

EVALUATION OF THE GENETIC STABILITY OF *In vitro*-PROPAGATED *Paulownia tomentosa* USING DNA-BASED MARKERS

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The genus *Paulownia* (Scrophulariaceae) includes nine species of fast-growing trees, indigenous to China and East Asia. It could be considered as a low demand water plant, in spite of not growing in barren zones (Caparros *et al.*, 2008). Most species of *Paulownia* are extremely fast growing and the tree can be harvested in 6-7 years old. A full grown *Paulownia* can reach a height of 10 to 20 meters and grows up to 3 meters in one year under ideal conditions. A 10-years old tree can measure 30-40 cm diameter at breast height and can have a timber volume of 0.3-0.5 m³ (Flynn and Holder, 2001). It may grow in intensive plantations with about 2000 trees per ha. The wood of *Paulownia* is soft, lightweight, ring porous straight grained, and mostly knot free wood with a satiny luster. Average specific gravity of the wood is reported as 0.35 g cm⁻³ (Kalaycioglu *et al.*, 2005; Flynn and Holder, 2001). *Paulownia* wood is used for a variety of applications such as furniture, construction, musical instrument, shipbuilding, aircraft, packing boxes, coffins, paper, plywood, cabinet-making, and molding (Flynn and Holder, 2001). *Paulownia* wood is marketed primarily for specialty solid wood products, oriented strand board, veneer, and for pulp

to produce fine papers (Rai *et al.*, 2000). Its stem bark has been used in Chinese herbal medicine as a component of remedies for infectious diseases such as gonorrhoea and erysipelas (Asai *et al.*, 2008). Aside from its continuing ornamental use, the species has value for its small sawn timbers that are in demand for specialty products. It is also used among Chinese folks to treat bronchitis, dysentery, bacterial dysentery, acute enteritis, parotitis and acute conjunctivitis, etc. (Liao *et al.*, 2008).

Micropropagation is one of the viable alternatives for large-scale multiplication of *Paulownia sp.* (Ipekci *et al.*, 2001). Over the years, *Paulownia sp.* has been propagated by direct or indirect organogenesis using various explants, including stem tips, leaf, capitulum etc. (Marcotrigiano and Stimart, 1983; Rao *et al.*, 1996). True-to-type clonal fidelity, *i.e.*, genetic stability, stability is one of the most important pre-requisites in the micropropagation of crop species. The occurrence of cryptic genetic defects arising *via* somaclonal variation in the regenerates can seriously limit the broad utility of the micropropagation system (Salvi *et al.*, 2001). It is, therefore, imperative to

establish genetic uniformity of micropropagated plants to confirm the quality of the plantlets for its commercial utility.

Polymerase chain reaction (PCR)-based techniques such as random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) are immensely useful in establishing the genetic stability of *in vitro*-regenerated plantlets in many crop species (Lakshmanan *et al.*, 2007; Joshi and Dhawan, 2007). RAPD and ISSR markers are very simple, fast, cost-effective, highly discriminative and reliable. They require only a small quantity of DNA sample and they do not need any prior sequence information to design the primer. They do not use radioactive probes as in restriction fragment length polymorphism (RFLP) (Lakshmanan *et al.*, 2007). Thus, they are suitable for the assessment of the genetic stability of *in vitro*-raised clones. In this study, the assessed the clonal stability of the *in vitro* were assessed *Paulownia tomentosa* plants using RAPD and ISSR markers was carried out. This is very useful for establishing a particular micropropagation system for the production of genetically identical and stable plants before it is released for commercial purposes.

MATERIALS AND METHODS

Plant materials and explant source

Shoot apexes from axillary buds of *Paulowina tomentosa* plants (3- to 4 year-old) grown at faculty of agriculture, Ain Shams university, Cairo, Egypt, were used

as a source of vegetative materials. The shoot tips (50-100 mm length) were firstly soaked for 30 Sec. in 70% ethanol solution. Further, the explants were disinfested in 0.1% (w/v) mercuric chloride (HgCl₂) solution for 15 min and then rinsed three times in sterile distilled water. The twig is cut into smaller segments (≈ 0.5 cm each) having one node in each segment. Nodal explants were used as source of explants (Rout *et al.*, 2001).

Culture medium

Murashige and Skoog (MS) basal medium supplemented with different concentrations of 6-Benzyladenine (BA) (Table 1). For induction of rooting, the *in vitro* raised shoots measuring about 2-3 cm growing on multiplication medium (MS + 4.44 μ M BA + 0.5 μ M NAA + 3% (w/v) sucrose) were excised and cultured on half-strength basal MS medium supplemented with IBA (0.6 μ M) and 2.5% (w/v) sucrose (Rout *et al.*, 2001).

Culture condition

All cultures were incubated under 16 h photoperiod under light intensity 1500 lux provided by cool, white fluorescent lamps at 25 \pm 2°C. The cultures were maintained by regular sub culturing at 3-weeks intervals to new medium with the same composition.

Acclimatization

Plantlets obtained *in vitro* were removed from culture vessels and washed with tap water, then disinfected by immer-

sion in Benlate solution (1.0 g/L) as a fungicide for 5 min. The plantlets were transferred to plastic pots (6 cm width) containing peat moss and sand (1:1). Pots were covered with transparent polyethylene pages for two weeks and gradually they removed in the greenhouse before subsequent transfer to the field (Taha *et al.*, 2008)

DNA isolation

Fresh leaves of plantlets obtained *in vitro* and field grown mother plants were collected from culture vessels, and then washed with tap water. The Leaf samples were frozen in liquid nitrogen and stored at -20°C until used. DNA was isolated from 50 mg of grind leaves using Qiagen Kit for DNA extraction. The extracted DNA was dissolved in 100 µl of elution buffer.

Random amplified polymorphic DNA (RAPD) and Inter simple sequence repeats (ISSR)-PCR techniques

Twelve arbitrary 10-base primers (Operon Technologies Inc., Alameda, California) were used for Polymerase Chain PCR reactions (25 µl) with random 10-nt primers were contained 25 to 50 mg of DNA (1 µl of diluted DNA), 1.5 µl of 10× reaction buffer, 200 µM dNTPs (Sigma Chemical Co., St. Louis), 0.8 µM primer (Operon Technologies, OP-A10, OP-A12, OP-A19, OP-B07, OP-B12, OP-C03 and OP-C04 have sequence 5' TCG GCC ATA G 3', 5'-TCG GCG ATA G-3', 5'-CAA ACG TCG G-3', 5'-GGT GAC GCA G-3', 5'-CCT TGA CGC A-3', 5'-

GGG GGT CTT T-3' and 5'-CCG CAT CTA C-3', respectively), and 0.6 units of *Taq* polymerase. Mixtures were overlain with 40µl of sterile light mineral oil and placed in a thermo cycler (Perkin Elmer 2400) programmed for 45 cycles: 1st cycle of 3.5 min at 94°C, 1 min at 37°C and 2 min at 72°C; then 44 cycles each of 1 min at 94°C, 1 min at 37°C, 2 min at 72°C followed by one final extension cycle of 7 min at 72°C. For ISSR primers (814A, 844A, HB08 and HB09 have sequence 5'CTC TCT CTC TCT CTC TTG3', 5'CTC TCT CTC TCT CTC TAC 3', 5' GAG AGA GAG AGA GG 3' and 5' GTG TGT GTG TGT GC 3', respectively), The ISSR conditions were for 36 cycles., the initial strand separation cycle at 94°C for 4 min, followed by 35 cycles including a denaturation step at 94°C for 1 min then an annealing step at 57°C for 1 min and polymerization step at 72°C for 2 min with a final extension step at 72°C for 10 min. Amplified products were electrophoresed in a 1.2% (w/v) agarose (Sigma, USA) gels with TAE buffer, stained with ethidium bromide, and photographed under ultraviolet (UV) light. The size of the amplification products were estimated for a 100 bp (100 bp to 3.0 Kb) ladder (Fermentas Inc.), all the reactions was repeated at least thrice.

Statistical analysis

Usually, 10 cultures were raised per each treatment and each experiment was conducted at least three times. The cultures were examined periodically and the morphological changes were recorded

on the basis of visual observations. The effects of different treatments were quantified as the percent of explants showing multiplication, mean number of multiple shoots/culture, rooting and number of roots/shoot percent. The data were statistically analysed by Duncan's multiplexer test (Harter, 1960). Data were analyzed with Statistical Analysis System software version 8.0 (SAS Institute, 1999).

Data scoring and analysis

Consistent, well-resolved fragments in the size range of 67 bp to 2.3 kb were scored by Bio Rad Gel Doc. Each band was treated as a marker. The scoring of bands was done at the basis of their presence (1) or absence (0) in the gel. The genetic associations were evaluated by calculating the Jaccard's similarity coefficient for pair-wise comparisons based on the proportion of shared bands produced by the primers.

RESULTS AND DISCUSSION

The present investigation was carried out to explore the morphogenic potential of *Paulownia tomentosa* from nodal explants using growth regulators. Out of the different cytokinins tested, BA was the most effective for inducing bud dormancy break in axillary meristems (Table 1). Axillary bud was developed within 8-10 days of inoculation onto MS basal medium supplemented with 4.44 μM BA. The maximum bud proliferation was observed in MS medium supplemented with this concentration within two-weeks of cultur-

ing; the axillary meristems elongated up to 4.5-6.0 cm height (Fig. 1a). Prolonged culture on induction medium resulted in the elongation of shoots. BA was then tested in combination with NAA (0.5 μM) to enhance the rate of shoot multiplication. The medium containing BA (4.44 μM) with NAA (0.5 μM) proved to be the most effective treatment for promoting shoot multiplication (3-4 shoots per nodal explant, each having 2-3 nodes) after 2-week of culture (Fig. 1a). Additions of low concentration of auxin promoted shoot multiplication were reported by various authors for *Paulownia elongata* (Ipekci *et al.*, 2001) and *Madhuca longifolia* (Rout and Das, 1993). The multiplication frequency was maintained for prolonged period without loss of multiplication ability. This might be due to a better balance of the endogenous and exogenous growth regulators and ionic concentration of nutrient salts as reported earlier in other crops (Rout and Das, 1993). Microshoots derived from axillary meristems were separated and transferred to a rooting medium having half-strength basal MS medium supplemented with of IBA with 2% (w/v) sucrose. IBA induced rooting on (0.30, 0.60 and 1.33 μM) within 2 weeks of culture (Table 2). Each shoot formed 2-4 roots in the culture contain 0.60 μM IBA after 2 weeks of incubation (Fig. 1b). About 95% of the shoots were rooted on medium containing 0.60 μM IBA within two weeks of culture. These observations have been reported earlier in other tree species (Iriando *et al.*, 1995; Rout *et al.*, 1995).

Acclimatization

Rooted plantlets of *Paulownia* were transferred from the aseptic culture environment (*in vitro*) to two types of soils of peat moss, (Fig. 1c), mixture of peat moss and sand at the ratio 1:1. These processes were established in the greenhouse. After two weeks, the highest percentage of survival (90%) was obtained by using a soil mixture of peat moss and sand as shown in Fig. (1d & e) cultivation the transplant of *Paulownia* succeeded in soil under Egyptian environments.

Assessment of genetic stability using some DNA based markers

A total of 12 RAPD primers were used for initial screening with the mother plant of *Paulownia tomentosa* but only 7 RAPD primers gave clear and reproducible bands. The number of scorable bands for each RAPD primer varied from 5 (OP-A19) to 8 (OP-A10 and OP-C03) (Fig. 2 Table 3). The 7 RAPD primers produced 47 distinct and scorable bands. Each primer generated a unique set of amplification products ranging in size from 67 bp (OP-B07) to 2300 bp (OP-B12). No polymorphism was detected during the RAPD analysis of *in vitro*-raised clones. The absence of genetic variation using RAPD has been reported in micropropagated shoots of *Pinus thunbergii* (Goto *et al.*, 1998), *in vitro*-regenerated turmeric (Salvi *et al.*, 2001) and *in vitro*-raised bulblets of *Lilium* (Varshney *et al.*, 2001).

Out of seven ISSR primers used in the initial screening, only four primers produced clear and reproducible bands. The optimum annealing temperature for ISSR markers are 57°C. The four ISSR primers produced 22 distinct and scorable bands in the size range of 280 bp (ISSR-HB09) to 1390 bp (ISSR-844A) (Table 3). The number of scorable bands for each primer varied from 3 (ISSR-814A) to 8 (ISSR-HB09). All banding profiles from micropropagated plants were monomorphic and similar to those of the mother plant (Fig. 3). Similar results have been reported by Martin *et al.* (2004) in almond and Joshi and Dhawan (2007) in *Swertia chirayita*.

Two PCR-based techniques, RAPD and ISSR, were used to test clonal stability because of their simplicity and cost-effectiveness. The use of the two markers, which amplify different regions of the genome, allows better chances for the identification of genetic variations in the clones (Martin *et al.*, 2004). All banding profiles from micropropagated plants were monomorphic and similar to those of the mother plant (Fig. 2). A similarity matrix based on Jaccard's coefficient revealed that the pair-wise value between the mother plant and the plantlets derived from different explants was 1, indicating 100% similarity. Similar results were obtained by Sreedhar *et al.* (2007) during the clonal stability analysis of long-term micropropagated shoot cultures of vanilla (*Vanilla planifolia* Andrews) by RAPD and ISSR markers. Earlier, Reynoird *et al.*

(1993) also did not observe any phenotypic variations during vegetative and reproductive phases among the regenerates of gerbera.

The presence or absence of variations during *in vitro* propagation depends upon the source of explants and the method of regeneration (Goto *et al.*, 1998). The sub- and supra-optimal levels of plant growth substances, especially synthetic ones, have also been associated with somaclonal variation (Martin *et al.*, 2004). Even at optimal levels, long term multiplication may often lead to somaclonal or epigenetic variations in micropropagated plants, thus, questioning the very stability of their clonal nature. In our study, plantlets were obtained from the sprouting of dormant buds situated in the axils of the bracts. These findings support the fact that a meristem-based micro-propagation system is much more stable genetically than those in which regeneration occurs via the callus phase. Plants regenerated from adventitious buds around axillary buds or from other well developed meristematic tissue showed the lowest tendency for genetic variation (Joshi and Dhawan, 2007). Even plants derived from organised meristems are not always genetically true to the type in many crops (Devarumath *et al.*, 2002). Hence, it becomes imperative to regularly check the genetic purity of the micro-propagated plants in order to produce clonally uniform progeny while using different techniques of micropropagation. In this study, the true to the type nature of the *in vitro* raised clones was

confirmed using DNA-based markers. No variability was detected among the tissue culture-raised plantlets; hence, capitulum explants can be successfully employed for the commercial multiplication of *Paulownia* without much risk of genetic instability.

SUMMARY

The genetic stability of *in vitro*-raised *Paulownia tomentosa* clones was assessed by using random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSRs) markers. Out of 12 RAPD and seven ISSR primers screened, only eight RAPD and four ISSR primers produced clear, reproducible and scorable bands. The seven RAPD primers produced 47 distinct and scorable bands. The molecular size of the total 22 fragments generated by RAPD technique primers ranged from ≈ 97 -2300 bp.

The number of scorable bands for ISSR primers varied from 3 (ISSR-14) to 8 (ISSR-07). The molecular size of PCR products generated 22 fragments by these ISSR ranged from ≈ 280 to 1390 bp. The number of bands generated per primer was greater in ISSR than RAPD. All banding profiles from micropropagated plants were monomorphic and similar to those of the mother plant. A similarity matrix based on Jaccard's coefficient revealed that the pair-wise value between the mother and the *in vitro*-raised plantlets was 1, indicating 100% similarity. This confirmed the true-to-type nature of the *in vitro*-raised clones.

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Table (1): Effect of cytokinin (BA) on response of shoot multiplication of *P. tomentosa* within two weeks of culture; the data represent the mean of three independent experiments.

Concentration (μM)	Mean percentage \pm SE
2.22	52 \pm 1.1
4.44	88 \pm 1.4
6.66	62 \pm 1.1

Table (2): Effect of auxin (IBA) and (4.44 μM BA and 0.5 μM NAA) on rooting of excised shoots of *P. tomentosa* within two weeks of culture; the data represent the mean of three independent experiments.

Concentration of IBA (μM)	Mean percentage \pm SE	Number of roots/shoot \pm SE
0.30	68 \pm 1.2	3.4 \pm 0.3
0.60	95 \pm 1.3	8.5 \pm 0.4
1.32	56 \pm 1.4	6.5 \pm 0.2

Table (3): Summary of numbers of bands pattern resulted from different molecular systems electrophoretic of the three explant and mother plant of *P. tomentosa*.

DNA Markers	Primer	No. of bands	Lowest length (\approx bp)	Highest length (\approx bp)
RAPD	OP-A10	8	400	2160
	OP-A12	7	100	600
	OP-A19	5	250	2280
	OP-B07	7	67	500
	OP-B12	6	390	2300
	OP-C03	8	280	820
	OP-C04	6	200	730
ISSR	814A	3	510	1100
	844A	6	320	1390
	HB08	5	740	1320
	HB09	8	410	970



Fig. (1): Micropropagation of *Paulownia tomentosa*:

- a- Formation of multiple shoots regenerated from axillary buds of a nodal explant cultured on MS medium supplemented with $4.44 \mu\text{M}$ BA + $0.53 \mu\text{M}$ NAA after two weeks of culture,
- b- Rooting of microshoot on 1/2 strength MS basal medium supplemented with $0.60 \mu\text{M}$ IBA + 2% (w/v) sucrose after two weeks of culture,
- c- Raised plantlet transplanted in soil one week after acclimatization,
- d- Two week after transplant and
- e- Five month after transplant.

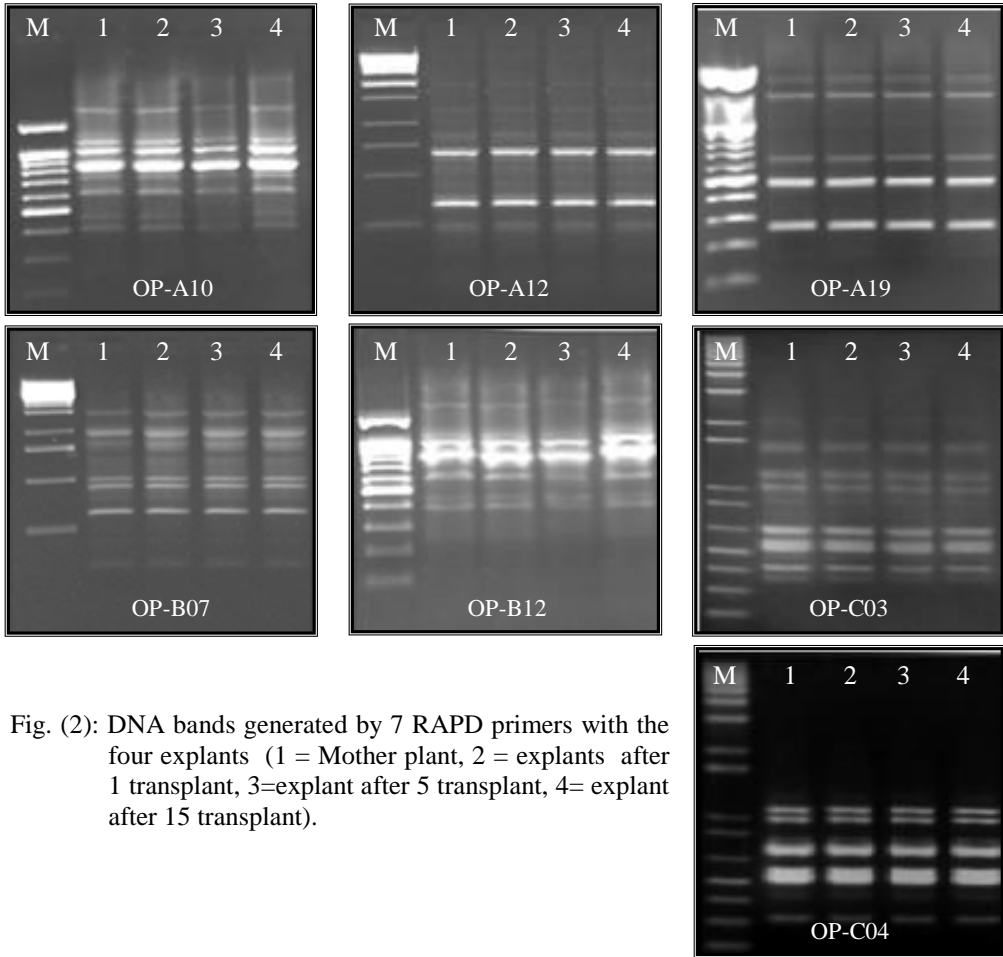


Fig. (2): DNA bands generated by 7 RAPD primers with the four explants (1 = Mother plant, 2 = explants after 1 transplant, 3=explant after 5 transplant, 4= explant after 15 transplant).

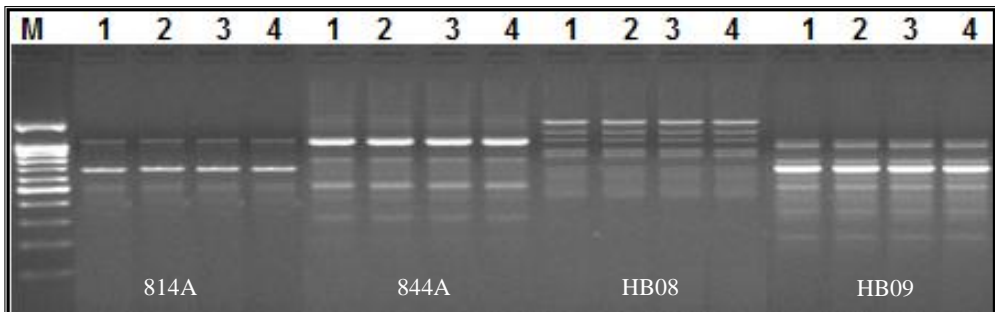


Fig. (3): DNA bands generated by four ISSR primers with the four explant (1=Mother plant, 2=explant after 1 transplant, 3= explant after 5 transplant, 4= explant after 15 transplant).