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MICROPROPAGATION

Genetic fidelity assessment of *in vitro*-regenerated plants of *Albizia julibrissin* using SCoT and IRAP fingerprinting

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Abstract A protocol was established for callus induction and plant regeneration of *Albizia julibrissin* Durazz., a multipurpose tree. Calli were induced on hypocotyl explants excised from 10- to 14-d-old *in vitro* seedlings cultured on Murashige and Skoog (MS) medium supplemented with α-naphthaleneacetic acid (NAA) alone or in combination with 6-benzylaminopurine (BA) or 6-furfurylaminopurine (kinetin). The highest frequency of organogenic callus (82.2± 3.6%) was obtained on MS medium with 10.8 μM NAA and 4.4 μM BA. Calli were then cultured on MS medium with BA or zeatin, singly or in combination, for shoot regeneration. Calli cultured on MS medium with 13.2 μM BA and 4.6 μM zeatin produced the highest frequency of adventitious shoot regeneration (75.3±6.3%). Maximum rooting of shoots (73.3±5%) was achieved using half-strength MS medium with 4.9 μM indole-3-butyric acid. The genetic fidelity of 12 plants acclimatized to the greenhouse was assessed based on analyses of start codon targeted (SCoT) polymorphism and inter-retrotransposon amplified polymorphism (IRAP). The 14 SCoT and 7 IRAP adapted primers produced 71 and 34 scoreable fragments, of which 33 (46%) and 12 (35%) were polymorphic, respectively. The *in vitro*-raised plants exhibited 0.129–0.438 genetic distance from the mother plant and 0.000–0.788 distance from one another according to the SCoT and IRAP analyses. Although the culture method described here may not be suitable for clonal propagation of elite genotypes, it can be used for conservation of this plant.

Keywords Adventitious shoot regeneration · *Albizia* · Genetic fidelity · IRAP · SCoT · Silk tree

Introduction

Silk tree or mimosa (*Albizia julibrissin* Durazz.: Fabaceae: Mimosoideae) is an important ecological and medicinal deciduous tree legume native to Azerbaijan, Iran, China, Nepal, Taiwan, Korea, and Japan (Cheatham *et al.* 1995; Orwa *et al.* 2009). Atmospheric nitrogen fixation, high frost tolerance, fast-growth, and response to coppicing make *A. julibrissin* a beneficial tree with many potential uses including soil fertility improvement and erosion control (Rhoades *et al.* 1998; Jordan 2004; Pitman 2008). In addition to the use of seeds as a source of oil and the foliage as fodder for livestock, goats, and other ruminants and wildlife (Wang *et al.* 2006; Nehdi 2011; Bouazza *et al.* 2012), butterflies and honeybees consume the nectar from the silk tree flowers. This tree has been extensively planted in gardens for ornamental purposes. The ornamental importance of this species has been limited by sensitivity to a soil-borne fungus, *Fusarium oxysporum* f. sp. *perniciosum*, which infects the root system, causing vascular wilting and eventual death (Phipps and Stipes 1976). High-quality timber from *A. julibrissin* can be used for building materials and furniture manufacturing (Zheng *et al.* 2004), and it could be a valuable resource for producing gum.

Flower heads of *A. julibrissin* are used in traditional medicine for the treatment of insomnia, anxiety, and depression (Kang *et al.* 2007), while an infusion made from the stem bark is used in folk medicine to treat sprains, fractures, abscesses,

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hemorrhoids, boils, and ulcers (Orwa *et al.* 2009; Kokila *et al.* 2013). Active natural products of great pharmaceutical interest include triterpenoid saponins (Kinjo *et al.* 1992; Liang *et al.* 2005; Han *et al.* 2011), phenolic glycosides (Jung *et al.* 2004), and flavonoids (Lau *et al.* 2007). It has been reported that two secondary metabolites from the tree pods have the potential to act as biocides and antioxidants (Lv *et al.* 2011). The triterpenoid julibroside J_{28} from the bark of the plant significantly inhibited the growth of three tumor cell lines *in vitro* (Liang *et al.* 2005). The anti-tumor properties of three minor saponin extracts (Zheng *et al.* 2006) and the cytotoxic effect of bark extracts on human acute leukemia Jurkat T cells have also been reported (Won *et al.* 2006). In addition, dried tissues of this plant contain 0.83% hyperoside and 0.9% quercitrin, which may play a role in anti-inflammation and intestinal repair, respectively (Ekenseair *et al.* 2006).

Silk tree is conventionally propagated by seed. However, seeds can be heavily infested by the beetle *Bruchidius terrenus* (Sharp) (Coleoptera: Chrysomelidae: Bruchinae) (Hoebeke *et al.* 2009; Hizal and Nihan Parlak 2013). Seed-based propagation is currently not providing sufficient material to conserve or improve the germplasm resource of this species in the declining Hyrcanian forests of northern Iran. Therefore, plant tissue culture may provide a viable approach for mass propagation, genetic conservation, and development of genetic engineering technology for the improvement of *A. julibrissin*, as well as for the commercial production of pharmaceutically important compounds. There is, however, limited information available on *in vitro* plant regeneration for this species. *A. julibrissin* can be regenerated via adventitious shoot morphogenesis from cotyledons, hypocotyls, and roots (Sankhla *et al.* 1993, 1994, 1995, 1996; Zhou *et al.* 2001) or through somatic embryogenesis (Burns and Wetzstein 1998).

The genetic fidelity or variability of *in vitro*-regenerated plants can be successfully verified by different DNA-based molecular markers, such as inter-simple sequence repeats (ISSR; Rawat *et al.* 2013), inter-retrotransposon amplified polymorphism (IRAP; Campbell *et al.* 2011), or start codon targeted (SCoT) polymorphism (Rathore *et al.* 2014). In this investigation, SCoT and IRAP were used to assess genetic constancy of *in vitro*-raised plants of *A. julibrissin*.

The objectives of our study were to develop an efficient adventitious shoot regeneration and rooting method from *in vitro* cultured hypocotyls excised from seeds of *A. julibrissin* and to assess the genetic stability or variability of regenerated plants.

Materials and Methods

Plant material and culture conditions. Mature pods of *A. julibrissin* were collected from a 40- to 50-yr-old tree growing in the Sisangan area of the Hyrcanian forest, Nowshahr, Iran. Seeds were excised from the pods and stored at 6°C in the dark until use. Seeds were rinsed under running tap water for 20 min, immersed in 90–95°C water (Fordham 1965) that was then allowed to gradually cool to room temperature, and incubated in the water overnight at 28°C in an incubator to stimulate germination. Treated seeds were surface disinfested by soaking in 70% (*v/v*) ethanol for 2 min, followed by immersion in 20% (*v/v*) bleach solution (5.25% sodium hypochlorite) containing three drops of Tween 20 per 100 ml for 10 min, followed by three rinses (3 min each) in sterile distilled water. Seeds were then germinated on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 3% (*w/v*) sucrose and 0.8% (*w/v*) agar (Duchefa, Haarlem, Netherlands, Plant Agar P1001). Hypocotyl segments were excised from 10- to 14-d-old *in vitro* seedlings for experiments. Unless noted otherwise, all *in vitro* material were cultured in 300-ml jam jars (120×80 mm) containing 50 ml medium, and maintained at 25±2°C under a 16-h photoperiod provided by cool-white fluorescent lamps (Philips, Eindhoven, Netherlands): 40 μmol m^{-2} s^{-1} for callus induction, 60 μmol m^{-2} s^{-1} for adventitious shoot regeneration and rooting. All media contained 3% (*w/v*) sucrose and 0.8% agar with the pH of the medium adjusted to 5.8 before autoclaving at 121°C for 20 min.

Callus induction and adventitious shoot regeneration. Hypocotyl segments (5–6 mm) were cultured horizontally on MS medium containing 0.2 g L^{-1} *myo*-inositol and supplemented with 5.4, 8.1, 10.8, 13.4, or 16.2 μM α-naphthaleneacetic acid (NAA) alone or in combination with 2.2 or 4.4 μM 6-benzylaminopurine (BA) or 2.3 or 4.7 μM 6-furfurylaminopurine (kinetin) for callus induction (Table 1). Medium without plant growth regulators (PGR) served as the control. Calli induced on hypocotyl explants from the different PGR treatments were subcultured twice to the same treatment medium every 4 wk. Replicate treatments were conducted using eight jars, each containing six explants, and the experiment was performed twice (n=96). The percentage of explants with callus and the number of total organogenic, white-friable calli per treatment were recorded 4- and 6-wk after culture initiation, respectively.

For shoot induction, an aliquot (150–200 mg) of callus obtained from hypocotyl sections was cultured on MS medium supplemented with 4.4, 8.8, 11.1, or 13.2 μM BA or 2.3, 4.6, or 6.8 μM zeatin alone or in combination (Table 2). Medium without PGRs was used as a control. After selection of the best PGRs for shoot regeneration (13.2 μM BA plus 4.6 μM zeatin; see Results), basal MS medium, woody plant medium (WPM; Lloyd and McCown 1981), and Gamborg (B5) medium (Gamborg *et al.* 1968) were supplemented with 0.2 or 0.3 g L^{-1} glycine and 0.2 or 0.3 g L^{-1} *myo*-inositol to

Table 1. Effect of plant growth regulators on callus induction from hypocotyl sections of *Albizia julibrissin*

Plant growth regulator (μM)			Callus induction (%)[z]	Organogenic callus (%)[y]
NAA	BA	KIN		
0	0	0	0±0j	0±0h
5.4			38.6±4.6i	9.3±2.5g
8.1			58.3±7.0fgh	15.7±2.9g
10.8			56.2±7.4fgh	16.8±2.7g
13.4			62.3±4.7efg	20.7±3.5fg
16.2			55.2±3.4fgh	18.7±2.6g
5.4	2.2		58.5±7.0fgh	49.8±7.0c
8.1	2.2		59.5±5.5fgh	49.0±5.5c
10.8	2.2		76.0±3.6cd	44.7±4.4cd
13.4	2.2		57.3±4.8fgh	34.2±1.9e
16.2	2.2		56.5±4.9fgh	35.3±3.0de
5.4	4.4		77.1±3.0bc	55.3±3.8bc
8.1	4.4		89.5±3.4ab	61.3±3.5b
10.8	4.4		100±0a	82.2±3.6a
13.4	4.4		100±0a	77.0±2.5a
16.2	4.4		65.6±4.5cde	53.1±2.6bc
5.4		2.3	63.6±4.4cde	14.6±3.0g
8.1		2.3	69.7±4.1cde	12.5±2.7g
10.8		2.3	67.7±4.5cde	18.7±4.0g
13.4		2.3	44.7±3.1hi	20.8±2.7fg
16.2		2.3	48.8±4.3ghi	19.8±2.6fg
5.4		4.7	60.5±5.3efg	32.2±2.4e
8.1		4.7	57.3±5.1fgh	20.8±3.4fg
10.8		4.7	47.8±4.4hi	16.6±3.8g
13.4		4.7	55.2±4.1fgh	30.1±2.1ef
16.2		4.7	38.5±3.1i	17.7±2.4g

Basal medium was MS medium containing 0.2 g L^{-1} *myo*-inositol. Results represent the means±standard error of two replicated experiments (96 explants per treatment). Means within a *column* followed by different *letter*(*s*) are significantly different at $P=0.05$ according to Duncan's multiple range test following analysis of variance

NAA α-naphthaleneacetic acid, *BA* 6-benzylaminopurine, *KIN* kinetin

[z] Data taken after 4 wk of culture

[y] Data taken after 6 wk of culture. Data indicate percentage of organogenic white-friable callus

determine the optimum basal medium and organic additives required for improvement of shoot regeneration and growth (Table 3). All treatments were performed twice with six replications and four callus aliquots per replication per treatment ($n=48$). The number of green shoot buds per callus was observed 2 wk after treatment. Data on the frequency of callus that formed shoots, number of shoots per callus, and number of internodes per shoot were recorded after 6 wk.

Root induction and plantlet acclimatization. Well-developed shoots (2–3 cm in length) were cultured on root induction medium consisting of half-strength MS medium or WPM containing 2.5, 4.9, or 7.4 μM indole-3-butyric acid (IBA) or 2.7, 5.4, or 8.1 μM NAA. Medium without PGRs was used as the control (Table 4). Percent root formation, number of roots per shoot, and root length (cm) were recorded 5 wk after culture of shoots on root induction medium. Shoots were cultured in 12 jars, each containing two shoots, and the entire experiment was performed twice ($n=48$). Rooted shoots were removed from the culture jars, rinsed with distilled water to remove remnants of agar from the roots, and transferred to plastic pots containing hardening medium (autoclaved perlite: garden soil [1:1 *v/v*]). To ensure high humidity, plantlets in pots were covered with transparent plastic bags for 4 wk. The potted plantlets were watered with half-strength MS medium without sucrose at weekly intervals. After 1 mo, bags were gradually removed to acclimatize the plants to *ex vitro* conditions. Acclimatized plantlets were transplanted after 1 mo to pots containing garden soil and maintained in the greenhouse under normal daylight conditions in Iran.

Data analysis. The experimental PGR treatments were conducted in a completely randomized design. All data were subjected to statistical analysis of variance (ANOVA) using SAS 9.1 (SAS Institute 2004), and when the ANOVA of treatment means was statistically significant, Duncan's multiple range test at 5% level of probability was used to distinguish differences between treatments.

Genomic DNA isolation and marker analyses. The developing and newly formed leaves from 12 regenerated plants along with leaves from the original tree were sampled (100 mg) and ground for genomic DNA extraction using the CTAB-based protocol of Doyle and Doyle (1990). Quality and quantity of isolated DNA were checked using a spectrophotometer at 260 and 280 nm (BioPhotometer, Eppendorf, Hamburg, Germany) and comparative loading in 1% (*w/v*) agarose gel with a known concentration of 100-bp DNA ladder (SinaClon, Karaj, Iran). Working samples of DNA aliquots were adjusted to 20–25 ng μL^{-1} final concentration in TE buffer and stored at −20°C until used. Eighteen SCoT and 10 IRAP primer sequences were screened, of which 14 and 7 adapted primers (Table 5), respectively, amplified scoreable and reproducible bands for evaluation of genetic variability of the plants.

SCoT amplification was performed in a 25-μL reaction mixture containing 50 ng template DNA, 12.5 μL 2x Master-Mix buffer (0.08 U μL^{-1} Taq polymerase, 3 mM $MgCl_2$, 0.4 mM of each dNTP (SinaClon), 0.8 μM of each SCoT primer, and sterile nuclease-free distilled water. The PCR program (Bio-Rad C-1000 Thermal Cycler) consisted of an initial denaturation for 5 min at 94°C; followed by

Table 2. Effect of plant growth regulators on adventitious shoot regeneration from white-friable callus induced on hypocotyl sections of *Albizia julibrissin*

Plant growth regulator (μM)		Mean no. green shoot buds per callus[z]	Shoot organogenesis (%)[y]	Mean no. microshoots per callus[y]
BA	Zeatin			
0	0	0±0h	0±0h	0±0h
4.4		1.8±0.3g	33.6±5.2g	1.1±0.3g
8.8		3.1±0.3ef	46.1±4.1def	2.0±0.2fg
11.1		3.3±0.2def	46.1±4.1def	1.6±0.2fg
13.2		3.0±0.3ef	39.8±3.8g	1.5±0.2fg
	2.3	3.6±0.4fg	52.3±7.5def	2.5±0.2ef
	4.6	2.8±0.5fgh	44.1±7.7efg	2.0±0.4fg
	6.8	3.1±0.3ef	44.0±5.3efg	2.5±0.2ef
4.4	2.3	3.3±0.5def	48.1±5.8def	2.1±0.3fg
8.8	2.3	4.0±0.6def	52.1±8.1def	3.0±0.4de
11.1	2.3	4.0±0.4def	56.5±5.3cde	4.5±0.4ab
13.2	2.3	4.1±0.5def	58.6±8.3cde	3.3±0.5cd
4.4	4.6	4.8±0.6abc	71.0±8.3ab	4.6±0.3a
8.8	4.6	4.8±0.3abc	64.8±3.8bcd	4.1±0.3bc
11.1	4.6	5.0±0.4ab	69.0±6.9ab	3.5±0.6cd
13.2	4.6	5.5±0.4a	75.3±6.3a	4.8±0.4a
4.4	6.8	5.1±0.5ab	66.8±7.6bc	4.1±0.3bc
8.8	6.8	4.5±0.2bcd	62.6±4.5cde	4.3±0.3bc
11.1	6.8	5.0±0.3ab	69.0±7.1ab	4.5±0.4ab
13.2	6.8	4.3±0.3cde	58.5±6.1cde	4.1±0.4bc

Basal medium was MS medium. Results represent the means±standard error of two replicated experiments (n= 48; about 250 mg callus was cultured as one explant). Means within a *column* followed by different *letter*(*s*) are significantly different at P=0.05 according to Duncan's multiple range test following analysis of variance

BA 6-benzylaminopurine

[z] Data taken after 2 wk of culture

[y] Data taken after 6 wk of culture

36 cycles of 60, 60, and 120 s at 94, 50, and 72°C, respectively; with a final extension at 72°C for 10 min. PCR amplification reactions for IRAP analysis consisted of 25-ng template DNA, 10 μL 2× Master-Mix buffer (0.08 U μL^{-1} Taq polymerase, 3 mM $MgCl_2$, 0.4 mM of each dNTP (SinaClon), 0.4 μM of each IRAP primer, and sterile nuclease-free distilled water to a final volume of 20 μL. The PCR program consisted of an initial denaturation for 3 min at 94°C; followed by 36 cycles of 30 s at 94°C, 1.5 min annealing at 55°C and 2 min extension at 72°C; followed by a final extension at 72°C for 10 min. Mineral oil (7 μL) was added to each microcentrifuge tube for PCR reactions. A negative-control PCR reaction with sterile distilled water in place of DNA template was conducted to visualize possible self-polymerization or DNA contamination. Additionally, a positive PCR reaction with a known DNA template and the specific forward and reverse primers was also conducted to check the stability of PCR conditions. All SCoT and IRAP amplifications were performed with two independent PCR reactions. Amplified PCR products were visualized under ultraviolet light using a gel documentation system (Bio-Rad Molecular Imager XR^+ system) after electrophoresis (1.2% agarose [*w/v*] at a constant voltage of 80 V for 70 min with a 1× TAE buffer followed by staining with 1 μg ml^{-1} ethidium bromide).

SCoT and IRAP bands were visually scored as 1 (presence) or 0 (absence) for each reaction. Because these marker systems are dominant, each band was representative of a bi-allelic locus (Williams *et al.* 1990). In scoring, bands with equal molecular weight from the same primer were considered as amplicons of the same locus. The size of the DNA amplification products were estimated comparatively using a 100-bp molecular weight marker and the Gel Doc system software (Bio-Rad; Image Lab Software v. 4.0). Ambiguous bands with low visual intensity were not scored. The level of polymorphism was calculated as the ratio of the number of polymorphic bands to the total number of bands and expressed as a percentage. Binary matrices of SCoT and IRAP scoring data were constructed. Using

Table 3. Effect of basal medium supplemented with glycine and *myo*-inositol on adventitious shoot organogenesis from callus induced on hypocotyl sections of *Albizia julibrissin*

Basal medium containing 13.2 μM BA plus 4.6 μM zeatin			Shoot organogenesis (%)[z]	Mean no. green shoot buds per callus[y]	Mean no. shoots per callus[z]	Mean no. internodes per shoot[z]
MS (G+M)	WPM (G+M)	B5 (G+M)				
0.2+0.2			71.1±7.6bc	6.0±0.5ab	3.0±0.3b	3.1±0.3b
0.2+0.3			62.8±7.2bc	5.8±0.6ab	2.6±0.5b	3.1±0.3b
0.3+0.2			73.1±3.8ab	5.1±0.3abc	3.6±0.3ab	3.0±0.3b
0.3+0.3			87.6±4.5a	6.5±0.4a	4.1±0.3a	3.1±0.3b
	0.2+0.2		54.3±2.7c	4.3±0.3cd	1.6±0.2c	2.5±0.3b
	0.2+0.3		58.5±7.7bc	4.3±0.5cd	2.6±0.3b	2.6±0.2b
	0.3+0.2		58.6±5.2bc	4.1±0.3cd	3.3±0.3ab	2.8±0.3b
	0.3+0.3		60.6±5.9bc	4.8±0.4bc	3.5±0.2ab	4.3±0.2a
		0.2+0.2	56.6±10.0bc	3.3±0.5d	2.8±0.4b	2.5±0.4b
		0.2+0.3	66.8±4.1bc	3.0±0.3d	3.1±0.3ab	2.8±0.3b
		0.3+0.2	66.8±4.1bc	3.1±0.5d	3.0±0.3b	2.8±0.5b
		0.3+0.3	69.0±2.6bc	4.3±0.5cd	3.3±0.3ab	3.0±0.4b

Results represent the means±standard error of two replicated experiments (each treatment had six replicates with eight explants per replication; about 250 mg callus was cultured as one explant). Means within a *column* followed by different *letter*(*s*) are significantly different at $P=0.05$ according to Duncan's multiple range test following analysis of variance

BA 6-benzylaminopurine, *B5* Gamborg *et al.* medium, *G* glycine, *M myo*-inositol, *MS* Murashige and Skoog, *WPM* woody plant medium

[z] Data taken after 6 wk of culture

[y] Data taken after 2 wk of culture

the program GenAlEx 6.5 (Peakall and Smouse 2012), Nei's pairwise genetic distance (D_{ij}; Nei 1987) among all possible pairs of the 12 *in vitro* plants and the mother plant was calculated as $D_{ij}=1-S_{ij}$ [$S_{ij}=2N_{ij}/(N_i+N_j)$, where N_{ij} is the number of common amplicons in genotypes *i* and *j*, and N_i and N_j are the total number of amplicons in genotypes *i* and *j*, respectively.

Table 4. Effect of induction basal medium and auxin concentration on root formation of *Albizia julibrissin* microshoots

Basal medium	Plant growth regulator (μM)		Rooting frequency (%)	Mean no. roots per shoot	Mean root length (cm)
	IBA	NAA			
h-MS	0	0	0±0f	0±0e	0±0f
	2.5		58.5±4.2b	4.5±0.4bc	2.0±0.2de
	4.9		73.3±5.0a	7.1±0.3a	3.3±0.2a
	7.4		54.5±4.2bc	5.1±0.3b	2.5±0.1bc
		2.7	29.3±2.7e	4.0±0.5cd	2.1±0.1d
		5.4	41.8±4.1cd	4.8±0.4bc	2.5±0.1bc
		8.1	44.1±4.2cd	4.6±0.5bc	2.3±0.2bcd
WPM	0	0	0±0f	0±0e	0±0f
	2.5		52.3±5.9bcd	4.6±0.4bc	2.5±0.2bc
	4.9		58.5±4.2b	5.3±0.3b	2.7±0.1b
	7.4		48.1±6.0bcd	4.6±0.4bc	2.2±0.1cd
		2.7	44.0±5.3cd	4.5±0.2bc	2.3±0.1bcd
		5.4	46.0±5.2bcd	4.7±0.2bc	2.1±0.2d
		8.1	39.8±3.8de	3.3±0.4d	1.7±0.2e

Data taken after 5 wk of culture. Results represent the means±standard error of two replicated experiments ($n=48$ per treatment). Means within a *column* followed by different *letter*(*s*) are significantly different at $P=0.05$ according to Duncan's multiple range test following analysis of variance

h-MS half-strength Murashige and Skoog medium, *IBA* indole-3-butyric acid, *NAA* α-naphthaleneacetic acid, *WPM* woody plant medium

Table 5. SCoT and IRAP primers and sequences, number and size range of generated fragments, and number of polymorphic bands in *Albizia julibrissin in vitro*-regenerated plants

Primer	5′→3′ sequence	Size (kb)	Total no. SB	No. PB	PB (%)
SCoT					
SCoT2	CAACAATGGCTACCACCA	0.6–1.3	4	1	25
SCoT3	CAACAATGGCTACCACCG	0.3–1.0	5	3	60
SCoT5	CAACAATGGCTACCACGA	0.5–1.1	5	0	0
SCoT6	CAACAATGGCTACCACGC	0.1–0.9	5	4	80
SCoT9	CAACAATGGCTACCAGCA	0.6–1.3	6	3	50
SCoT12	ACGACATGGCGACCAACG	0.3–0.9	5	3	60
SCoT14	ACGACATGGCGACCACGC	0.2–1.1	6	3	50
SCoT16	ACCATGGCTACCACCGAC	0.3–1.1	6	3	50
SCoT22	AACCATGGCTACCACCAC	0.3–1.0	5	4	80
SCoT25	ACCATGGCTACCACCGGG	0.3–1.2	4	0	0
SCoT27	ACCATGGCTACCACCGTG	0.3–1.1	5	3	60
SCoT28	CCATGGCTACCACCGCCA	0.2–1.1	4	0	0
SCoT30	CCATGGCTACCACCGGCG	0.2–0.8	5	3	60
SCoT35	CATGGCTACCACCGGCCC	0.2–1.1	6	3	50
IRAP					
Hana	CACGATTCACCTTAATATCTGACA	0.5–1.4	5	3	60
Gaga	GGGAACCAACCGTCACA	0.4–1.5	5	0	0
5′LTR1	TTGCCTCTAGGGCATATTTCCAACA	0.3–1.3	6	2	33
LTR6149	CTCGCTCGCCCACTACATCAACCGCGTTTATT	0.5–1.0	4	3	75
LTR6150	CTGGTTCGGCCCATGTCTATGTATCCACACATGTA	0.4–1.3	5	1	20
3′LTR	TGTTTCCCATGCGACGTTCCCCAACA	0.5–1.2	4	2	50
Nikita	CGCATTTGTTCAAGCCTAAACC	0.6–1.4	5	1	20

Summary of data

Marker system	No. primer	Total no. SB	Mean no. SB per primer	Total no. PB	Mean no. PB per primer	% P
SCoT	14	71	5.1	33	2.4	46.48
IRAP	7	34	4.9	12	1.7	35.29

IRAP inter-retrotransposon amplified polymorphism, *SB* scoreable bands, *SCoT* start codon targeted, *PB* polymorphic bands, *P* polymorphism

Results and Discussion

Callus induction. Hot-water-scarified seeds of *A. julibrissin* germinated at 80–90% on MS medium lacking PGRs and produced *in vitro* seedlings within 7–10 d. Callus was induced from the cut ends of the hypocotyl explants, began to form 8–10 d after initial culture (Fig. 1*a*), and covered the entire surface of the explants within 3–4 wk (Fig. 1*b*, *c*). From 38.5–100% hypocotyl explants formed callus and organogenic callus formation varied from 9.3–82.2% (P=0.05) depending on the type and concentration of PGRs (Table 1). Medium devoid of PGRs (control) failed to induce callus even after 6 wk of culture. Media without cytokinin (BA or kinetin), but with 5.4–16.2 μM NAA, showed the highest percentage (62.3%) of callus induction on MS medium with 13.4 μM NAA. Callus induction frequencies were significantly increased when NAA was combined with either BA or kinetin (Table 1). Maximum callus formation (100%) was achieved on MS medium with 10.8 or 13.4 μM NAA plus 4.4 μM BA (Table 1). MS medium containing only 5.4 μM NAA yielded mostly yellowish-white, soft callus that was not capable of shoot regeneration (Fig. 1*b*). Calli induced and maintained for 6–8 wk on MS medium with 10.8 μM NAA plus 4.4 μM BA were white and friable (Fig. 1*c*), and 82.2% were organogenic on the basis of callus and shoot bud appearance (Fig. 1*d*). Callus induced on MS medium with NAA in combination with kinetin (38.5–69.7% callus induction) produced a low percentage of organogenic callus (12.5–32.2%).

Many leguminous trees are recalcitrant to *in vitro* culture (Anis *et al.* 2005), and limited information is available on callus induction and subsequent adventitious shoot regeneration in *A. julibrissin*. Hypocotyl explants excised from 10- to 12-d-old *in vitro* seedlings of *A. julibrissin* and cultured on B5 medium with 0–4 μM paclobutrazol, uniconazole, or prohexadione calcium also produced callus at high frequency (90–100%) (Sankhla *et al.* 1993). The roots from 8- to 10-d-old

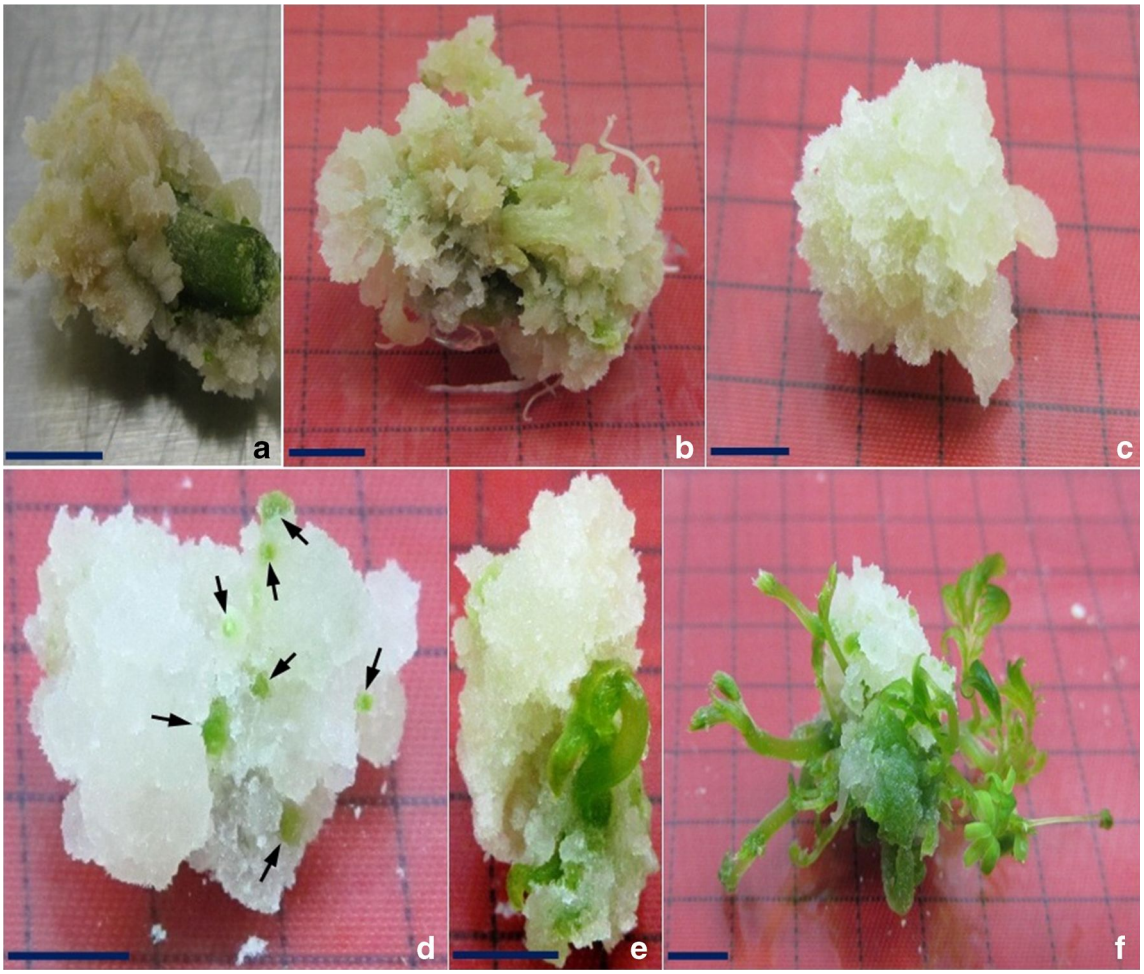

Figure 1. Callus induction and microshoot organogenesis from hypocotyl sections of *Albizia julibrissin*. *a*, *b* Non-organogenic yellowish-white, soft callus from hypocotyl cultured on Murashige and Skoog (MS) medium with 16.2 and 5.4 μM α-naphthaleneacetic acid (NAA) after 2 wk and 6 wk, respectively. *c* Organogenic white-friable callus from hypocotyl on MS medium with 10.8 μM NAA plus 4.4 μM 6-benzylaminopurine (BA) after 6 wk. *d* Callus proliferation and green shoot bud formation from organogenic white-friable callus on MS medium with 13.2 μM BA plus 4.6 μM zeatin after 2 wk. *Arrows* indicate green shoot buds. *e*, *f* Adventitious microshoot formation from organogenic white-friable callus after 3- and 4-wk, respectively, on MS medium with 13.2 μM BA plus 4.6 μM zeatin. *Bars*=5 mm.

intact *in vitro*-grown silk tree seedlings split and formed prolific callus on MS medium containing 0.1–1 μM thidiazuron (TDZ) (Sankhla *et al.* 1994). Roots excised from 15- to 20-d-old *in vitro A. julibrissin* seedlings and cultured on B5 medium with the ethylene precursors/generators 1-amino-cyclopropane-1-carboxylic acid and 2-chloroethylphosphonic acid formed callus, but subsequent shoot formation was inhibited (Sankhla *et al.* 1995). Excised root segments from 15- to 20-d-old *in vitro* silk tree seedlings cultured on B5 medium containing NAA, indole-3-acetic acid (IAA), IBA, kinetin, BA, 6-(γ,γ-dimethylallylamino)purine (2iP), zeatin, or TDZ formed some callus after 1 wk in culture (Sankhla *et al.* 1996). As in our study, a combination of an auxin with a cytokinin has been widely used for successful induction of organogenic callus in many commercially or medicinally important regeneration systems. For example, Sha Valli Khan *et al.* (2002) reported 64% induction of white, friable callus on *Bixa orellana* seeds cultured on MS medium with 5 μM NAA and 2.5 μM BA.

Adventitious shoot regeneration. Both yellowish-white and white-friable callus types were cultured separately on MS medium either without PGRs as a control, or with different concentrations and combinations of BA and zeatin, for shoot organogenesis (Table 2). Ten days after culture on shoot regeneration medium containing PGRs individually or in combination, white-friable calli proliferated rapidly and green shoot buds appeared (Fig. 1*d*). The yellowish-white callus type, however, failed to exhibit shoot differentiation and did not show further growth. Shoot regeneration medium lacking PGRs did not induce shoot organogenesis from the organogenic white-friable callus. The number of green shoot buds, shoot organogenesis frequency, and number of

microshoots per callus varied by PGR type, concentration, and combination (Table 2). Compared to MS medium containing BA or zeatin alone, the combination of these PGRs promoted shoot regeneration. MS medium with 13.2 μM BA plus 4.6 μM zeatin produced the highest number of green shoot buds (5.5±0.4) per callus. The best shoot regeneration was achieved on this medium with 75.3% of the cultures responding and the highest mean number of shoots (4.8± 0.4) per callus (Table 2).

BA, either alone or in combination with other PGRs, has been very effective for the induction of callus or shoot organogenesis in several *Albizia* species. Sankhla *et al.* (1994) reported good adventitious shoot regeneration (16±2 shoots) on roots of intact seedlings of *A. julibrissin* cultured on MS medium with B5 vitamins and 10 μM BA, as compared to 5.2± 0.3 shoots on excised root explants cultured on B5 medium with 10 μM BA (Sankhla *et al.* 1996). The addition of 3 mg L^{-1} hymexazol to MS medium containing 2.22 μM BA and 0.05 μM NAA was reported to increase adventitious shoot development on cotyledons and hypocotyls of *A. julibrissin* (Zhou *et al.* 2001). Mamun *et al.* (2004) reported 100% callus induction on *Albizia lebbeck* nodal sections cultured on MS medium with 8.88 μM BA plus 1.07 μM NAA, with the best shoot regeneration (7.3 per callus) occurring on MS medium with 11.1 μM BA plus 1.07 μM NAA. However, cotyledonary explants (83.3%) regenerated the greatest number of shoots on MS medium with only 8.88 μM BA. Root explants from 15-d-old *in vitro A. lebbeck* seedlings cultured on MS medium with 7.5 μM BA plus 0.5 μM NAA produced 16±1.9 shoot buds per root explant (Perveen *et al.* 2011). Organogenic callus induced on nodal explants of *A. lucida* regenerated shoots when cultured on MS medium containing 8.88 μM BA plus 0.54 μM NAA (Saha *et al.* 2013). Hypocotyls of *Albizia odoratissima* cultured on MS medium with 7.5 μM BA and 0.5 μM NAA in the light differentiated adventitious shoots from callus, whereas hypocotyls cultured in the dark produced non-morphogenic calli (Rajeswari and Paliwal 2008). In our study, we found that increasing zeatin to 6.8 μM in combination with 13.2 μM BA decreased the percentage of shoot organogenesis to 58.5% (Table 2). Vengadesan *et al.* (2003), also showed that the number of adventitious shoots regenerated from cotyledon-derived callus in the leguminous tree *Acacia sinuata* was significantly decreased (to 8.5 per callus) with 5 μM zeatin in combination with 13.3 μM BA, as compared to 25.3 shoots regenerated when 2.5 μM zeatin plus 13.3 μM BA was used. Origination of adventitious shoots from callus (Fig. 1*e*, *f*) and the 6 wk duration required for complete formation of shoots (Fig. 2*a*, *b*) indicated indirect organogenesis in our study.

Shoot growth on the regeneration medium was asynchronous and poor. Therefore, experiments were conducted to compare the efficiency of different basal media (MS medium, WPM, and B5 medium) containing 13.2 μM BA plus 4.6 μM zeatin, supplemented with glycine and *myo*-inositol for improvement of adventitious shoot regeneration and growth (Table 3). The frequency of shoot regeneration, number of green shoot buds, number of shoots per callus, and number of internodes per shoot varied depending on the basal medium as well as on the concentrations of glycine and *myo*-inositol (Table 3). The highest frequency of shoot-producing callus (87.6±4.5%; Fig. 2*c*), highest average number of green shoot buds (6.5±0.4), and maximum mean number of shoots per callus (4.1±0.3) were observed on MS medium containing 0.3 g L^{-1} glycine plus 0.3 g L^{-1} *myo*-inositol, while the highest average number of internodes per shoot (4.3±0.2) was obtained on WPM with 0.3 g L^{-1} glycine and 0.3 g L^{-1} *myo*-inositol (Table 3; Fig. 2*d*). The relatively improved elongation of shoots on WPM may be a result of the lower concentration of ionic nutrients than in the other full-strength media. This finding is in accordance with results by Vengadesan *et al.* (2003) where half-strength MS medium favored adventitious shoot formation from callus of *Acacia sinuate*. In contrast, Perveen *et al.* (2011) reported that full-strength MS medium was more effective than other media for increasing shoot number and shoot length of *A. lebbeck* using root explants. Glycine and *myo*-inositol at 0.3 g L^{-1} each were found to be a significant factors in enhancing the percentage of shoot organogenesis and mean number of shoots per callus as compared to MS medium with only 0.2 g^{-1} L glycine but did not increase the number of shoots per callus in our study. The addition of glycine and *myo*-inositol at 0.3 g L^{-1} yielded an optimum response in over 60% of cultures, regardless of the basal medium (Table 3). Although elongation of shoots was improved on medium augmented with these organics, only one or two shoots per callus elongated and exhibited continued growth, while other microshoots failed to elongate or died.

Rooting of adventitious shoots and plantlet acclimatization. Shoots failed to form roots on rooting medium lacking PGRs. Adventitious roots became visible at the base of shoots within 3 wk after culture on medium containing IBA or NAA. The best rooting percentage (73.3%), mean number of roots per shoot (7.1±0.3), and mean root length (3.3±0.2 cm) were obtained when shoots were cultured on half-strength MS medium with 4.9 μM IBA (Table 4; Fig. 2*e*). In comparison, a lower number of roots and shorter roots were obtained when IBA was replaced with NAA (Table 4). *In vitro* rooting experiments carried out with other leguminous species including *A. julibrissin* (Sankhla *et al.* 1994; Tudor Radu and Radomir 2010), *A. chinensis* (Sinha *et al.* 2000), *A. procera* (Swamy *et al.* 2004), *A. odoratissima* (Rajeswari and Paliwal 2006), *A. lebbeck* (Perveen *et al.* 2011), *Acacia sinuata* (Vengadesan *et al.* 2000), and *Cassia siamea* (Parveen *et al.* 2010) all showed successful root formation on half- or full-strength MS medium with various concentrations of IBA. Sankhla *et al.* (1994) reported that

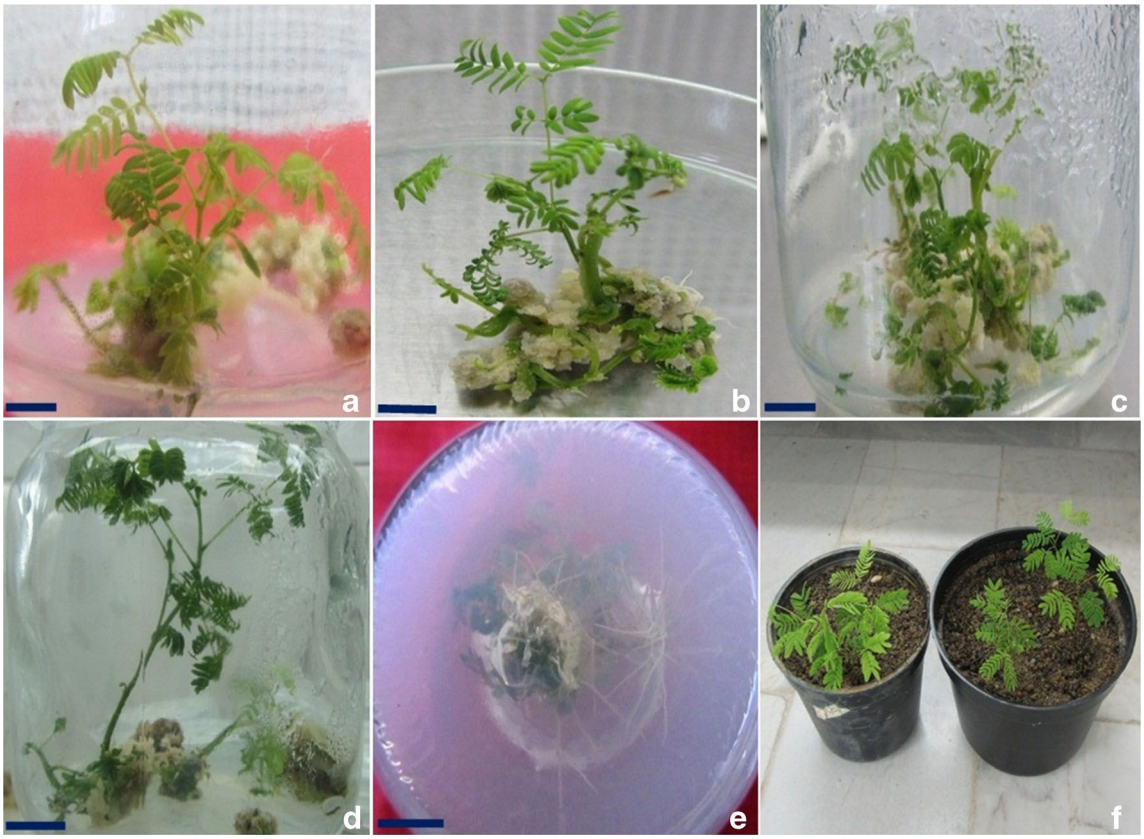

Figure 2. *In vitro* organogenesis from white-friable callus of *Albizia julibrissin*. *a*, *b* Adventitious shoot formation from callus cultured on Murashige and Skoog (MS) medium containing 13.2 μM 6-benzylaminopurine (BA) plus 4.6 μM zeatin (*a*) and 11.1 μM BA plus 4.6 μM zeatin (*b*), after 6 wk. *c* Induction of multiple shoots on MS medium with 13.2 μM BA, 4.6 μM zeatin, 0.3 g L^{-1} glycine, and 0.3 g L^{-1} *myo*-inositol after 6 wk. *d* *In vitro* elongated shoot on woody plant medium (WPM) with 13.2 μM BA, 4.6 μM zeatin, 0.3 g L^{-1} glycine, and 0.3 g L^{-1} *myo*-inositol after 6 wk. *e* Root formation induced on half-strength MS medium with 4.9 μM indole-3-butyric acid. *f* *In vitro*-regenerated plants approximately 2 mo after acclimatization. *Bars*=5 mm.

A. julibrissin shoots induced on roots of *in vitro* intact seedlings by kinetin or BA rooted faster than TDZ-induced shoots, when cultured on MS medium with or without 4.9 μM IBA. Tudor Radu and Radomir (2010) obtained 85% rooting of *A. julibrissin* "micro cuts" on half-strength MS medium with 0.98 μM IBA versus 40% rooting with 3.94 μM IBA. Perveen *et al.* (2011) reported the best rooting (66%) of *A. lebbeck* microshoots on half-strength MS medium with 2 μM IBA. Normal growth and development of rooted plantlets in our study was observed 5 wk after transfer to the greenhouse (Fig. 2*f*). Over 60% of *in vitro* rooted plants were successfully acclimatized to the greenhouse.

Genetic variability of regenerated plants. SCoT is a simple, gene-targeted, and cost-effective DNA-based marker system whereby primers target the short conserved region flanking the ATG translation start codon of plant genes (Collard and Mackill 2009). SCoT polymorphism has proven to be informative, effective, and reproducible for the evaluation of genetic stability of plants (Gorji *et al.* 2011; Rathore *et al.* 2014). The retrotransposon-based IRAP marker system reveals insertional polymorphisms of retrotransposon regions of DNA by a simple PCR of single primers followed by electrophoresis for analysis of the PCR amplicons. An investigation with barley *in vitro*-regenerated plants showed that the IRAP method was an informative tool for the detailed elucidation of mutation profiles that occur under *in vitro* conditions (Campbell *et al.* 2011).

The genetic variability of *A. julibrissin* regenerated plants was assessed initially with 18 SCoT and 10 IRAP screened primers, of which 14 and 7 primers, respectively, produced intense and re-amplifiable bands. A total of 71 amplicons were generated from PCR amplification with SCoT primers using amplicons ranging in size from 100 to 1300 bp. Depending on the primer sequence, the scoreable bands from SCoT primers ranged from four (SCoT2, SCoT25, and SCoT28) to six (SCoT9, SCoT14, SCoT16, and SCoT35) with an average of five bands per primer (Table 5). The number of polymorphic bands from each SCoT primer also varied from zero (SCoT5, SCoT25, and SCoT28) to four (SCoT6, 80% and SCoT22, 80%). SCoT6 and SCoT22 each amplified five bands in total, so the polymorphism for these two markers

was 4/5 or 80%. The banding pattern of the regenerated *A. julibrissin* genotypes and the mother plant obtained with the SCoT22 primer are shown in Fig. 3*a*. Of the 34 total scoreable bands obtained with IRAP primers, six (maximum) and four (minimum) scoreable bands were generated from primers 5′LTR1, and LTR6149 and 3′LTR, respectively, with an average of 4.9 scoreable bands per primer. Among these primers, 75% polymorphism was observed for primer LTR6149 (shown in Fig. 3*b*), while no polymorphism was recorded for primer Gaga (Table 5).

Investigation of genetic variability generated by *in vitro* conditions using more than one marker technique has been suggested to be advantageous because each marker may elucidate changes in different regions of the species' genomic DNA (Palombi and Damiano 2002; Martins *et al.* 2004). A comparative summary of our results with the SCoT and IRAP marker systems is presented in Table 5. Genetic similarity revealed between *in vitro*-regenerated plants and the mother plant was higher with the SCoT system than with the IRAP system (Fig. 4). This observation confirms that *in vitro* culture conditions can affect different genome regions of regenerated plantlets, which indicated that the use of at least two marker techniques was more effective than any one technique for screening the genome variability in tissue culture plants (Martins *et al.* 2004; Rathore *et al.* 2011).

The SCoT and IRAP profiles were used individually to estimate pairwise genetic distances among the 12 *in vitro* plants and between those plants and the mother plant (Table 6). According to both SCoT and IRAP analyses, overall, the *in vitro* plants and the mother plant showed a relatively high degree of genetic dissimilarity. SCoT-based genetic distance values between *in vitro* plants varied from 0.095 to 0.788, with an average of 0.352 (Table 6). IRAP-based genetic distance values among the *in vitro* plants ranged from 0.000 to 0.693, with an average of 0.292 (Table 6). The calculated genetic distance between *in vitro* plants and the mother plant were 0.129–0.405 for SCoT analysis and 0.182–0.438 for IRAP analysis (Table 6, Fig. 4). It was obvious from these

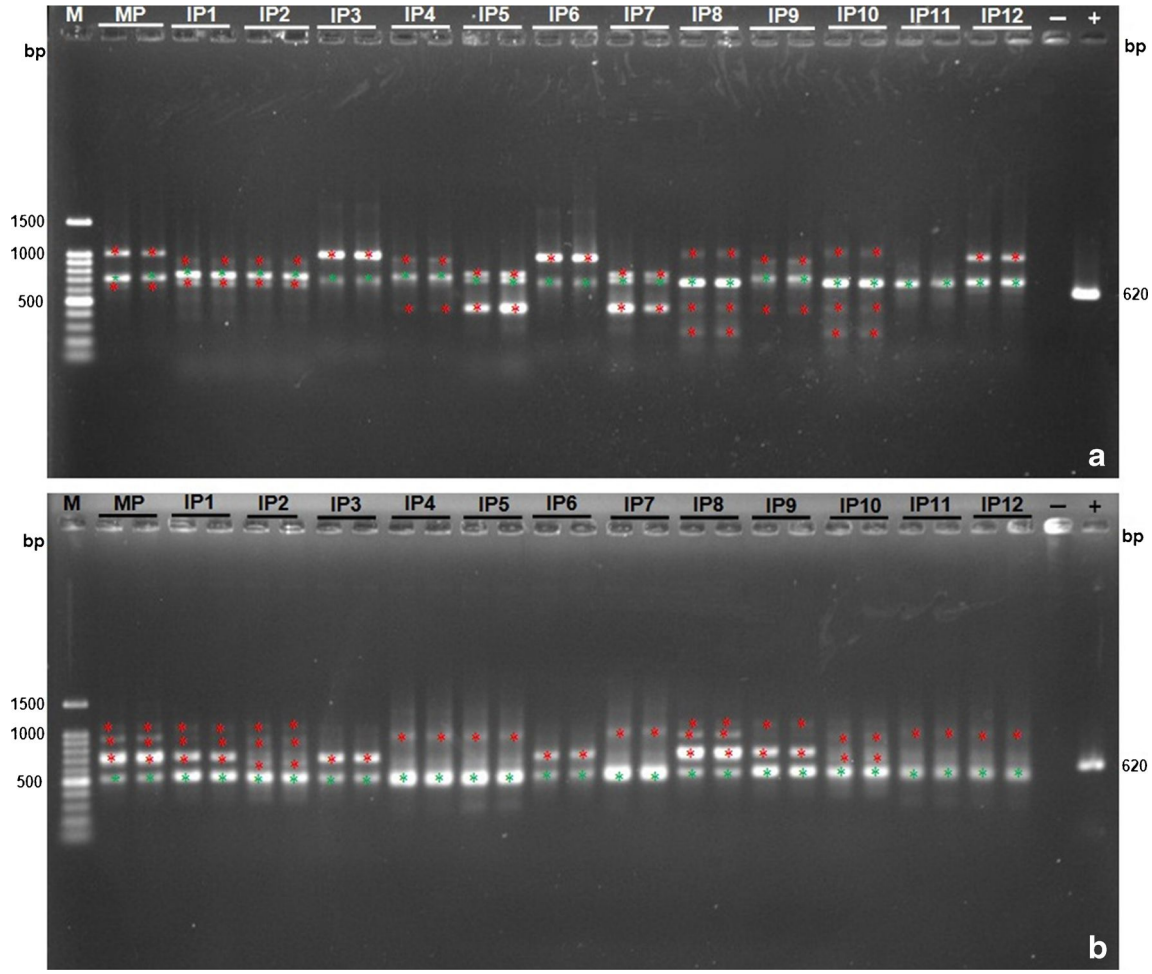

Figure 3. Genetic variability of *in vitro*-regenerated plants (*IP*-1–*IP*12) of *Albizia julibrissin* compared with each other and with the mother plant (*MP*). *a* Banding pattern of SCoT amplification products with primer SCoT22. *b* Banding pattern of IRAP amplification products with primer LTR6149. (*M* 100-bp molecular marker). *Asterisk* indicates some of the scored SCoT or IRAP amplified bands (*red*: polymorphic bands; *green*: monomorphic bands).

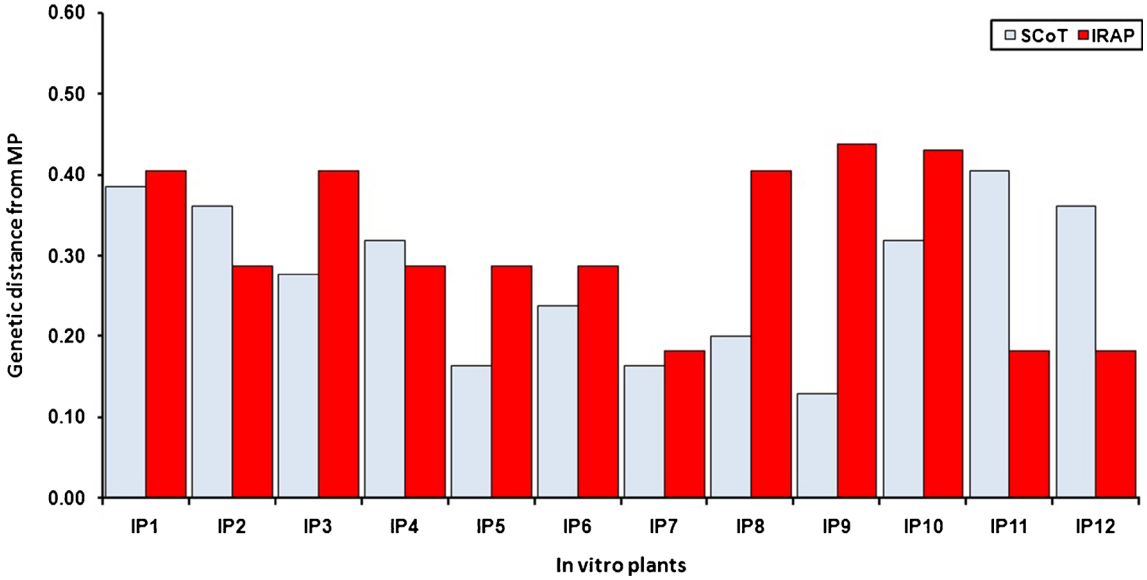

Figure 4. Histogram of the pairwise Nei's genetic distance values between the *in vitro*-raised plants (*IP*-1–*IP*12) and the mother plant (*MP*) based on SCoT and IRAP fingerprinting data.

observations that there was a relatively high level of genetic divergence of *in vitro*-regenerated plants from the original mother plant. This high frequency of genetic variation may be a result of an accumulation of somatic mutations during the tissue culture period, induced by several determining factors including the various concentrations and types of PGRs that were applied (Martin *et al.* 2006; Bairu *et al.* 2011), and the outcrossing nature of seed production in this species. The induction of somaclonal variation in plants regenerated from a single mother plant under *in vitro* conditions has been routinely observed (Rathore *et al.* 2011; Rawat *et al.* 2013). DNA-based methods such as simple sequence repeat (SSR), ISSR, random amplified polymorphic DNA (RAPD), SCoT, IRAP, and amplified fragment length polymorphism (AFLP) analysis have been used to evaluate the genetic stability of regenerants (Rahman and Rajora 2001; Palombi and Damiano 2002; Campbell *et al.* 2011; Rawat *et al.* 2013). Considering the importance of tissue culture techniques in

Table 6. Pairwise genetic distance matrix showing data from SCoT and IRAP analyses for *in vitro*-raised plants and the mother plant of *Albizia julibrissin*

	MP	IP1	IP2	IP3	IP4	IP5	IP6	IP7	IP8	IP9	IP10	IP11	IP12	Ave.
MP	**0.00**	0.405	0.288	0.405	0.288	0.288	0.288	0.182	0.405	0.438	0.431	0.182	0.182	
IP1	0.385	**0.00**	0.288	0.405	0.539	0.288	0.087	0.182	0.182	0.288	0.288	0.182	0.405	
IP2	0.361	0.405	**0.00**	0.288	0.182	0.182	0.182	0.087	0.288	0.405	0.405	0.087	0.288	
IP3	0.278	0.405	0.278	**0.00**	0.539	0.087	0.288	0.182	0.405	0.539	0.539	0.182	0.405	
IP4	0.318	0.552	0.606	0.318	**0.00**	0.405	0.405	0.288	0.539	0.693	0.405	0.288	0.288	
IP5	0.164	0.552	0.501	0.238	0.278	**0.00**	0.182	0.087	0.288	0.405	0.405	0.087	0.288	
IP6	0.238	0.552	0.405	0.318	0.201	0.201	**0.00**	0.087	0.087	0.182	0.182	0.087	0.288	
IP7	0.164	0.361	0.318	0.238	0.361	0.129	0.278	**0.00**	0.182	0.288	0.288	0.000	0.182	
IP8	0.201	0.318	0.361	0.361	0.405	0.238	0.318	0.095	**0.00**	0.087	0.288	0.182	0.405	
IP9	0.129	0.405	0.278	0.278	0.318	0.164	0.164	0.095	0.129	**0.00**	0.405	0.288	0.539	
IP10	0.318	0.452	0.606	0.606	0.452	0.361	0.361	0.361	0.318	0.238	**0.00**	0.288	0.539	
IP11	0.405	0.788	0.724	0.501	0.452	0.361	0.361	0.361	0.318	0.318	0.552	**0.00**	0.182	
IP12	0.361	0.606	0.663	0.452	0.405	0.318	0.405	0.318	0.161	0.278	0.501	0.164	**0.00**	**0.292**[z]
Ave.													**0.352**[y]	

SCoT-based genetic distances are given below the bold diagonal; IRAP-based genetic distances are given above the bold diagonal

MP mother plant, *IP in vitro*-regenerated plant (*IP*1–*IP*12), *Ave.* average

[z] Average genetic distance between pairs of *in vitro*-raised plants according to IRAP analysis

[y] Average genetic distance between pairs of *in vitro*-raised plants according to SCoT analysis

genetic improvement and production of secondary metabolites, such genetic variation may be useful in selecting variants for large-scale production of pharmaceutically important compounds (Bairu *et al*. 2011). Similarly, a high level of somaclonal variation has been observed in other callus-based regeneration systems (Al-Zahim *et al.* 1999; Rahman and Rajora 2001; Rathore *et al.* 2011). For example, callus-tissue-derived plants of the medicinally important herb *Picrorhiza kurroa* showed a relatively high frequency of genomic variations when subjected to RAPD and ISSR genome analysis (Rawat *et al.* 2013).

Conclusions

This report demonstrated reliable organogenic callus induction and subsequent plant regeneration from *in vitro*-derived hypocotyl explants of *A. julibrissin*. The appropriate concentrations and combination of PGRs required for the different stages of *in vitro* development were defined. This investigation provided the first data on genetic variability assessment of *in vitro*-regenerated plants of this important species with SCoT and IRAP marker analyses. Our findings of relatively high levels of genetic variation at both the start codon and in retrotransposon regions may aid in producing phenotypic variability for selection of clones for the large-scale production of important medicinal metabolites of this woody tree species. This protocol may also be of use in genetic engineering studies of this economically and medicinally beneficial tree legume.

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