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 gonorrhea and erysipelas (Asai 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 Paulownia 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 μM BA + 0.5 μM NAA + 3% (w/v) sucrose) were excised and cultured on half-strength basal MS medium supplemented with IBA (0.6 μM) 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±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 were 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 ul 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’-CAAC GAC 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 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 multiplerange 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 morphgenic 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-
**Acclimatization**

Rooted plantlets of *Paulowina* 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 *Paulowina* 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.
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(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 to1390 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 ±SE |
|--------------------|----------------------|
| 2.22               | 52 ± 1.1             |
| 4.44               | 88 ± 1.4             |
| 6.66               | 62 ± 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 ±SE | Number of roots/shoot ±SE |
|---------------------------|----------------------|---------------------------|
| 0.30                      | 68 ± 1.2             | 3.4 ± 0.3                 |
| 0.60                      | 95 ± 1.3             | 8.5 ± 0.4                 |
| 1.32                      | 56 ± 1.4             | 6.5 ± 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 (≈bp) | Highest length (≈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 μM BA + 0.53 μM NAA after two weeks of culture,

b- Rooting of microshoot on 1/2 strength MS basal medium supplemented with 0.60 μM 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).