981) containing 3 mg l^{-1} gibberellic acid (GA_3, Sigma). (WPM1)

~~Dormancy breaking~~ Breaking dormancy of the seeds Report in %M

For breaking the dormancy of the seeds, four methods were employed. In the first method, the seeds were stored at 4°C for 2 d ~~without hot treatment~~, and then cultured on WPM1 after disinfection. In the second method, the seeds were ~~dipped into~~ placed in 40°C water for 2 h and ~~subsequently~~ then stored at 4°C for 2 d, ~~and then cultured~~ followed by culture on WPM1 after disinfection. In the third method, the seeds were cultured on (WPM0) after incubation in 500 mg l^{-1} GA_3 for 3 d at 4°C. In the fourth method, the seeds were cultured on WPM1 immediately after the thick peel was ~~pared off~~ removed. One hundred seeds per treatment with three replicates were evaluated.

~~WPM0 was woody plants medium (Lloyd and McCown 1980)~~ WPM without glycine and plant ~~hormone~~ growth regulators.

Culture establishment and multiplication of the axillary shoots

After culturing in ~~the modified~~ WPM with 3 mg l^{-1} GA_3 for ~~two~~ 2 months, nodal explants were prepared from the seedlings in vitro and transferred to initiation medium. All initiation media ~~we tested were based on modified~~ was WPM, but additionally contained one of the following: 0.01, 0.2, 0.5, 1.0 mg l^{-1} zeatin (ZT, Sigma) or 0.01, 0.2, 0.5, 1.0 mg l^{-1} N^6-benzyladenine (BA, Sigma), and 0.04 mg l^{-1} indole-3-butyric acid (IBA,

Sigma, St. Louis). After 30 days of incubation the elongated shoots (longer than 6 cm, 3-4 branches) were divided into nodal segments, each bearing a single axillary bud. These segments were then transplanted onto multiplication medium consisting of modified WPM supplemented with ZT (0.01, 0.1, 0.5, 1.0 mg l^{-1}) or BA (0.01, 0.1, 0.5, 1.0 mg l^{-1}) and 0.02 mg l^{-1} IBA, or supplemented with 1.0 mg l^{-1} BA, 1.0 mg l^{-1} ZT and 0.2 mg l^{-1} IBA. After four weeks of culture the average number of shoots was assessed, and all obtained shoots were separated and incubated further on WPM0 to increase elongation.

Subsequently, the elongated shoots were tested for rooting in media MS1, MS2, MS3, MS4 and MS5. The rooted Nanjing linden plantlets were transferred to small pots containing a mixture of sterilized peat and perlite (1:1 v/v), and after an acclimatization period of four weeks were transplanted into the field.

Medium MS1, MS2, MS3, MS4 and MS5 contained half-strength MS (Murashige and Skoog, 1962) with different concentrations of IBA (0.5, 1.0, 2.0, 3.0, 5.0 mg l^{-1}).

Culture conditions

All media in this study, excepting rooting medium, were supplemented with 30 g l^{-1} sucrose, and solidified with 7.5 g l^{-1} agar (Sanland Chemical Co. Ltd., USA). Then the pH of the media was adjusted to 5.8 before autoclaving at 121°C, 151 kPa for 15min. All cultures were incubated at 25±1°C with 14h light at a photosynthetic photon flux density of 50 mol m^{-2} s^{-1}.

Statistical analysis

Each treatment comprised either 100 seeds or 20 explants, and each was repeated three times. Data on the number of shoots obtained per explant, germination rate of seeds, response in explants and number of nodes per shoot 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 analysis and LSD's multiple range rest. For paired-comparisons between treatments, post-hoc analysis and independent-samples t test were used.

RESULTS AND DISCUSSION

Report results to one decimal place.

Disinfestation ~~Disinfection~~ of seeds

Treating the seeds with 5.65% sodium hypochlorite for 6 min and 0.1% $HgCl_2$ for 6 min resulted in 95.7% ~~95.67%~~ of contamination-free seeds, while ~~through~~ the treatment with 2.83% sodium hypochlorite for 6 min and 0.2% $HgCl_2$ for 6 min produced 36.3% ~~36.33%~~ ~~of~~ contamination-free seeds ~~were obtained~~. The treatments employing 5.65% (m/v) sodium hypochlorite for 12 min, and 0.1% (m/v) mercuric chloride ($HgCl_2$) for 12 min resulted in 96.3% ~~96.33%~~ and 91.3% ~~91.33%~~ bacterial contamination, respectively. We found that the ~~disinfectant effect of the~~ combination of sodium hypochlorite and $HgCl_2$ was significantly better than sodium hypochlorite or $HgCl_2$ alone ($F = 229.40$, $p < 0.001$). The ~~disinfectant~~ 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 sodium hypochlorite and high concentration $HgCl_2$ ($t = 12.59$, $p < 0.001$), which agrees with the results of other studies (He et al. 2011).

Breaking dormancy ~~Dormancy breaking~~ of seeds

~~In this study, we identified~~ The breaking of dormancy was ~~dormancy breaking~~ of seeds as the most important limiting factor for successful, large scale Nanjing linden *in vitro* propagation. ~~As the existence of~~ A long dormancy period in linden seed had been reported previously (Chalupa ~~Vladimir~~ 1990, Chalupa ~~Vladimir~~ 1984, Yang et al. 2011), Therefore, a preliminary study was carried out to determine a procedure that would allow an effective ~~dormancy breaking~~ by breaking dormancy. The germination rates of seeds treated with the four treatments to break dormancy showed considerable

Table 1 can be deleted.

variation (Table 1). Seeds ~~treated with refrigeration~~ (stored at 4°C for 2 d without hot-treatment) showed ~~28.33~~% germination rate after ~~two~~ weeks and ~~45.00~~% after ~~two~~ months. Seeds treated with a combination of ~~hot-treatment (dipped into~~ 40°C water for 2 h and ~~refrigeration~~ (stored at 4°C for 2 d) showed ~~30.00~~% germination after ~~two~~ weeks and ~~49.67~~% after ~~two~~ months of culture, which ~~is~~ in both cases not significantly different from the ~~refrigeration-only~~ treatment ($t = 0.791$, $p = 0.437$ and $t = 1.306$, $p = 0.262$; respectively). Seeds that were incubated in 500 mg l^{-1} GA_3 for 3 d and cultured on basal medium without GA_3 showed only ~~10.33~~% and ~~16.33~~% germination after ~~two~~ weeks and ~~two~~ months, respectively. However, completely untreated seeds that were directly ~~incubated~~ on WPM1 after ~~their~~ extraction from fruits, showed a germination rate of ~~33.33~~% after ~~two~~ weeks, and ~~65.33~~% after ~~two~~ months of culture, which ~~is~~ in both cases higher than all other three treatments ($t = 11.663$, $p < 0.001$; $t = 32.870$, $p < 0.001$). During germination the seeds started to develop roots after ~~four~~ weeks and a shoot was observable after ~~five~~ weeks. When the seedlings had developed into small plants after ~~two~~ months (Fig. 1A), the nodal explants were prepared from the shoots (Fig. 1B).

Initiation of explants

Overall ZT performed better than BA with regards to ~~the effectiveness of~~ initiating axillary buds ~~as~~ explants (66-98% vs. 50-~~70~~% responded buds), with differences becoming significant for concentrations at 0.5 mg l^{-1} ($t = 8.50$, $p = 0.001$) and 1.0 mg l^{-1} ($t = 3.13$, $p = 0.035$). This ~~is~~ in accordance with results ~~reported~~ for other woody species (~~e.g.~~ Tatsuhito et al. 1975, Kyong et al. 2001, Simon et al. 1993, Gubis et al. 2004). Of the eight media formulations tested, WPM8 supplemented with

0.5 mg l^{-1} ZT and 0.04 mg l^{-1} IBA yielded the best results as initiation medium for Nanjing linden explants (Table 2, Fig. 1C). ~~Not only showed~~ explants exhibited the highest induction response (98%), ~~but~~ And shoots also showed significantly faster growth, resulting in an average length of 6.8 cm and 4.3 nodes per shoot.

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 ~~is~~ was the most widely used ~~growth hormone~~ plant growth regulator in tissue culture ~~techniques~~ ~~concerning the~~ for propagation of woody species, as it ~~has~~ was proven to be an effective agent for shoot multiplication (Nour ~~et al.~~ and Thorpe 1993, Al-Juboory et al. 1998, Lucchesini and Mensuali-Sodi 2004, Sghir et al. 2005, Reed ~~et al.~~ and Abdelnour-Esquivel 1991). In the present study, the average number of branches per explant after ~~four~~ 4 weeks of ~~incubation~~ culture was significantly higher ~~when grown~~ on medium containing both ZT and BA, as opposed to containing only one of the substances, ZT or BA ($F = 4.471$, $p = 0.003$; see Table 3). ~~Additionally~~, In addition the observed number of nodes correlated in the same way ~~as~~ the plants grown on medium containing ZT and BA ~~having~~ had a significantly higher average number of nodes per branch as plants grown on medium with one of the two substances missing ($F = 3.524$, $p = 0.009$; Table 3). The highest average number of branches per explant (2.5), as well as the highest average number of nodes per branch (4.5), was obtained on WPM18 with 0.5mg l^{-1} ZT and 0.5mg l^{-1} BA combined with 0.02mg l^{-1} IBA (~~2.5 and 4.5, respectively~~; Table 3).

We conclude that the combination of both cytokinins, ZT and BA, ~~is~~ was a successful strategy to enhance shoot multiplication, as long as certain concentrations ~~are~~ were not exceeded.

The shoots obtained after the first multiplication step were cut into short nodal segments and were placed on WPM0, and ~~incubated~~ cultured for ~~one~~ 1 month, for further elongation.

~~*Shoot rooting* and acclimatization~~ Rooting of shoots

For rooting experiments, shoots 4-6 cm ~~long~~ in length were selected from healthy cultures. Low salt medium containing low concentration of auxin (IBA, IAA or NAA) has been widely used as the basic rooting medium for several woody plants (~~Rochelle~~ Beasley and ~~Paula~~ Pijut 2013, Azad et al. 2005, Brissette et al. 1990, Rugini 1984). ~~Furthermore,~~ Earlier studies on *in vitro* rooting of linden indicated that half-strength basal medium had a higher instance of rooting success than full-strength basal medium (~~Vladimir~~ Chalupa 1984, Liu et al. 2009). Rooting induction was observed in rooting medium after ~~two~~ 2 weeks of culture (Figure 1E). Over ~~91.67~~ 91.7% rooting was observed at 3 mg l^{-1} IBA ~~concentration,~~ and ~~30.67~~ 30.7%, ~~50.00~~ 50%, ~~70.33~~ 70.3% and ~~88.00~~ 88% rooting were observed at 0.5, 1.0, 2.0 ~~and~~ or 5.0 mg l^{-1} IBA ~~concentration~~ (Figure 2). The rooting effect of IBA was better in MS4 containing 3 mg l^{-1} and MS5 containing 5 mg l^{-1} than in MS1 containing 0.5 mg l^{-1} ($t=32.25$, $p<0.001$, $t=34.40$, $p<0.001$). This result of a higher instance of rooting at high concentrations of auxins contrasts with results obtained for *T. cordata*, where a high proportion of rooting was induced at much lower concentrations (~~Vladimir~~ Chalupa 1984). Plants ~~in which~~ with roots ~~had successfully been induced~~ were transplanted to pots containing a mixture of sterilized peat and perlite (1:1 v/v) and were then grown for 3-4 weeks in a greenhouse under high humidity conditions. The survival rate ~~for this late step in the protocol~~ was always very high, with 95-100% of the root-induced ~~explants~~ shoots surviving. ~~The~~ T transfer to the field ~~would be~~ was initiated by slowly reducing humidity levels, and thereby acclimatizing the plants to outside conditions, and finally transplantation to the field.

Were plants actually transplanted to the field? If so, please show a photo.

The present study introduces a new customized protocol for the *in vitro* propagation of Nanjing linden (*Tilia miqueliana*). By fine tuning culture media used for ~~the~~ several steps, we established an overall improved method for generating clonal plants from explants obtained from axial buds of seedlings. It may also be possible to use this protocol to obtain clones from mature tissues of *T. miqueliana* through micropropagation, and we hope this will contribute to a better supply of this highly valued species for landscaping purposes.

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* Report all results to one decimal place *
* Report all plant growth regulators in micro Molar (μM)

Table 1 Not necessary as all this information is in text

Table1. Germination rate of Nanjing linden seeds after application of different dormancy breaking treatments.

Treatment	% of seed germinated	
	After 2 weeks	After 2 months
stored at 4℃ for 2 d without hot-treatment	28.33±0.58 b	45.00±1.00 b
incubated in 40℃ water for 2 h	30.00±3.61 bc	49.67±6.11 b
incubated in 500 mg l^{-1} gibberellic acid for 3 d	10.33±1.53 a	16.33±2.08 a
Cultured on modified WPM with 3 mg l^{-1} gibberellic acid	33.33±3.06 c	65.33±1.53 c

One hundred seeds per treatment with three replicates were evaluated. Data for germination of seeds were scored after two weeks and two months of culture.

Explant response

Table2. Effects of ZT and BA on axillary bud initiation in Nanjing linden

Medium NO.	Plant growth regulators (mg l^{-1}) BA/ZT	IBA	Responded explants(%) ~~(means ± SD)~~	Length of shoots (cm) ~~(means ± SD)~~	No. of nodes per shoot ~~(means ± SD)~~
WPM2	BA 0.01	0.04	50±8.7 a	4.3±0.41 a	2.4±0.30 a
WPM3	BA 0.20	0.04	73±2.9 b	4.5±0.31 a	2.8±0.10 ab
WPM4	BA 0.50	0.04	70±5.0 b	5.1±0.39 ab	2.1±0.28 a
WPM5	BA 1.00	0.04	68±7.6 b	4.3±0.24 a	2.8±0.20 ab
WPM6	ZT 0.01	0.04	66±15.2 b	5.5±0.26 bc	3.3±0.33 bc
WPM7	ZT 0.20	0.04	80±5.0 bc	5.8±0.44 bc	3.6±0.21 c
WPM8	ZT 0.50	0.04	98±2.9 d	6.8±0.12 d	4.3±0.44 d
WPM9	ZT 1.00	0.04	92±10.4 cd	6.0±0.54 c	3.8±0.60 cd

Align colums

Data recorded after three weeks of culture in initiation medium. Twenty explants were cultured for each treatment with three replicates. Means followed by ~~the same~~ letters are significantly different at $p \leq 0.05$, according to LSD's multiple range test.

± SD different were

Table 3. Effects of ZT and BA on shoot multiplication in Nanjing linden

Explant response

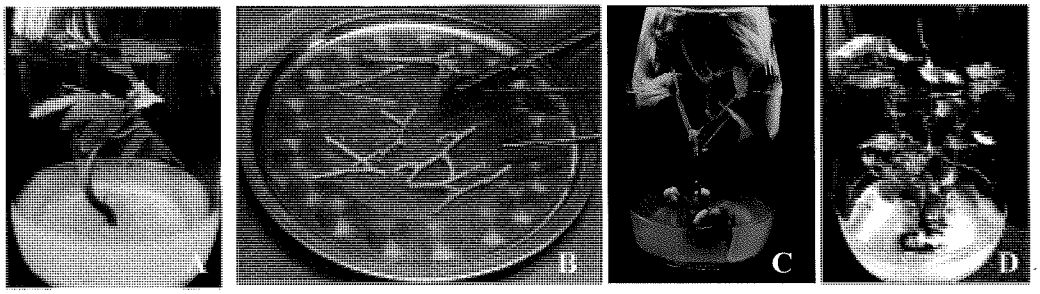
Medium NO.	Plant growth regulators (mg l^{-1}) BA	ZT	IBA	Responded shoots (%) ~~(mean ± SD)~~	No. of branches ~~(mean ± SD)~~	No. of nodes per branch ~~(mean ± SD)~~
WPM10	0.01	0.00	0.02	80±8.6 a	1.0±0.00 a	4.4±0.55 b
WPM11	0.10	0.00	0.02	83±7.6 ab	1.0±0.00 a	3.8±0.44 ab
WPM12	0.50	0.00	0.02	88±6.2 ab	1.5±0.46 ab	4.0±0.60 ab
WPM13	1.00	0.00	0.02	77±15.2 a	2.0±0.41 ab	4.8±0.15 b
WPM14	0.00	0.01	0.02	78±12.6 a	1.1±0.13 a	3.0±0.35 a
WPM15	0.00	0.10	0.02	85±10.0 ab	1.9±0.36 ab	4.3±0.83 ab
WPM16	0.00	0.50	0.02	90±10.0 ab	1.5±0.50 ab	3.4±0.20 ab
WPM17	0.00	1.00	0.02	80±0.0 a	2.0±0.88 ab	3.6±0.55 ab
WPM18	0.50	0.50	0.02	98±2.8 b	2.5±0.53 b	4.5±0.30 b
WPM19	1.00	1.00	0.20	92±14.4 ab	1.1±0.08 a	3.8±0.74 ab

Align colums

Plants were measured after three weeks of culture in initiation medium. Twenty explants were cultured for each treatment with three replicates. Means followed by ~~the same~~ letters are significantly different at $p \leq 0.05$, according to LSD's multiple range test.

± SD different were

All tables must stand alone from the text. Spell out abbreviations in a footnote for each table. e.g. WPM, BA, ZT, IBA.



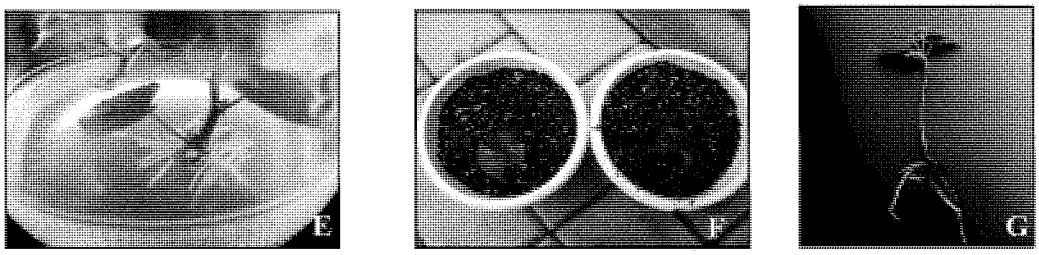


Fig.1. Micropropagation of Nanjing linden (*T. miqueliana*) A) Seedling developed from disinfected seed, B) Nodal explants used for culture, C) Elongated shoots on elongation medium supplemented with 0.04 mg l^{-1} IBA, 0.5mg l^{-1} ZT, D) Multiplication of axillary shoots, E) Roots induced on rooting medium, F) Plantlets transferred into small pots, G) Fully ~~established pot~~ Rooted plants of Nanjing linden.

disinfested

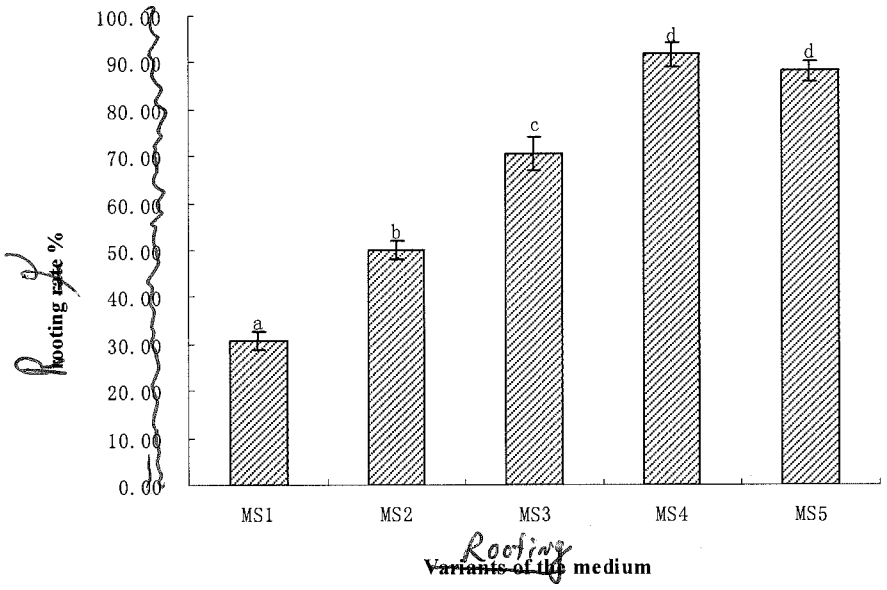


Fig. 2. Effects of the plant growth regulators on the rooting rate of shoots. MS1: 0.5 mg l^{-1} IBA, MS2:1.0 mg l^{-1} IBA, MS3: 2.0 mg l^{-1} IBA; MS4: 3.0 mg l^{-1} IBA, M5: 5.0 mg l^{-1} IBA. Treatments with different letters are significantly different at $p \leq 0.05$ according to LSD's multiple range tests; Vertical bars represent mean values of three replications ± SD.