Acta Physiol Plant (2009) 31:33–41
DOI 10.1007/s11738-008-0196-9

ORIGINAL PAPER

Adventitious bud regeneration from leaf explants of *Platanus occidentalis* L. and genetic stability assessment

Yuehua Sun · Yanling Zhao · Xiaojuan Wang · Guirong Qiao · Guoliang Chen · Ye Yang · Jing Zhou · Liang Jin · Renying Zhuo

Received: 11 March 2008 / Revised: 21 May 2008 / Accepted: 11 June 2008 / Published online: 8 August 2008

Abstract The aim of this work is to develop a method of plant regeneration from leaf explants of *Platanus occidentalis* L. successfully. Woody plant medium (HortScience 16:453–459, 1981) and Murashige and Skoog (Physiol Plant 15:473–497, 1962) medium were used as induced and rooted basal medium, respectively. The effects of combinations of 6-BA, IBA, NAA and KT with different concentrations on adventitious bud regeneration from *P. occidentalis* leaf explants were compared. The results showed that the highest shoot regeneration frequency (90%) and maximum number (13.72 ± 0.44) of shoots per explant was recorded on WPM medium supplemented with 22.20 mmol l^{-1} 6-BA and 0.49 mmol l^{-1} IBA. A 40-day-old explants were much more productive for shoot formation than others in this study. The regenerated shoots were cultured on MS medium supplemented with 1.33 mmol l^{-1} 6-BA, 0.16 mmol l^{-1} NAA and 2% (w/v) adenine, after 2-week shoots were transferred to 1/2 MS medium supplemented with 0.49 mmol l^{-1} IBA for rooting. Hardened plantlets via acclimatization were transferred to pots and transplanted to the soil finally. To ascertain whether tissue culture had effects on the genetic stability of plantlets regenerated, the genetic diversity was assessed using RAPD marker. A total of 96 bands ranging from 0.5 to 2.2 kb with an average of 6.4 bands per primer, were obtained using 15 primers. Amplified products exhibited few of polymorphic patterns across all the plants of *P. occidentalis* and the overall frequency of detection of somaclonal polymorphisms was lower than 0.0104%.

Keywords *Platanus occidentalis* L. · Leaf explant · Plant regeneration · Tissue culture · Genetic stability · RAPD

Abbreviations

MS	Murashige and Skoog
WPM	Woody plant medium
6-BA	6-Benzyladenine
KT	Kinetin
IBA	Indole-3-butyric acid
NAA	Naphthaleneacetic acid
RAPD	Random amplified polymorphic DNA

Introduction

Platanus occidentalis is a common tree and one of the largest in the eastern deciduous forests of American. Other names are American planetree, buttonwood, American sycamore and buttonball-tree. It is a fast-growing and long-lived tree of lowlands and old fields. *P. occidentalis* is valuable for timber and is also widely planted as a shade tree because of its distinctive white, exfoliating bark and broad, dense crown. Furthermore, it provided with the

Yuehua Sun, Yanling Zhao, and Xiaojuan Wang contributed equally to this work.

Communicated by E. Lojkowska.

Y. Sun · Y. Zhao · G. Qiao · G. Chen · Y. Yang · J. Zhou · R. Zhuo (✉)
Key Lab of Tree Genomics, The Research Institute of Subtropical of Forest, Chinese Academy of Forest, Fuyang, Hangzhou, Zhejiang 311400, China
e-mail: zhuory@gmail.com

Y. Sun · X. Wang · G. Chen · L. Jin
College of Pastoral Agriculture Science and Technology, Lanzhou University, Lanzhou, Gansu 730020, China

ability of adapting for various environments, within the range of *P. occidentalis*, average annual temperatures vary from 4 to 21°C, with average annual extremes from 41 to 34°C; the lowest temperature recorded was −40°C. Average annual precipitation varies from 760 to 2030 mm, and the frost-free period is from 100 to 300 days.

However, *P. occidentalis* also has demerits, its pollen and seed hairs disperse widely in the spring, which not only pollutes the environment but also affects human health (Subiza et al. 1994; Varela et al. 1997). This disadvantage, together with the destruction caused by diseases and insects (Walter 1946; Walter et al. 1952; Matasci and Gessler 1997), has badly tarnished its good image. Application of plant genetic transformation may help to solve these problems by introducing desirable traits such as sterility and disease or insect resistance into this species (Shuerman and Dandekar 1993; Strauss et al. 1995). To support such a transformation strategy, the establishment of an efficient in vitro plant regeneration system is necessary. Recent years, more and more reports about tissue culture and genetic transformation of *Platanus* species were appeared. Plant regeneration from mesophyll protoplasts of *P. orientalis* was successful (Wei and Xu 1991). Micropropagation of *Platanus acerifolia* in vitro (Liu et al. 2002a, b; Liu and Bao 2003; Wang et al. 2004; He et al. 2006; Fan et al. 2004; Li et al. 2007a, b), *Agrobacterium*-mediated genetic transformation of *P. acerifolia* (Li et al. 2007a, b) and colchicine-induced chromosome doubling in *P. acerifolia* (Liu et al. 2007) were also reported. However, data on tissue culture of *P. occidentalis* were very limited (Zou and Shi 2005, 2006). So it was necessary for *P. occidentalis* to detect a more effective regenerated system.

Another important aspect of micropropagation protocols is the genetic integrity of the plants. In vitro plants are usually susceptible to genetic changes due to culture stress (Dunstan and Thorpe 1986; Cecchini et al. 1992; Cullis and Cleary 1986; Rani and Raina 1998). Several techniques such as cytological, isozymes and molecular markers have been employed to detect variation if any or to confirm the genetic stability of micropropagated plants (Gupta and Varshney, 1999). Usefulness of RAPD and cytological analysis in detection of variation in micropropagated plants has been amply demonstrated in large array of plants by many workers (Bohanec et al. 1995; Ezura and Oasawa 1994; Isabel et al. 1993; Rani et al. 1995; Rani and Raina 1998; Zahim-Al et al. 1999).

In this paper, we reported an efficient regeneration system via multiple shoot bud induction from optimal age leaf explants in *P. acerifolia.* We have also aimed at assessing the molecular fidelity of in vitro plants through RAPD analysis.

Materials and methods

Plant material

Dry seeds of *P. occidentalis* that imported from Louisiana, South America, were collected and stored at −40°C. Seeds were geminated on the paper napkin absorbing enough fresh water in a container, up-growth until 3 weeks. Exactly 2–3 cm seedlings pulled out from the paper napkin were thoroughly washed under running tap water, then surface disinfected with 75% (v/v) ethanol for 30 s, followed with 0.1% (w/v) mercuric chloride for 5 min, and finally rinsed five times with sterile distilled water to remove traces of mercuric chloride. The surface-disinfected roots of seedlings were resected using an axenic scalpel and the remanent parts were incubated aseptically in culture tubes containing half-strength Murashige and Skoog (MS) (Murashige and Skoog 1962) basal medium supplemented with auxin indole-3-butyric acid (IBA) 0.49 mmol l^{-1} to obtain the intact plantlets with enough roots. After new roots reaching 1–2 cm, the integrated plantlets were transferred to MS media for up-growth.

Culture media and conditions

Murashige and Skoog and WPM (namely Wood Plant Medium, McCown and Loyd 1981) media containing 3% (w/v) sucrose (2% in root medium) and 0.6% agar with different concentration plant growth regulators. The cultures were incubated at 25 ± 2°C with 50 μmol m^{-2} s^{-1} irradiance provided by cool fluorescent tubes (40 W) and were exposed to a photoperiod of 16 h and 55 ± 5% of relative humidity (RH).

Plant regeneration from leaf explant

The expanding leaves (different age 20, 30, 40, 50 and 60 days) of plantlets were excised from the shoot and wounded by three to four transverse cuts across the mid-vein on the abaxial side without severing it, removing the petioles and leaf tips (leaves were in uniform size at the same age) . Leaves were placed firmly on the medium surface with the adaxial side touching the medium in 90-mm petri dishes containing 25 ml WPM medium allied with different concentrations and combinations of various plant growth regulators (Table 1). Then petri dishes were sealed with clean film and incubated in culture room for dark culture. Seven days later, all petri dishes were moved to a photoperiod of 16 h culture shelf for another 3 weeks.

Table 1 The different concentrations and combinations of various plant growth regulators used on organogenesis of leaf explants of American plane tree (*Platanus occidentalis* L.)

Number	6-BA + IBA (mmol l^{-1})	6-BA + NAA (mmol l^{-1})	KT + IBA (mmol l^{-1})
1	4.44 + 0.49	4.44 + 2.69	9.08 + 2.46
2	6.66 + 0.49	8.88 + 2.69	13.62 + 2.46
3	11.10 + 0.49	13.32 + 2.69	18.16 + 2.46
4	13.32 + 0.49	17.76 + 2.69	22.70 + 2.46
5	15.54 +0.49	22.20 + 2.69	27.24 + 2.46
6	17.76 + 0.49	26.64 + 2.69	31.78 + 2.46
7	22.20 + 0.49	31.08 + 2.69	36.32 + 2.46
8	26.64 +0.49	35.52 + 2.69	40.86 + 2.46
9	35.52 + 0.49	/	/

Shoot growth and elongation

To facilitate shoot growth, a two-step culture procedure was adopted. The first step involved the induction of shoots from leaf explants on WPM medium augmented with different cytokinin and auxin for 4 weeks in all, then followed by step two in which cytokinins and auxins induced regenerated shoots were subculture on WPM medium containing half concentration cytokinins and auxins than those in the first step. After 2 weeks, the regenerated shoots transferred to MS medium containing 1.33 mmol l^{-1} 6-BA, 0.161 mmol l^{-1} NAA and 2% (w/v) adenine, for elongation and growth.

To test the effects of explants from different age plants on regeneration frequency, leaves were selected as the *P. occidentalis* planted for 20, 30, 40, 50 and 60 days, respectively. The numbers of shoots per explant induced on optimal medium above were recorded.

In vitro root induction

The elongated shoots (2–3 cm) with more than two fully expanded leaves were excised, and rooting induction was adopted on half-strength MS medium with 0.49, 0.98, 1.48, 1.97 and 2.46 mmol l^{-1} IBA, respectively, and reduced the concentrations of sucrose from induced medium 3 (w/v) to 2% (w/v). After 3 weeks, percentages of rooted shoots and mean number of roots per shoot were recorded.

Hardening and transplanting to soil

In vitro regenerated plantlets were washed carefully in running tap water to remove the traces of agar, followed by the transfer to culture tubes with stopple containing fresh water or quarter-strength liquid MS nutrients without sucrose for 6–7 days. Hardened plantlets were transferred to pots containing autoclaved soil and soilrite (1:1, w/w) and were covered with polybags for 1 week to maintain high relative humidity. The plants were transferred to the greenhouse and kept under periodic mist (15 times every day, 5 s each time) for the first week.

Genetic stability assessment

To detect the effect of plant growth regulators in media on the genetic stability of plantlets cultured in vitro, a comparison of genetic diversity of plantlets (70 individuals from seven populations) cultured in vitro for several months on media associating with different cytokinins and auxins based on RAPD patterns was carried out (Table 2).

Genomic DNA of 70 individual plants was extracted from seven populations which were regenerated from same mother plant with CTAB method described as Doyle and Dickson (1987). Quality and quantity of DNA preparations were checked by standard spectrophotometry and the samples were diluted to a concentration of 25 ng μl^{-1} before use. PCR reaction was carried out in a DNA Thermal Cycle (PE9600, USA). Each 25 µl reaction mix contained 1× PCR reaction buffer (10 mmol l^{-1} Tris–HCl, 50 mmol l^{-1} KCl, pH 8.3), 3 mmol l^{-1} $MgCl_2$, 0.5 U of DNA polymerase, 200 µmol l^{-1} each of dATP, dTTP, dCTP and dGTP (all reagents from Dingguo, Beijing), 0.6 µmol l^{-1} primer, and approximately 50 ng of template DNA.

A total of 60 primers were screened for RAPD analysis. Out of these, 15 primers, s54, s55, s60, s69, s79, s82, s84, s99, s189, s194, s222, s234, s235, s1099, and s1229, were used for further analyses. The PCR conditions were as follows: initial extended step of denaturation at 94°C for 3 min followed by 38 cycles of denaturation at 94°C for 30 s, annealing at 36°C for 30 s, elongation at 72°C for 1.5 min, followed by an extended elongation step at 72°C for 10 min. Reaction products was electrophoresed on 1.2% agarose gel at 160 V followed by staining with ethidium bromide and photographed under ultraviolet light using a Digital Imaging System.

Statistical analysis

All experiments were repeated three times with five replicates (petri dish) per repeat. Per petri dish contained four explants. The data on shoot formation and rooting were

Table 2 Seven populations generated in vitro with different hormone combinations for RAPD analysis

Population	Plant growth regulator 6-BA + IBA (mmol l^{-1})	Number of individual
1	35.52 + 0.492	10
2	26.64 + 0.492	10
3	22.20 + 0.492	10
4	17.76 + 0.492	10
5	13.32 + 0.492	10
6	11.10 + 0.492	10
7	4.44 + 0.492	10

Population 1 (contain 10 plantlets) regenerated from WPM medium supplement with 35.52 mmol l^{-1} 6-BA + 0.492 mmol l^{-1} IBA

analyzed by SAS version 9.1.3, and the data on RAPD result were analyzed by Popgene version 1.3.1.

Results

Plant regeneration, shoot multiplication and elongation

During dark culture for 7 days, a series of eyeable changes of explants occurred. After 2 days of incubation, a majority of the explants became verdancy and swelled. At the sixth day, explants became semitransparent with bright surface; some other visible tiny outshoots also appeared on explant surface (Fig. 1a). Three days on photoperiod shelf, the color of some explant cuts or intact explants become red (Fig. 1b). About 3 weeks, adventitious buds were differentiated from some explants with a little or no callus (Fig. 1c). The range of percentage of shoot regenerating explants was 17–90% (Fig. 2) and the average number of shoots per explant varied significantly at different concentrations plant growth regulator (Fig. 3). The frequency of axillary shoot proliferation and the number of shoots per explant increased with concentration of plant growth regulators up to the optimum level. In the combination of different concentrations 6-BA and 2.46 mmol l^{-1} IBA, the shoot regeneration was inhibited with the increase of 6-BA from 4.44 to 13.32 mmol l^{-1}. When the concentration of 6-BA was 13.32 mmol l^{-1}, the explants were mainly induced roots but not buds (Fig. 1d). When the concentrations of 6-BA decreased from 13.32 up to 22.20 mmol l^{-1}, the shoot regeneration frequency (90%) and shoots number per explants (13.72 ± 0.44) were up to the summit at simultaneity. However, shoot regeneration frequency was restrained with continuous presence of 6-BA (Fig. 2). Within a range of various concentrations of KT and IBA, the highest shoot regeneration frequency (83%) and maximum mean number of shoots (7.6 ± 0.16) was recorded at 22.70 mmol l^{-1} KT and 2.46 mmol l^{-1} IBA after 5 weeks induction (Figs. 2, 3). In the case of combination of 6-BA (4.44–35.52 mmol l^{-1}) and NAA (2.69 mmol l^{-1}), the highest regeneration frequency (88%) and maximum mean number of shoots (13.5 ± 0.35) were recorded at the 22.20 mmol l^{-1} concentrations of 6-BA.

After 6 weeks on WPM-induced medium, adventitious buds were transferred to MS medium containing 1.33 mmol l^{-1} 6-BA and 0.16 mmol l^{-1} NAA, 3% (w/v) sucrose, 0.6% (w/v) agar, with 2% (w/v) adenine for 2 weeks. During this period, the low concentrations of hormones accelerated the shoots developing rapidly, the state were displayed in Fig. 1e.

Compared with other younger or older explants, 40-day-old explants were much more productive for shoot formation with the highest shoot regeneration frequency (90%) and maximum number (13.72 ± 0.44) (Fig. 4).

Rooting, hardening and transplanting to soil

The regenerated shoots were transferred to rooting media for root induction. It was found that the best rooting response occurred in medium supplemented with 0.49 mmol l^{-1} IBA after 3 weeks of culture (Fig. 1f) (data not shown). The plantlets were transferred to pots for hardening and acclimatized, and then transferred to the glasshouse for up-growth (Fig. 1g).

Genetic stability assessment

A total of 96 bands ranging from 0.5 to 2.2 kb with an average of 6.4 bands per primer, were obtained using 15 primers. Overall frequency of detection of somaclonal polymorphisms was lower than 0.0104%. The results of genetic distance between seven populations generated in vitro were displayed in Table 2, and examples of RAPD profiles of plants cultured on WPM medium amended with 17.76 mmol l^{-1} 6-BA and 0.49 mmol l^{-1} IBA are shown (Fig. 5)

Discussion

Plant regeneration from leaf explants

Adventitious buds from leaves of *P. occidentalis* has obtained successfully by using 6-BA and IBA as a potent cytokinin and auxin. Axillary shoot proliferation from leaves of seedlings was well documented for several tree species such as *Acacia nelotica* (Dewan et al. 1992), *Sterculia urens* (Purohit and Dave 1996), *Conyza canadensis* (Distabanjong et al. 1997), *Dalbergia sissoo* (Pradhan et al. 1998), *Acacia sinuate* (Vengadesan et al. 2002), and *Sesbania rostrata* (Jha et al. 2004). Leaves failed to

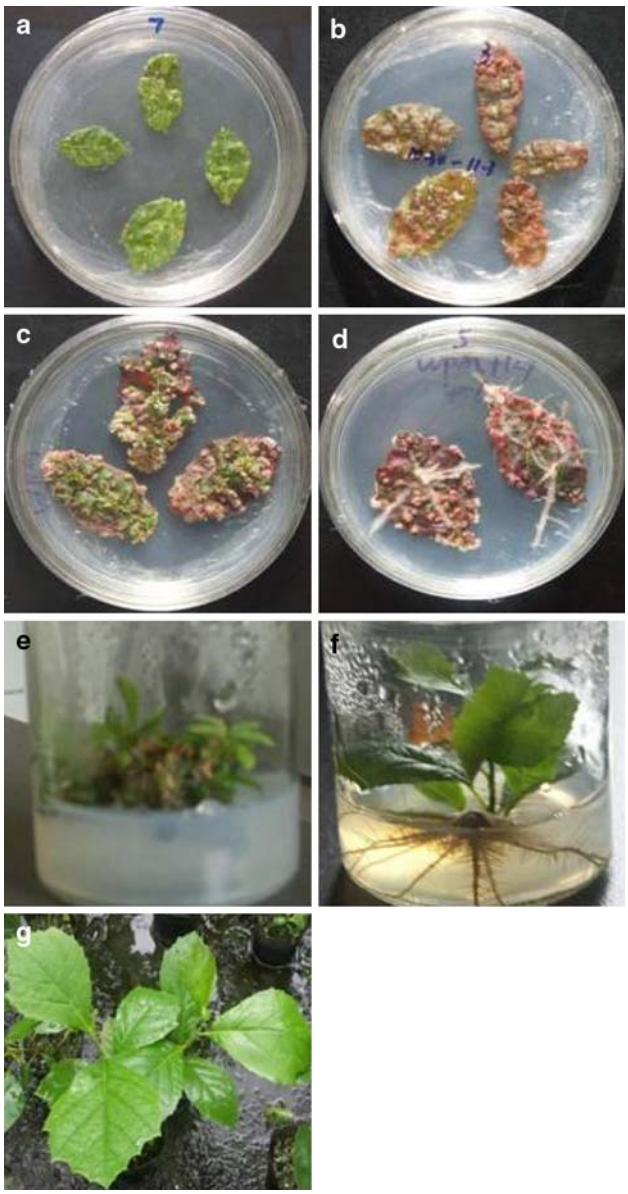

Fig. 1 Plant regeneration of *Platanus occidentalis* in vitro from leaf explants. **a** Dark culture for 7 days. **b** Explants becoming red. **c** Callus formation and adventitious bud regeneration from wounded cultured for 30 days. **d** Explants with roots. **e** Shoot elongation and multiplication on MS medium supplemented with mmol l^{-1} 6-BA and 0.16 mmol l^{-1} NAA. **f** Rooted plantlets cultured for 4 weeks on root induction medium. **g** Regenerated plant of *Platanus occidentalis* L., 2 months after transfer to greenhouse

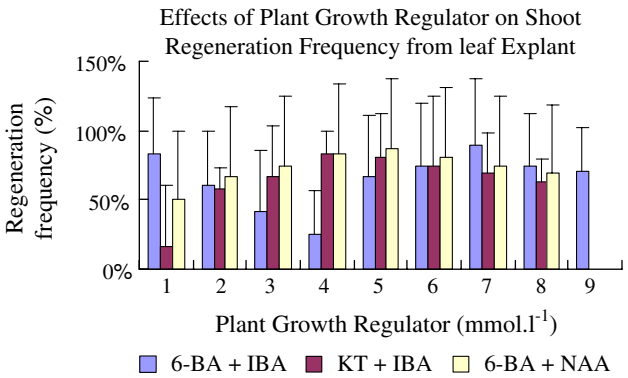

Fig. 2 Regeneration frequencies from leaf explant of *Platanus occidentalis* L. cultured on mediums supplemented with various combinations of plant growth regulators for 6 weeks

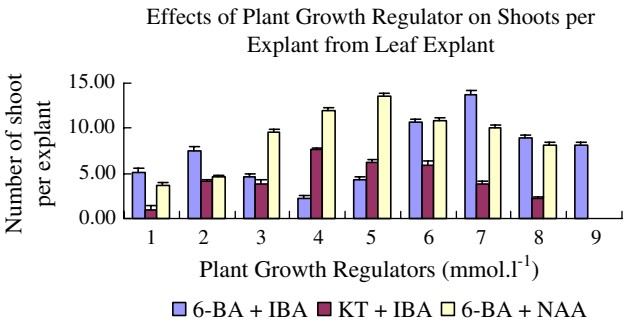

Fig. 3 Adventitious buds per explant of *Platanus occidentalis* L. cultured on mediums supplemented with various combinations of plant growth regulators for 6 weeks

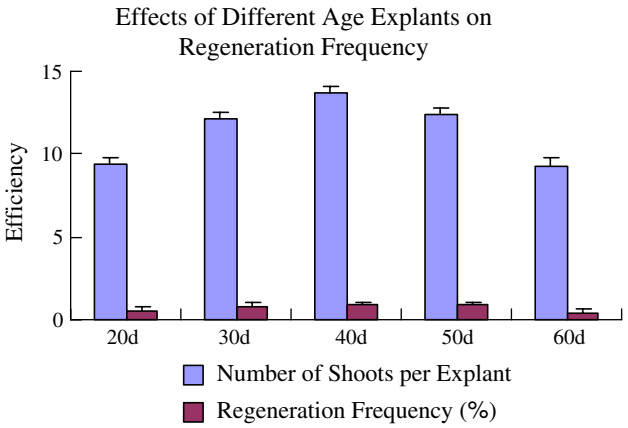

Fig. 4 Regeneration frequencies and adventitious buds per explant of *Platanus occidentalis* L. cultured onWPM basal medium supplemented with optimal combination of plant growth regulators (22.20 mmol l^{-1} 6-BA and 0.49 mmol l^{-1} IBA) for 6 weeks

differentiation without plant growth regulator, and addition of cytokinins and auxins to WPM or MS medium was essential to induce multiple shoots. Buds of the explants were differentiated on WPM medium containing different concentrations of cytokinins (6-BA or KT) and auxins (IBA or NAA). The dosage of cytokinin in the culture medium is known to be critical for shoot organogenesis. In the present study, 6-BA was found to be more potent compared to other cytokinins used. Superiority of 6-BA for shoot induction may be attributed to the ability of plant tissues to metabolize 6-BA more readily than other synthetic growth regulators, or to the ability of 6-BA to induce production of natural hormones, such as zeatin within the

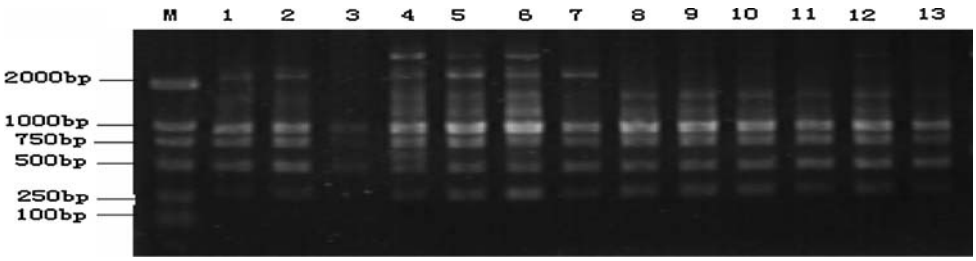

Fig. 5 The RAPD pattern amplified with S84 primer, *lane M* is molecular marker (DL2000), *lanes 1–7* were regenerants from pop1; *lanes 8–13* regenerants from pop4 (the same details as Table 2)

tissue (Zaerr and Mapes 1982). The promotory effect of 6-BA in inducing multiple shoots has been previously reported in *Garcinia mangostana* (Goh et al. 1990; Huang et al. 2000). Both direct and indirect shoot regeneration require plant cells to undergo dedifferentiation and differentiation, both of which are known to be affected by not only exogenous plant growth regulators but also endogenous content of the hormones (Trigiano and Gray 2000).

Prior incubation in the dark is very important process for dedifferentiation and differentiation of *P. occidentalis.* Incubation in the dark has been reported to increase shoot regeneration in various plant species, such as blueberry (Billings et al. 1988), pear (Chevreau et al. 1989), apple (Fasolo et al. 1989), campanula (Sriskandarajah et al. 2004), watermelon (Compton 1999), Zhanhua winter jujube (Gu and Zhang 2005) and quince (Baker and Bhatia 1993). Our results indicated that an incubation period of 7 days in the dark is optimal for shoot regeneration of *P. occidentalis* (data not presented). Incubation in the dark may delay degradation of endogenous and/or exogenous plant growth regulators (Rusli and Pierre 2001). In addition, dark treatment may reduce the levels of cell wall thickness and cell wall deposits (cellulose and hemicellulose), facilitating translocation of plant growth regulators in plant cells (Herman and Hess 1963).

Besides media, different explants selected also could influence the rate of explant regeneration in woody plants. It was reported that *Decalepis arayalpathra* had higher frequencies of sprouting in basal nodes than in terminal nodes (Sudha et al. 2005). Different age explants may have different levels of endogenous hormones and, therefore, the age of explants would have a critical impact on the regeneration success, those results has been reported in other plants, including *Cercis canadensis* (Distabanjong and Geneve 1997) and *Morus alba* (Thomas 2003). In our study, when 40-day-old and other younger or older leaves were compared, it was clear that 40-day-old explants were much more productive for shoot formation than others. Similar studies have also been reported in *Prunus* (Mante et al. 1989), *Lachenalia* (Niederwieser and Van Staden 1990), *Cydonia oblonga* (Baker and Bhatia 1993), *Aegle marmelos* (Islam et al. 1993) and *Malus* (Famiani et al. 1994).

Shoot growth and elongation

This stage was crucial for adventitious buds to continue multiplicative growth. If the explants with adventitious buds were cultured continuously on the first stage-induced medium, the explants became brownness and no vigor on account of lacking nutrition; On the other hand, the congregation of high concentration's growth regulators in shoot explants restrained buds growth further. Inhibition of shoot elongation may be because of the high cytokinin activity (Huetteman and Preece 1993). To solve these problems and further enhance the number of shoots per explant, tissue was repeatedly subcultured on another fresh shoot induction medium supplement with less half-growth regulators than induced medium in first stage culture 2 weeks later. Thus, by adopting this strategy, more shoots per explant were harvested. This type of culture procedure of using primary (shoot induction) and secondary (shoot elongation) medium was successfully applied to a number of plant species, including *Malus domestica* (Fasolo et al. 1989), *Cajanus cajan* (Eapen et al. 1998), *Nothapodites foetida* (Satheeshkumar and Seeni 2000), *Rosa damascena* (Kumar et al. 2001), *Morus alba* (Thomas 2003), and *Acacia sinuata* (Vengadesan et al. 2002).

In vitro root induction and hardening

The efficient rooting of regenerated shoots and the survival of plantlets in the soil are the important final steps for successful micropropagation. Rooting of shoots was achieved in the presence of auxins. In general, both NAA and IBA induce rooting response, in this study, however, IBA was proved better as it induced longer and thinner roots in higher number of explants compared to thick and stunted roots produced on NAA supplemented media. Similar results were reported in Mangosteen (Goh et al. 1990), apple (De Klerk et al. 1997) and green ash (Kim et al. 1998). Primary and lateral root growth was promoted by IBA, but strongly inhibited by NAA, such as in Ohwi (Liu et al. 2002a, b) and Karwinskia (Kollárová et al. 2004). The superior effects of IBA on the root and shoot development in these plant species, might be due to several factors, such as its preferential uptake, transport and

Table 3 Nei's unbiased measures of genetic distances between seven populations generated in vitro

POP ID	1	2	3	4	5	6	7
1							
2	0.0192						
3	0.0337	0.0115					
4	0.0175	0.0059	0.0194				
5	0.0202	0.0056	0.0174	0.0119			
6	0.0215	0.0058	0.0134	0.0095	0.0114		
7	0.0240	0.0042	0.0160	0.0062	0.0100	0.0061	

The population is regenerated from different PGRs combination as same details as table 2

stability over NAA and subsequent gene activation (Ludwig-Muller 2000).

Hardening of plantlets is essential for survival of plantlets under ex vitro conditions. For hardening, medium with reduced mineral salt and sucrose concentration was used as it probably forced the regenerants to rely on their own photosynthetic apparatus for nutrition (Kozai et al. 1988).

Genetic stability assessment

In vitro clonal propagation techniques provide the ability to efficiently regenerate and maintain large numbers of elite genotypes, including putative transformants. However, genotypic instability is commonly observed in plants derived from tissue culture and is at least partly due to in vitro-induced stress (Evans et al.1984; Larkin and Scowcroft1981). This phenomenon of somaclonal variation may be either exploited within a breeding process, to increase genetic variability (Karp 1995), or is undesirable for long-term genotype preservation (Larkin and Scowcroft 1981). The molecular basis of somaclonal variation is not precisely known, but both genetic and epigenetic mechanisms have been proposed. Somaclonal variation may arise as a result of point mutations, rearrangements in nuclear or organellar DNA, the activation of mobile elements, ploidy or epigenetic changes causing deviations from the desired phenotype quality standard (Phillips et al. 1994; Jaligot et al. 2000). RAPD technique, being simple and cost effective, has been used to assess the genetic variations of tissue culture plants in numerous studies (Martins et al. 2004), including *Curcuma longa* (Salvi et al. 2001), turmeric (Tyagi et al. 2007), and other plants (Munthali et al. 1996; Tyagi et al. 2007). Genetic variability of in vitro propagated clones is that because plantlets derived from in vitro culture can exhibit somaclonal variation which is often heritable and therefore results in stable genetic changes (Larkin and Scowcroft 1981; Munthali et al. 1996). Actually, chromosome instability and somaclonal variation are more frequently associated with disorganized callus growth which produces adventitious buds, as opposed to the relative stability of organized cultures derived from axillary meristems (Bayliss 1980). We found that overall frequency of somaclonal polymorphisms was lower than 0.01%, which demonstrated that the new method presented in this study was valuable to be extended, especially for further genetic transformation of *P. occidentalis* (Table 3).

Conclusion

This study presented a simple and efficient method for plant regeneration *in vitro* using leaf explants of *P. occidentalis*. And the genetic stability of plantlets regenerated was demonstrated using RAPD markers. The new method presented in this paper can probably be extended to other relative species of *P. occidentalis*.

Acknowledgments This work was supported by 948 Program (No. 2005-4-58) of State Forestry Administration People's Republic of China.

References

Baker BS, Bhatia SK (1993) Factors affecting adventitious shoot regeneration from leaf explants of quince (*Cydonia oblonga*). Plant Cell Tissue Organ Cult 35:273–277. doi:10.1007/BF00037281

Bayliss MW (1980) Chromosomal variation in plant tissue culture. Int Rev Cytol Suppl 11A:113–144

Billings SG, Chin CK, Jelenkovic G (1988) Regeneration of blueberry plantlets from leaf segments. HortScience 23:763–766

Bohanec B, Jakse M, Ihan A, Javornik B (1995) Studies of gynogenesis in onion (*Allium cepa*). Plant Sci 104:215–224. doi:10.1016/0168-9452(94)04030-K

Cecchini E, Natah L, Cavallini A, Durante M (1992) DNA variation in regenerated plants of Pea. Theor Appl Genet 84:874–879. doi:10.1007/BF00227399

Chevreau E, Skirvin RM, Abu Quaid HA, Korban SS, Sullivan JG (1989) Adventitious shoot regeneration from leaf tissues of three pear (Pyrus sp.) cultivars in vitro. Plant Cell Rep 7:688–691

Compton ME (1999) Dark pretreatment improves adventitious shoot organogenesis from cotyledons of diploid watermelon. Plant Cell Tissue Organ Cult 58:185–188. doi:10.1023/A:1006364013126

Cullis CA, Cleary W (1986) DNA variation in Flax tissue culture. Can J Genet Cytol 28:247–251

De Klerk G, ter Brugge J, Marinova S (1997) Effectiveness of indoleacetic acid, indolebutyric acid and naphthaleneacetic acid during adventitious root formation in vitro in Malus 'Jork 9'. Plant Cell Tissue Organ Cult 49:39–44. doi:10.1023/A:1005850222973

Dewan A, Nanda K, Gupta SC (1992) In vitro micropropagation of *Acacia nilotica* subsp. Indica Brenen via cotyledonary nodes. Plant Cell Rep 12:18–21. doi:10.1007/BF00232415

Distabanjong K, Geneve R (1997) Multiple shoot formation from cotyledonary node segments of Eastern redbud. Plant Cell Tissue Organ Cult 47:247–254. doi:10.1007/BF02318979

Doyle JJ, Dickson EE (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19:11

Dunstan DI, Thorpe TA (1986) Plant regeneration and genetic variability. In: Vasil IK (ed) Cell culture and somatic cell genetics of plants, vol 3. Academic Press, Orlando, pp 223–241

Eapen S, Tivarekar S, George L (1998) Thidiazuron-induced shoot regeneration in pigeonpea (*Cajanus cajan* L.). Plant Cell Tissue Organ Cult 53:217–220. doi:10.1023/A:1006060318752

Evans DA, Sharp WR, Medina-Filho HP (1984) Somaclonal and gametoclonal variation. Am J Bot 77:759–774. doi:10.2307/2443467

Ezura H, Oasawa K (1994) Ploidy of somatic embryos and the ability to regenerate plantlets in melon (*Cucumis melo* L.). Plant Cell Rep 14:107–111. doi:10.1007/BF00233771

Famiani F, Ferradini N, Staffolani P, Standardi A (1994) Effect of leaf excision time and age, BA concentration and dark treatments on in vitro shoot regeneration of M26 apple rootstock. J Hortic Sci 69(4):679–685

Fan GQ, Li M, He YQ, Jiang JP (2004) Somatic embryogenesis of platanus orientalis and its plantlet regeneration. Scientia Silvae Sinicae 40(3):71–75 (in Chinese with English abstract)

Fasolo F, Zimmerman RH, Fordham I (1989) Adventitious shoot formation on excised leaves of in vitro grown shoots of apple cultivars. Plant Cell Tissue Organ Cult 16:75–86. doi:10.1007/BF00036516

Goh HKL, Rao AN, Loh CS (1990) Direct shoot bud formation from leaf explant of seedlings and mature mangosteen (*Garcinia mangostana* L.) trees. Plant Sci 68:113–121. doi:10.1016/0168-9452(90)90159-L

Gu XF, Zhang JR (2005) An efficient adventitious shoot regeneration system for Zhanhua winter jujube (*Zizyphus jujuba* Mill.) using leaf explants. Plant Cell Rep 23:775–779. doi:10.1007/s00299-005-0920-5

Gupta PK, Varshney RK (1999) Molecular markers for genetic fidelity during micropropagation and conservation. Curr Sci 76:1308–1310

He XL, Liu GH, She JM, Ni WC (2006) Adventitious shoot regeneration from in vitro cultured leaves of *Platanus orientalis* Willd. Jiangsu J Agric Sci 22(3):225–228 (in Chinese with English abstract)

Herman DE, Hess CE (1963) The effect of etiolation upon the rooting of cuttings. Proc Int Plant Prop Soc 13:42–62

Huang LC, Huang BL, Wang CH, Kuo CI, Murashige T (2000) Developing an improved in vitro propagation system for slow-growing species using *Garcinia mangostana* L. (mangosteen). In Vitro Cell Dev Biol 36:501–504

Huetteman CA, Preece JE (1993) Thidiazuron: a potent cytokinin for woody plant tissue culture. Plant Cell Tissue Organ Cult 33:105–119. doi:10.1007/BF01983223

Isabel N, Tremblay L, Michaud M, Tremblay FM, Bousquet J (1993) RAPDs as an aid to evaluate the genetic integrity of somatic embryogenesis derived populations of *Picea mariana*. Theor Appl Genet 86:81–87. doi:10.1007/BF00223811

Islam R, Hossain M, Joarder OI, Karim MR (1993) Adventitious shoot formation on excised leaf explants of in vitro grown seedlings of Aegle marmelos Corr. J Hortic Sci 68:95–498

Jaligot E, Rival E, Beulé T, Dussert S, Verdeil JL (2000) Somaclonal variation in oil palm (*Elaeis guineensis* Jacq.): the DNA methylation hypothesis. Plant Cell Rep 19:684–690. doi:10.1007/s002999900177

Jha AK, Prakash S, Jain N, Nanda K, Gupta SC (2004) Micropropagation of *Sesbania rostrata* from the cotyledonary node. Biol Plant 48:289–292. doi:10.1023/B:BIOP.0000033458.88441.67

Karp A (1995) Somaclonal variation as a tool for crop improvement. Euphytica 85:295–302. doi:10.1007/BF00023959

Kim MS, Klopfenstein NB, Cregg BM (1998) In vitro and ex vitro rooting of micropropagated shoots using three green ash (*Fraxinus pennsylvanica*) clones. New For 16:43–57. doi:10.1023/A:1006564423011

Kollárová K, Lisková D, Kákoniová D, Lux A (2004) Effect of auxins on *Karwinskia humboldtiana* root cultures. Plant Cell Tissue Organ Cult 79:213–221. doi:10.1007/s11240-004-0662-z

Kozai T, Koyama Y, Watanabe I (1988) Multiplication of potato plantlets in vitro with sugar free medium under high photosynthetic photon flux. Acta Hortic 230:121–127

Kumar A, Sood A, Palni UT, Gupta AK, Palni LMS (2001) Micropropagation of *Rosa damascena* Mill from mature bushes using thidiazuron. J Hortic Sci Biotechnol 76:30–34

Larkin PJ, Scowcroft WR (1981) Somaclonal variation—a novel source of variability from cell cultures for plant improvement. Theor Appl Genet 60:197–214. doi:10.1007/BF02342540

Li ZN, Fang F, Liu GF, Bao MZ (2007a) Stable agrobacterium-mediated genetic plane tree (*Platanus acerifolia* Willd.). Plant Cell Rep 26:641–650. doi:10.1007/s00299-006-0271-x

Li ZN, Liu GF, Fang F, Bao MZ (2007b) Adventitious shoot regeneration of *Platanus acerifolia* Willd. Facilitated by Timentin: an antibiotic for suppression of *Agrobacterium tumefaciens* in genetic transformation. For Stud China 9(1):14–18. doi:10.1007/s11632-007-0003-5

Liu GF, Bao MZ (2003) Adventitious shoot regeneration from in vitro cultured leaves of London plane tree (*Platanus acerifolia* Willd.). Plant Cell Rep 21:640–644

Liu C, Zhu J, Liu Z, Li L, Pan R, Jin L (2002a) Exogenous auxin effects on growth and phenotype of normal and hairy roots of *Puerarialobata* (Willd.) *Ohwi*. Plant Growth Regul 38:37–43. doi:10.1023/A:1020904528045

Liu GF, Huang J, Chen LQ, Bao MZ (2002b) Plant regeneration from excised hypocotyl explants of *Platanus acerifolia* Willd. In Vitro Cell Dev Biol Plant 38:558–563. doi:10.1079/IVP2002350

Liu GF, Li ZN, Bao MZ (2007) Colchicine-induced chromosome doubling in *Platanus acerifolia* L. and its effect on plant morphology. Euphytica 157:145–154. doi:10.1007/s10681-007-9406-6

Ludwig-Muller J (2000) Indole-3-butyric acid in plant growth and development. Plant Growth Regulation 32:219–230

Mante S, Scorza R, Cordts JM (1989) Plant regeneration from cotyledons of *Prunus persica*, *Prunus domestic* and *Prunus cerasus*. Plant Cell Tissue Organ Cult 19:1–11. doi:10.1007/BF00037771

Martins M, Sarmento D, Oliveira MM (2004) Genetic stability of micropropagated almond plantlets, as assessed by RAPD and ISSR markers. Plant Cell Rep 23:492–496. doi:10.1007/s00299-004-0870-3

Matasci M, Gessler C (1997) A fungus menaces the London planetree. Acta Vet Hung 45(2):69–75

McCown BH, Loyd G (1981) Woody plant medium (WMP)—a mineral nutrient formulation for microculture of wood plant species. HortScience 16:453–459

Munthali MT, Newbury HJ, Ford-Lloyd BV (1996) The detection of somaclonal variants of beet using RAPD. Plant Cell Rep 15:474–478. doi:10.1007/BF00232977

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:473–497. doi:10.1111/j.1399-3054.1962.tb08052.x

Niederwieser JG, Van Staden J (1990) The relationship between genotype, tissue age and endogenous cytokinin levels on adventitious bud formation on leaves of Lachenalia. Plant Cell Tissue Organ Cult 22:223–228. doi:10.1007/BF00033640

Phillips RL, Kaeppler SM, Olhoft P (1994) Genetic instability of plant tissue cultures: break down of normal controls. Proc Natl Acad Sci USA 91:5222–5226. doi:10.1073/pnas.91.12.5222

Pradhan C, Kar S, Pattnaik S, Chand PK (1998) Propagation of *Dalbergia sissoo* Roxb through in vitro shoot proliferation from cotyledonary nodes. Plant Cell Rep 18:122–126. doi:10.1007/s002990050543

Purohit SD, Dave A (1996) Micropropagation of *Sterculia urens* Roxb. An endangered tree species. Plant Cell Rep 15:704–706. doi:10.1007/BF00231929

Rani V, Raina SN (1998) Genetic analysis of enhanced axillary branching derived *Eucalyptus tereticornis* Smith and *E. camaldulensis* Dehn. plants. Plant Cell Rep 17:236–242. doi:10.1007/s002990050385

Rani V, Parida A, Raina SN (1995) Random amplified polymorphic DNA (RAPD) markers for genetic analysis in micropropagated plants of Populus deltoids Marsh. Plant Cell Rep 14:459–462. doi:10.1007/BF00234055

Rusli I, Pierre CD (2001) Factors controlling high efficiency adventitious bud formation and plant regeneration from in vitro leaf explants of roses (*Rosa hybrida* L.). Sci Hortic (Amsterdam) 88:41–57 10.1016/S0304-4238(00)00189-8

Salvi ND, George L, Eapen S (2001) Plant regeneration from leaf base callus of turmeric and random amplified polymorphic DNA analysis of regenerated plants. Plant Cell Tissue Organ Cult 66:113–119. doi:10.1023/A:1010638209377

Satheeshkumar K, Seeni S (2000) In vitro multiplication of Nothapodites foetida (Wight.) Sleumer through seedling explant culture. Indian J Exp Biol 38:273–277

Shuerman PL, Dandekar AM (1993) Transformation of temperate woody crops: progress and potentials. Sci Hortic (Amsterdam) 55:101–124. doi:10.1016/0304-4238(93)90027-N

Sriskandarajah S, Frello S, Jørgensen K, Serek M (2004) Agrobacterium tumefaciens-mediated transformation of *Campanula carpatica*: factors affecting transformation and regeneration of transgenic shoots. Plant Cell Rep 23:59–63. doi:10.1007/s00299-004-0797-8

Strauss SH, Rottmann WH, Brunner AM (1995) Sheppard LA Genetic engineering of reproductive sterility in forest trees. Mol Breed 1:5–26. doi:10.1007/BF01682086

Subiza J, Cabrera M, Valdivieso R, Subiza JL, Jerez M, Jimenez JA et al (1994) Seasonal asthma caused by airborne *Platanus pollen*. Clin Exp Allergy 24(12):1123–1129

Sudha CG, Krishnan PN, Pushpangadan P, Seeni P (2005) In vitro propagation of *Decalepis arayalpathra*, a critically endangered ethnomedicinal plant. In Vitro Cell Dev Biol Plant 41:648–654. doi:10.1079/IVP2005652

Thomas TD (2003) Thidiazuron induced multiple shoot induction and plant regeneration from cotyledonary explants of mulberry. Biol Plant 46:529–533. doi:10.1023/A:1024807426591

Trigiano RN, Gray DJ (2000) Plant tissue culture: concepts and laboratory exercises, 2nd edn. CRC Press, Boca Raton, FL, USA

Tyagi RK, Agrawal A, Mahalakshmi C, Hussain Z, Tyagi H (2007) Low-cost media for in vitro conservation of turmeric (*Curcuma longa* L.) and genetic stability assessment using RAPD markers. In Vitro Cell Dev Biol Plant 43:51–58

Varela S, Subiza J, Subiza JL, Rodriguez R, Garcia B, Jerez M et al (1997) Platanus pollen as an important cause of pollinosis. J Allergy Clin Immunol 100(1):748–754. doi:10.1016/S0091-6749(97)70268-9

Vengadesan G, Ganapathi A, Prem Anand R, Anbazhagan RV (2002) In vitro propagation of *Acacia sinuata* (Lour.) Merr. via cotyledonary nodes. Agrofor Syst 55:9–15. doi:10.1023/A:1020269022363

Walter JM (1946) Canker stain of plane trees. Circular no. 742. United States Department of Agriculture, Washington, DC

Walter JM, Rex EG, Schreiber R (1952) The rate of progress and destructiveness of canker stain of plane trees. Phytopathology 42:236–239

Wang L, Li HS, Lin N, Cui DC (2004) Establishment of leaf regeneration system in *Platanus acerifolia*. Scientia Silvae Sinicae 40(1):58–63 (in Chinese with English abstract)

Wei ZM, Xu ZH (1991) Mesophyll protoplast culture and plant regeneration of oriental planetree (*Platanus orientalis*). Acta Bot Sin 33(1):813–818

Zaerr JB, Mapes MO (1982) Action of growth regulators. In: Bonga JM, Durzan DJ (eds) Tissue culture in forestry. Martinus Nijhoff/Dr. W. Junk Publishers, The Hague, pp 231–255

Zahim-Al AM, Ford-Lloyed BV, Newbury HJ (1999) Detection of somaclonal variation in garlic (*Allium sativum* L.) using RAPD and cytological analysis. Plant Cell Rep 18:473–477. doi:10.1007/s002990050606

Zou YM, Shi JS (2005) Establishment of high frequency regeneration system of adventitious bud of *Platanus occidentalis* Linn. J Nanjing For Univ 29(4):15–19 (Natural Sciences Edition)

Zou YM, Shi JS (2006) Tissue culture and mass propagation of *Platanus occidentalis* Linn. J Nanjing For Univ 30(6):61–65 (Natural Sciences Edition)