Tree Genetics & Genomes (2016) 12: 100
DOI 10.1007/s11295-016-1057-0

ORIGINAL ARTICLE

Evaluation of genetic homogeneity of in vitro-raised plants of *Tecomella undulata* (Sm.) Seem. using molecular markers

Sidhika Chhajer[1] · **Rajwant K. Kalia**[1]

Received: 25 June 2016 / Revised: 1 September 2016 / Accepted: 13 September 2016 / Published online: 19 September 2016

Abstract *Tecomella undulata* (Sm.) Seem (family Bignoniaceae) is an economically and pharmaceutically important timber tree of arid regions of India. Overexploitation of natural stands coupled with minimal conservation and reforestation efforts has led to its incorporation in list of endangered species. This monotypic genus can be propagated only through seeds as no methods are available for its vegetative propagation. Therefore, protocol for multiplication of *T. undulata* via direct regeneration using nodal segments from mature trees has been standardized. Authentication of genetic homogeneity of these in vitro-raised plants is necessary for commercial-scale application of the developed micropropagation protocol. PCR-based molecular markers which have emerged as simple, fast, reliable, and labor-effective tools for testing the genetic homogeneity of in vitro-raised plants were used in the present study. Arbitrary (random amplified polymorphic DNA, RAPD), semi-arbitrary (inter-simple sequence repeat, ISSR; start codon targeted (SCoT) polymorphism), and sequence-based (simple sequence repeat, SSR) markers were used. DNA samples of shoots maintained in vitro for 2 years collected after every 4 subculture cycles (of 3 weeks each) and field-transferred plantlets were compared with the mother tree DNA using 131 primers (25 each of RAPD, ISSR, SCoT and 56 SSR). Scorable unambiguous and reproducible DNA fragments were produced by 77 (21 RAPD, 20 ISSR, 22 SCoT and 14 SSR) primers. A total of 71, 93, 94, and 42 distinct and scorable DNA fragments were produced by RAPD, ISSR, SCoT, and SSR primers respectively with an average of 3.38, 4.65, 4.27, and 3.0 DNA fragments per primer. The true-to-type nature of the in vitro-raised plants of *T. undulata* undergoing up to 32 subculture passages over a period of approximately 2 years was authenticated by monomorphic DNA fragments amplified with all primer combinations. Therefore, the developed micropropagation protocol can be safely used on a commercial scale for multiplying *T. undulata* plants.

Keywords Genetic fidelity testing · In vitro cultures · Micropropagation · Molecular markers · Nodal segments · Rohida

Abbreviations

AFLP	Amplified fragment length polymorphism
BAP	6-benzyl-amino-purine
CTAB	Cetyl trimethyl ammonium bromide
dNTPs	Deoxyribonucleotide triphosphates
FYM	Farmyard manure
IBA	Indole-3-butyric acid
ISSR	Inter-simple sequence repeat
MS	Murashige and Skoog's medium
NAA	α-Naphthalene acetic acid
PCR	Polymerase chain reaction
RAPD	Random amplified polymorphic DNA
SAMPL	Selectively amplified microsatellite polymorphic loci
SCoT	Start codon targeted polymorphism
SSR	Simple sequence repeat
SH	Schenk and Hilderbrandt medium

Communicated by M. Wirthensohn

✉ Rajwant K. Kalia
rajwantkalia@yahoo.com; rajwant.kalia@icar.gov.in

[1] Division III (PIP & PM), Central Arid Zone Research Institute (CAZRI), Jodhpur, Rajasthan 342003, India

Introduction

Tecomella undulata (Sm.) Seem (family Bignoniaceae), commonly known as *Rohida*, *Desert Teak*, or *Marwar Teak*, is a medicinally and economically important timber tree of hot arid regions (state flower of Rajasthan, India). It has been used in indigenous medicinal systems for a wide range of therapeutic activities like hepatoprotective, antibacterial, antimicrobial and antifungal immunomodulatory, anticancer, cytotoxic, analgesic, anti-inflammatory, anti-obesity, etc. (Kalia et al. 2014). Its other uses include phytoremediation of chromium-contaminated soil (Mathur et al. 2010) and rehabilitation of lignite mine backfills (Kumar et al. 2011). It is a widely accepted tree species in arid zone agroforestry but is heading towards extinction due to overexploitation and has been designated as "threatened" in Rajasthan, India (Tripathi and Jaimini 2002). The older trees are dying due to infestation of wood by borers and wood-decaying fungi while young plantlets are seldom found growing in the field. It is usually desirable to multiply elite trees having desirable silvicultural traits; however, no vegetative propagation methods are available for this species. In vitro propagation has emerged as a powerful technique for large-scale propagation of this slow-growing tree. Attempts have been made to propagate this tree through in vitro approaches using seedling (Nandwani et al. 1995, 1996; Aslam et al. 2006; Singh et al. 2009; Varshney and Anis 2012) as well as mature explants (Arya and Shekhawat 1986; Rathore et al. 1991; Bhansali 1993; Kumari and Singh 2012; Tyagi and Tomar 2013; Chhajer and Kalia 2016).

In vitro methods are being used routinely for mass multiplication of selected genotypes, conservation of rare and threatened species, and species with no or recalcitrant seeds and large-scale production of phytochemicals (Pal et al. 2009; Kalia 2015). However, occurrence of somaclonal variation during in vitro propagation is a serious limitation in the practical applications of the plant tissue culture technique. The primary factors controlling induction of somaclonal variation in vitro include culture method, explants source, ploidy level, and in vitro culture age (Rani and Raina 2000); high concentrations of growth regulators used (Venkatachalam et al. 2007); cell cycle disturbances caused by exogenously applied growth regulators (Peschke and Phillips 1992); increased mutation rate per cell-generation over time and accumulation of mutations over a period of time (Rodrigues et al. 1998); alteration in DNA methylation patterns, DNA damage, and mutation (Phillips et al. 1994); and alteration of cell's ability to repair damaged and mutated DNA (Leroy et al. 2000). The variability detected is a combined effect of genetic and epigenetic variations occurring during the course of culture and the genetic heterogeneity of the cells of explants (Bhojwani and Dantu 2013).

Assessment of genetic homogeneity of in vitro-raised plants at initial stage of ex vitro transfer can help in minimizing the propagation of somaclones. Therefore, suitability of developed micropropagation protocol must be scrutinized critically especially in perennials where commercial success of micropropagation depends solely on the maintenance of clonal uniformity (Heinz and Schmidt 1995). Morphological and physiological traits have been used for fidelity testing in many species (Agnihotri et al. 2009; Singh et al. 2012a, b); however, these morphological evaluations require field maintenance of plants till maturity with extensive observations. Further, the morphological and physiological differences may disappear over the growing seasons or the initially uniform looking plants may behave differently during flowering/fruiting stages due to genetic aberrations. Also, some changes induced during in vitro culture may not be apparent under ex vitro conditions (Palombi and Damiano 2002). Nowadays, more efficient DNA markers are available which can detect variations irrespective of the age or tissue of the plant, growth stage, or the prevailing environmental conditions. In the last two decades, many DNA-based markers like RAPD, ISSR, SCoT, AFLP, SAMPL, and SSR have been successfully used for assessment of genetic homogeneity of

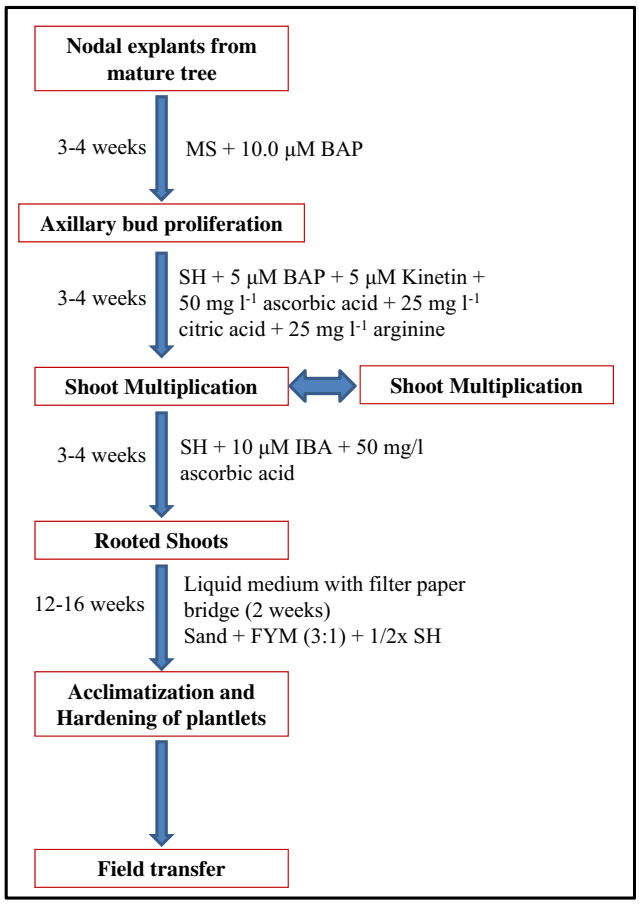

Fig. 1 Micropropagation protocol for in vitro multiplication of *Tecomella undulata* through axillary bud proliferation from mature nodal explants

Table 1 PCR conditions for different molecular markers assayed

A

Molecular marker	DNA	$MgCl_2$	Taq Buffer	dNTPs	Primer	Taq polymerase	Total volume
RAPD	50 ng	1.5 mM	1×	0.2 mM	1.0 μM	0.6 U	25 μl
ISSR	70 ng	1.5 mM	1×	0.2 mM	1.0 μM	0.6 U	25 μl
SCoT	70 ng	1.5 mM	1×	0.2 mM	1.0 μM	0.6 U	25 μl
SSR	100 ng	1.5 mM	1×	0.2 mM	0.5/0.5 μM	0.9 U	25 μl

B

Molecular marker	PCR program	Detection system
RAPD	5 min 95 °C, [1 min 95 °C/1 min 36–38 °C/2 min 72 °C] × 40 cycles, 10 min 72 °C	2 % Agarose gel
ISSR	5 min 95 °C, [45 s 95 °C/1 min 42–45 °C/1 min 72 °C] × 40 cycles, 10 min 72 °C	2 % Agarose gel
SCoT	5 min 95 °C, [45 s 95 °C/1 min 50–55 °C/1:30 min 72 °C] × 40 cycles, 10 min 72 °C	2 % Agarose gel
SSR	5 min 95 °C, [1 min 95 °C/1 min 48–55 °C/2 min 72 °C] × 40 cycles, 10 min 72 °C	4 % Agarose gel

A PCR reaction components, *B* PCR programs used for each marker system

tissue culture-raised plants. It has been suggested to use a combination of two or more marker types for genetic fidelity testing of plants so as to target a larger part of the genome under study (Singh et al. 2013a, b). Therefore, in the present study, four marker systems viz. RAPD, ISSR, SCoT, and SSR were used to assess genetic homogeneity of tissue culture-raised plants of an endangered tree species, *T. undulata*. To the best of our knowledge, there is no report on the application of ISSR, SCoT, and SSR markers in the analysis of genetic homogeneity of micropropagated plants of *T. undulata*.

Materials and methods

Plant material and culture conditions

The plant material consisted of in vitro-raised shoots maintained on cytokinin-supplemented multiplication medium for 3 months to 2 years and in vitro-raised plants transferred to the field after rooting, hardening, and acclimatization. These plants were regenerated via enhanced axillary branching of nodal explants collected from a 31-year-old tree of

Fig. 2 Micropropagation of *Tecomella undulata* through axillary bud proliferation from mature nodal explants **a** mother tree, **b** nodal explants, **c** induction of axillary buds, **d** multiplication of shoots, **e** rooting, and **f** 1-month-old pot-transferred plantlet

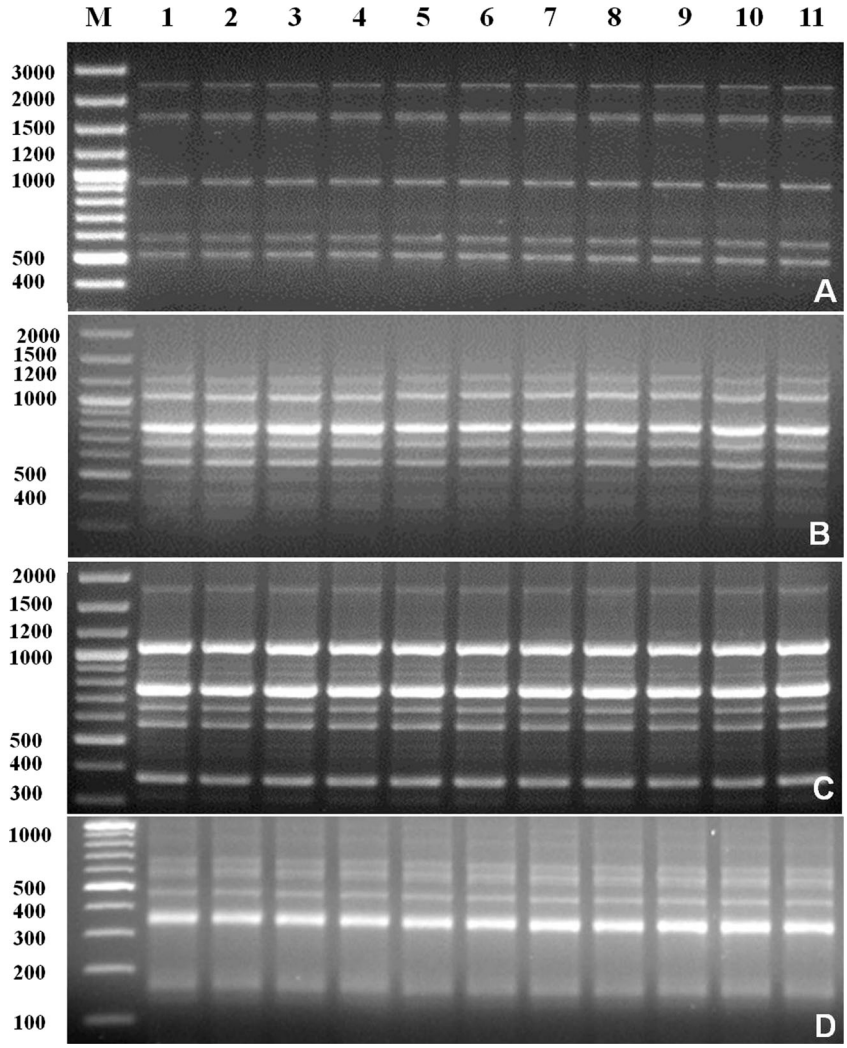

Fig. 3 Genetic homogeneity testing of in vitro-raised shoots/plants of *Tecomella undulata*. Amplification of DNA with **a** RAPD (OPA 04), **b** ISSR (ISSR 44), **c** SCoT (SCoT 28), and **d** SSR (Jc 28) primers. *Lanes: M* 100 bp plus DNA ladder; *1* mother plant, *2–9* in vitro cultured shoots from the 4th (3 month) to 32nd (24 months) passage after every 4 subculture cycles, *10–11* in vitro-raised plants transferred to pots (4-months old)

T. undulata (Chhajer and Kalia 2016). The micropropagation procedure consisted of culturing the nodal explants on BAP-supplemented MS medium, transfer of shoot clumps to multiplication medium (SH + 5 μM BAP + 5 μM Kinetin), rooting of shoots on auxin-supplemented media (SH + 10 μM IBA), and finally hardening and acclimatization of in vitro-raised plants in the greenhouse before field transfer. Details of the micropropagation procedure are summarized in Fig. 1. The shoot cultures were maintained continuously on multiplication medium for approximately 2 years (32 passages) with regular subculturing to fresh medium every 3 weeks. Shoots were randomly collected from 5 culture flasks after every four subcultures starting from the 4th to 32nd subculture passage for testing the effect of continuous cytokinin exposure duration on genetic homogeneity of shoots. In addition, leaf samples of two randomly selected field-transferred plants (4 months after transfer) were also collected for analysis.

Table 2 Summary of results obtained with various molecular markers assayed for evaluating the genetic homogeneity of micropropagated plants of *Tecomella undulata*

Molecular marker	Number of primers used	Primers showing amplification	Total number of bands	Average number of bands
RAPD	25	21	71	3.38
ISSR	25	20	93	4.65
SCoT	25	22	94	4.27
SSR	56	14	42	3.00

DNA extraction and PCR amplification

Total genomic DNA of the mother plant, in vitro-raised shoots, and field-transferred plants was extracted using the modified CTAB method (Doyle and Doyle 1990). The yield and purity of the isolated DNA was determined electrophoretically on 0.8 % agarose gel and spectrophotometrically at the wavelength of 260 nm using Biophotometer (D30, Eppendorf, Germany).

Table 3 Random amplified polymorphic DNA (RAPD) primers used for testing the genetic homogeneity of micropropagated plants of *Tecomella undulata*

Sr. no.	Primer code	Primer sequence (5′–3′)	Annealing temperature (°C)	Number of scorable/ monomorphic bands	Size range of amplification product (bp)
1.	OPA 02	TGCCGAGCTG	38	5/5	500–1300
2.	OPA 03	AGTCAGCCAC	37	4/4	500–1300
3.	OPA 04	AATCGGGCTG	36	5/5	500–3000
4.	OPA 07	GAAACGGGTG	36	1/1	700
5.	OPA 09	GGGTAACGCC	37	2/2	300–1000
6.	OPA 11	CAATCGCCGT	37	2/2	1000–1500
7.	OPA 13	CAGCACCCAC	38	3/3	500–2000
8.	OPB 05	TGCGCCCTTC	37	2/2	600–1400
9.	OPB 06	TGCTCTGCCC	37	5/5	500–1400
10.	OPB 07	GGTGACGCAG	38	2/2	400–600
11.	OPB 08	GTCCACACGG	38	4/4	700–1300
12.	OPB 17	AGGGAACGAG	37	2/2	500–1500
13.	OPB 18	CCACAGCAGT	37	3/3	200–1500
14.	OPC 02	GTGAGGCGTC	36	2/2	350–1100
15.	OPC 04	CCGCATCTAC	36	5/5	200–2000
16.	OPC 05	GATGACCGCC	37	2/2	1000–1500
17.	OPC 06	GAACGGACTC	37	3/3	350–1400
18.	OPD 02	GGACCCAACC	38	6/6	500–2000
19.	OPD 05	TGAGCGGACA	37	1/1	250
20.	OPN 04	GACCGACCCA	38	6/6	300–1500
21.	OPZ 18	AGGGTCTGTG	37	6/6	200–1800

In order to target a wider coverage of the genome, four sets of primers including arbitrary (RAPD), semi-arbitrary (ISSR, SCoT), and sequence based (SSR) markers were used in the present study. Twenty-five primers each of RAPD, SCoT (Integrated DNA Technologies Inc.), and ISSR (Life-Technologies, India Pvt. Ltd.) and 56 SSR (Sigma Aldrich Chemicals Pvt. Ltd., Banglore, India) were used for genetic homogeneity testing. In this study, 25 out of 36 SCoT primers (developed by Collard and Mackill (2009)) were screened to evaluate the genetic fidelity of micropropagated plants. Due to absence of SSR sequences of *T. undulata* in the public domain, cross-species SSR markers available from other members of the family Bignoniaceae (*Arrabidaea chica* (Figueira et al. 2010—9 primers); *Incarvillea sinensis* (Yu et al. 2011—8 primers); *Jacaranda copaia* (Vinson et al. 2008—8 primers), (Jones and Hubbell 2003—10 primers); *Tabebuia aurea* (Braga et al. 2007—21 primers)) were used.

DNA amplification was performed in an Eppendorf thermocycler (Mastercycler Nexus GSX1). Details of PCR reaction mix and PCR thermal cycler conditions are given in Table 1A and B, respectively. The agarose (Amresco, USA) gels with 1× TAE buffer were stained with ethidium bromide (10 μl/100 ml gel; Sigma-Aldrich Chemicals Pvt. Ltd., Banglore, India), run under a steady voltage of 110 V for 120 min and then photographed under the gel documentation system (Alphaimager EC, Protein Simple, CA, USA). In all agarose gels, GeneRuler 100 bp plus DNA ladder (Thermo Scientific, EU, Lithuania) was used as size marker.

To ensure the consistency of results, all reactions were performed twice. Only consistently produced, unambiguous, and well-resolved amplicons produced through amplification of RAPD, ISSR, SCoT, and SSR markers were scored manually. All markers were treated as dominant for the purpose of scoring which was done on the basis of presence ("1") or absence ("0") of amplicons in the gel. NTSYS-pc version 2.1 (Rohlf 2000) was used to evaluate genetic associations by calculating the Jaccard's similarity coefficient for pairwise comparisons based on the proportion of shared bands produced by the primers.

Results and discussion

Micropropagation protocol was developed for clonal multiplication of *T. undulata* via axillary bud proliferation from mature nodal explants (Figs. 1 and 2; Chhajer and Kalia 2016). Morphological aberrations like leaf size, shape and color, branching pattern, or change in multiplication rate were not observed during propagation of shoots maintained on cytokinin-enriched medium for over 2 years. In vitro

Table 4 Inter-simple sequence repeat (ISSR) primers used for testing the genetic homogeneity of micropropagated plants of *Tecomella undulata*

Sr. no.	Primer code	Primer sequence (5′–3′)	Annealing temperature (°C)	Number of scorable/ monomorphic bands	Size range of amplification product (bp)
1	ISSR-13	GCTT+$(GACA)_3$	42.0	5/5	450–1400
2	ISSR-31	$(GT)_8$ + T	43.0	6/6	350–1600
3	ISSR-32	$(TC)_8$ + A	42.0	4/4	500–2000
4	ISSR-33	$(TC)_8$ + C	42.0	2/2	800–1600
5	ISSR-34	$(TC)_8$ + G	42.0	2/2	800–1300
6	ISSR-35	$(AC)_8$ + G	43.0	6/6	500–1500
7	ISSR-37	$(TG)_8$ + G	45.0	4/4	300–1200
8	ISSR-38	$(CA)_8$ + AT	45.0	8/8	200–1200
9	ISSR-39	$(CA)_8$ + GC	42.0	4/4	400–800
10	ISSR-40	$(AC)_8$ + CA	42.0	5/5	350–850
11	ISSR-41	$(AC)_8$ + TG	42.0	6/6	400–1500
12	ISSR-42	$(TG)_8$ + GC	42.0	5/5	300–1300
13	ISSR-43	$(AC)_8$ + TT	42.0	7/7	250–1200
14	ISSR-44	$(AC)_8$ + TA	42.0	5/5	550–1200
15	ISSR-45	$(AG)_8$ + AT	44.0	6/6	350–1200
16	ISSR-46	$(AG)_8$ + AA	42.0	3/3	450–1500
17	ISSR-47	$(AG)_8$ + TA	42.0	8/8	300–1500
18	ISSR-48	$(AG)_8$ + TT	44.0	3/3	650–1600
19	ISSR-49	$(ACTG)_4$	43.0	2/2	600–1000
20	ISSR-50	$(AGTC)_4$	42.0	2/2	900–1500

regeneration through axillary bud proliferation has minimum risk of generation of somaclonal variations as organized (pre-existing) meristems develop into shoots without involving dedifferentiation or redifferentiation of cells or tissues into meristems (de novo organogenesis). Morphological stability of the in vitro-raised plants was also reported by Singh et al. (2012b, 2013b) in *Dendrocalamus asper;* however, Lakshmanan et al. (2007) documented morphological variation (e.g., hyperhydricity) in banana due to use of high levels of cytokinins. Release of cytotoxic by-products by various hormones in tissue culture media act as a stress which induces programmed loss of cellular controls (Phillips et al. 1994).

DNA-based molecular markers have emerged as a powerful technique for the purpose and therefore are being used in many crops and trees (Singh et al. 2013a, b; Rathore et al. 2014; Agarwal et al. 2015). Previously, only Kumari and Singh (2014) have reported the assessment of genetic stability of tissue culture-raised shoots of *T. undulata* using RAPD markers. However, chances of occurrence of mutations outside the priming sites of RAPD markers cannot be ruled out. A better analysis of genetic stability of plants can be achieved by using more than one DNA amplification technique allowing increased possibilities for identification of genetic variations. Therefore, a set of 131 primers involving four marker systems (RAPD, ISSR, SCoT, and SSR) were used in the present study. Screening for variations in the whole genome was ensured by selection of marker systems having the ability to target different regions of the genome including coding as well as non-coding regions. RAPD (arbitrary, dominant, decamers) and ISSR (semi-arbitrary, medium to highly reproducible, dominant, and more stringent) markers are capable to scan the whole genome randomly and quickly while SSRs, the highly polymorphic and reproducible markers being sequence based, can detect variation at pre-determined sites such as repetitive regions of the genome (Singh et al. 2013b). On the other hand, SCoT being a gene-targeted DNA marker based on the short conserved region flanking the ATG translation start codon (Collard and Mackill 2009) specifically targets only the coding regions. Rathore et al. (2011) and Lakshmanan et al. (2007) suggested the use of more than one marker system for better analysis of genetic stability of plants to target different regions of the genome. Cuesta et al. (2010) and Singh et al. (2013a, b) also used four marker systems for ascertaining the genetic fidelity of micropropagated *Pinus pinea* and bamboos, respectively.

The carry-over effect of growth regulators is a well-known phenomenon; therefore, the in vitro-raised shoots maintained for more than 2 years on cytokinin-enriched medium, and also plants after 4 months of field transfer, were tested to address the issue of carry-over effect of growth regulators on genetic integrity, if any. Shoots were collected after every 4 subculture cycles starting from the 4th cycle to the 32nd cycle (3–

Table 5 Start codon targeted (SCoT) primers used for testing the genetic homogeneity of micropropagated plants of *Tecomella undulata*

Sr. no.	Primer code	Primer sequence (5′–3′)	Annealing temperature (°C)	Number of scorable/ monomorphic bands	Size range of amplification product (bp)
1	SCoT 1	CAACAATGGCTACCACCA	51	3/3	300–1300
2	SCoT 2	CAACAATGGCTACCACCC	52	5/5	500–2000
3	SCoT 5	CAACAATGGCTACCACGA	51	2/2	1000–3000
4	SCoT 6	CAACAATGGCTACCACGC	51	2/2	1200–1500
5	SCoT 9	CAACAATGGCTACCAGCA	54	2/2	1100–1300
6	SCoT 11	AAGCAATGGCTACCACCA	53	3/3	900–2000
7	SCoT 12	ACGACATGGCGACCAACG	51	3/3	700–1200
8	SCoT 15	ACGACATGGCGACCGCGA	51	4/4	200–1500
9	SCoT 16	ACCATGGCTACCACCGAC	55	5/5	1000–2000
10	SCoT 18	ACCATGGCTACCACCGCC	51	8/8	300–1900
11	SCoT 19	ACCATGGCTACCACCGGC	51	4/4	200–1600
12	SCoT 20	ACCATGGCTACCACCGCG	51	4/4	400–1000
13	SCoT 21	ACGACATGGCGACCCACA	55	9/9	200–1300
14	SCoT 22	AACCATGGCTACCACCAC	51	3/3	600–1200
15	SCoT 23	CACCATGGCTACCACCAG	53	3/3	800–2000
16	SCoT 28	CCATGGCTACCACCGCCA	51	7/7	300–1900
17	SCoT 29	CCATGGCTACCACCGGCC	55	5/5	400–1500
18	SCoT 30	CCATGGCTACCACCGGCG	51	6/6	600–3000
19	SCoT 31	CCATGGCTACCACCGCCT	55	1/1	700
20	SCoT 32	CCATGGCTACCACCGCAC	51	7/7	500–2700
21	SCoT 34	ACCATGGCTACCACCGCA	51	2/2	1400–2800
22	SCoT 36	GCAACAATGGCTACCACC	54	6/6	600–2800

24 months). The fingerprinting profiles of the in vitro shoot cultures, regenerated plantlets, and the mother plant generated using the RAPD, ISSR, SCoT, and SSR markers are shown in Fig. 3, and their scoring data is summarized in Tables 2, 3, 4, 5, and 6. A total of 21 (out of 25) random decamer oligonucleotides showed amplification of 71 scorable DNA fragments, in the size range of 200 to 3000 bp (Table 3; Fig. 3a). The number of fragments for each primer varied from 1 to 6, with an average of 3.38 DNA fragments per RAPD primer. All the fragments were monomorphic in nature. Out of 25 ISSR primers used for the genetic homogeneity testing, 20 primers produced amplification with 93 scorable fragments in the size range of 200–2000 bp (Table 4). Figure 3b is a representative example of monomorphic fragments obtained with ISSR primers. More fragments were amplified by ISSR primers (4.65/primer) compared to RAPD (3.38/primer), which is in line with results of Singh et al. (2013a, b) but contrary to the results of Cuesta et al. (2010) wherein ISSR markers amplified fewer bands compared to RAPD markers. Singh et al. (2013a, b) and Cuesta et al. (2010) reported a DNA fragment size range of 200 to 3000 bp and 500 to 3000 bp respectively for both RAPD and ISSR primers while in the present study size of fragments varied from 200 to 3000 bp with RAPD and 200–2000 bp for ISSR primers.

SCoT primers (22 out of 25 tested) amplified a total of 94 DNA fragments with an average of 4.27 fragments per primer. The number of fragments in the selected primers varied from one (SCoT 31) to nine (SCoT 21) in the size range of 200 to 3000 bp (Fig. 3c; Table 5). No polymorphic fragments were observed with any of the SCoT primers used. This start codon targeted polymorphism detection technique reported by Collard and Mackill (2009) for the first time has been used in genetic fidelity testing in some recent reports only (Rathore et al. 2014; Agarwal et al. 2015). Rathore et al. (2014) reported amplification of 2–7 fragments (average 4.3/primer) with SCoT primers in the size range of 200–1500 bp in *Cleome gynandra* while Agarwal et al. (2015) reported amplification of 2–10 fragments (average 5.3/primer) in the size range of 100–1100 bp. Information generated by SCoT primers is usually derived either from the gene itself or its immediate flanking sequences compared to RAPD or ISSR where information is usually based on the non-coding regions of DNA. Therefore, SCoT marker technique being correlated to functional genes and their corresponding traits (Collard and Mackill 2009; Xiong et al. 2011) can be of better use for gene tagging studies. The microsatellite markers used in the present study were adopted from other members of the family Bignoniaceae (Figueira et al. 2010; Yu et al. 2011; Vinson et al. 2008; Jones and Hubbell 2003; Braga

Table 6 Simple sequence repeat (SSR) primers used for testing the genetic homogeneity of micropropagated plants of *Tecomella undulata*

Sr. no.	Primer[a]	Primer sequences (5′–3′)	Annealing temperature (°C)	Number of scorable/ monomorphic bands	Size range of amplification product (bp)
1.	In831	F: GGGAATTTCTCGATACGT R: TAGACCAACCATCAGGCT	48	2/2	200–700
2.	Jc 10	F: GAGGGGTTGACGCAGATTT R: GTGGTGAGATTCCTTCAAGCAT	52	4/4	250–1100
3.	Jc 15	F: AGCATGCGGACCTAGTCAAC R: TGGTTCCAACTGGGTTCTCT	50	2/2	100–260
4.	Jc 21	F: GCTCAACAAAGACTCGTAGGAA R: GAGAGAGAGAGAGAACAATAGCATTC	48	1/1	150
5.	Jc 28	F: AAGCCCACTATTGCATCCAG R: TGTGTAGTTCCTCTCTCTCTCTCTC	51	5/5	200–900
6.	Tau 07	F: CCATAAGCTGCATCAACACA R: ATCCTAAGATCGGTACTCCA	51	4/4	150–500
7.	Tau 14	F: GGTAACGGATTGCTGGTTGT R: CATTGCGAATGGCCTATGGT	52	5/5	150–900
8.	Tau 15	F: TTTGAGGGGTTGAAGCATTT R: CATTGTGGTCCCTCTCAACA	48	4/4	120–500
9.	Tau 16	F: GCTTGTAGCAACGTTAGGTTT R: TGTGCATTGTGACTACCAGCTA	49	3/3	100 - 500
10.	Tau 18	F: TGAATCGGATTAATCGTGGA R: GTGCATTGTGACTACCAGCTA	50	3/3	300–700
11.	Tau 21	F: CTTTTGGGGGTCTTTGGAAT R: TGAAAGAGACAGAGACAAAGATACA	52	1/1	200
12.	Tau 22	F: TATCTCTCCGCCGTACACCT R: CCAATCGAAGAGCCCATTTA	51	1/1	150
13.	Achi 8	F: TGTAGGCAATGATCCGAATAAG R: GGGACCCCTTTAGAAGTCG	50	4/4	150–300
14.	JACC 21	F: ATCTCCTCAAACACCCACAT R: TCATTGCCTCCAACACACTT	51	3/3	200–350

[a] Primer sources: In831—*Incarvillea sinensis* (Yu et al. 2011); Jc 10–18—*Jacaranda copaia* (Vinson et al. 2008); Tau 07–22—*Tabebuia aurea* (Braga et al. 2007); Achi 8—*Arrabidaea chica* (Figueira et al. 2010); JACC 21—*Jacaranda copaia* (Jones and Hubbell 2003)

et al. 2007) in the absence of *T. undulata* sequences in public domain. Amplification was obtained with 14 out of the 56 cross-species SSR markers used. A total of 42, all monomorphic, DNA fragments ranging in size from 100 to 1100 bp were produced (Fig. 3d; Table 6). Various workers have already proved the suitability of genomic and cross-species SSRs (Pandey et al. 2012; Singh et al. 2013a) for genetic fidelity studies.

Among the four marker systems used, ISSR primers produced the maximum average number (4.65) of DNA fragments per primer while SSR primers produced the minimum (3.0). The scoring data of well-resolved fragments of RAPD, ISSR, SCoT, and SSR markers when subjected to calculation of similarity matrix based on Jaccard's similarity coefficient showed that pair-wise value of the in vitro cultures, regenerated plantlets, and the mother plant was 1, indicating 100 % similarity. This study confirmed that *T. undulata* cultures can maintain genetic homogeneity and remain free from somaclonal variations over a culture period extending up to 2 years, when multiplied through axillary bud proliferation. Organized (pre-existing meristems) cultures especially shoot tips and axillary buds are known to maintain strict genotypic and phenotypic stability compared to de novo originating meristematic structures like adventitious buds differentiating from callus or directly from cultured tissues (Singh et al. 2013b). Propagation by axillary buds circumvents the dedifferentiation or redifferentiation of cells or tissues, avoiding genomic aberrations and consequently maintenance of clonal fidelity of in vitro-raised plantlets (Negi and Saxena 2010). Length of in vitro culture maintenance has been variable in different plants—4 years (almonds; Martins et al. 2004), 44 months (*Swertia chirayita,* Joshi and Dhawan 2007), and 2 years (*D. asper*; Singh et al. 2013b). Initiation of in vitro cultures of *T. undulata* is difficult being season specific, prone to persistent contamination, physiological status of explants, etc. (Chhajer and Kalia 2016). Therefore, retention of clonal uniformity for prolonged period under in vitro conditions has immense commercial significance. The developed protocol can ensure continuous supply of genetically uniform plants over a prolonged duration without resorting to initiation of fresh cultures frequently, thus reducing the overall cost of plant production at commercial scale.

The four marker systems (RAPD, ISSR, SCoT, and SSR) used in this study targeted different regions of the genome; thus, amplification of 300 distinct and scorable fragments by 58.8 % of the assayed markers assured an extensive screening of the genome of *T. undulata* at various stages of in vitro propagation. The 100 % similarity index obtained between the mother plant, in vitro cultures, and regenerated plantlets with all the four DNA markers used revealed that these PCR-based cost effective, quick to use, easy to apply, and highly polymorphic markers separable on agarose gel can be effectively used for routine and rapid genetic homogeneity testing of in vitro-raised clones. Arbitary primers like RAPD and ISSR have been extensively used in genetic fidelity studies while SSR primers being sequence-based species-specific markers have found limited use. The recently developed SCoT marker is another marker of choice being based on start codon of functional genes. Cuesta et al. (2010) recommended the use of RAPD primers after comparison of RAPD, ISSR, AFLP, and SAMPL markers for assessment of somaclonal variation in micropropagated plants of stone pine.

Conclusions

The present study describes the use of four PCR based marker systems (RAPD, ISSR, SCoT, and SSR) for assessment of genetic stability of in vitro-raised plants of *T. undulata*, an endangered timber tree of arid regions. Production of monomorphic bands by in vitro cultures maintained on cytokinin-enriched medium for 3 months to 2 years, 4 months of field-transferred tissue culture-raised plants and the mother tree with all the DNA-based markers, confirmed the true-to-type nature of the plants produced. Method of regeneration primarily account for the appearance of somaclonal variations in vitro. Plantlets derived from organized cultures like shoot tips and axillary buds are usually more stable genetically than those obtained through indirect organogenesis from unorganized callus. Therefore, the developed micropropagation protocol based on axillary shoot proliferation can be used for large-scale micropropagation of *T. undulata* without any risk of genetic instability appearing for at least 32 passages of 3 weeks each or up to 2 years of continuous subculturing, thus can help to fill the gap of demand and supply of planting material. Detection of somaclonal variations at an early stage can help in getting rid of genetically instable plants, thus ensuring filed maintenance of only clonally uniform plants till maturity. This is the first study reporting genetic homogeneity testing of *T. undulata* maintained in vitro for more than 2 years using four DNA marker systems.

Acknowledgment Research grant provided by the Department of Biotechnology, Government of India, New Delhi, under the project sanctioned vide order no. BT/PR6558/PBD/16/998/2012 to RKK is gratefully acknowledged.

Author contribution SC conducted all the experiments and RKK designed the experiments, arranged research grants, and prepared the manuscript.

Data archiving statement No data for archiving.

References

Agarwal T, Gupta AK, Patel AK, Shekhawat NS (2015) Micropropagation and validation of genetic homogeneity of *Alhagi maurorum* using SCoT, ISSR and RAPD markers. Plant Cell Tiss Organ Cult 120:313–323

Agnihotri RK, Mishra J, Nandi SK (2009) Improved *in vitro* shoot multiplication and rooting of *Dendrocalamus hamiltonii* Nees et Arn. Ex Munro: production of genetically uniform plants and field evaluation. Acta Physiol Plant 31:961–967

Arya HC, Shekhawat NS (1986) Clonal multiplication of tree species in Thar Desert through tissue culture. For Ecol Manag 16:201–208

Aslam M, Singh R, Negi PS, Bhakuni DS, Das SC (2006) Enhanced *in vitro* regeneration from cotyledonary node explants of *Tecomella undulata* (Smith) Seem. Proc Nat Acad Sci, India Sec B: Biol Sci 76:281–285

Bhansali RR (1993) Bud culture for shoot multiplication and plantlet formation of *Tecomella undulata* (Rohida) a wood tree of arid zone. Trop Sci 33:1–8

Bhojwani SS, Dantu PK (2013) Plant tissue culture: an introductory text. Springer India, New Delhi. doi: 10.1007/978-81-322-1026-9

Braga AC, Reis AMM, Leoi LT, Pereira RW, Collevatti RG (2007) Development and characterization of microsatellite markers for the tropical tree species *Tabebuia aurea* (Bignoniaceae). Mol Ecol Note 7:53–56

Chhajer S, Kalia RK (2016) Seasonal variation in the *in vitro* responses of mature nodes of *Tecomella undulata* (Sm.) Seem. The Ind For 142(9):827–832

Collard BCY, Mackill DJ (2009) Start codon targeted (SCoT) polymorphism: a simple, novel DNA marker technique for generating gene-targeted markers in plants. Plant Mol Biol Rep 27:86–93

Cuesta C, Ordas RJ, Rodriguez A, Fernandez B (2010) PCR-based molecular markers for assessment of somaclonal variation in *Pinus pinea* clones micropropagated *in vitro*. Biol Plant 54:435–442

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus 12:11–15

Figueira GM, Ramelo PR, Ogasawara DC, Montanari I Jr, Zucchi MI, Cavallari MM, Foglio MA (2010) A set of microsatellite markers for *Arrabidaea chica* (bignoniaceae), a medicinal liana from the neotropics. Am J Bot:e63–e64

Heinz B, Schmidt J (1995) Monitoring genetic fidelity vs somaclonal variation in Norway Spruce (*Picea abies*) somatic embryogenesis by RAPD analysis. Euphytica 85:341–345

Jones FA, Hubbell SP (2003) Isolation and characterization of microsatellite loci in the tropical tree *Jacaranda copaia* (Bignoniaceae). Mol Ecol Note 3:403–405

Joshi P, Dhawan V (2007) Assessment of genetic fidelity of micropropagated *Swertia chirayita* plantlets by ISSR marker assay. Biol Plant 51:22–26

Kalia RK (2015) Tissue culture based methodologies for conservation of agrobiodiversity. Desert Environ Newsl. CAZRI, Jodhpur. 17:5–6

Kalia RK, Rai MK, Sharma R, Bhatt RK (2014) Understanding *Tecomella undulata*: an endangered pharmaceutically important timber species of hot arid regions. Genet Res Crop Evol 61:1397–1421

Kumar S, Kumar P, Agrawal DK, Choudhary AK (2011) Rehabilitation of lignite mine backfill with indigenous desert tree, *Tecomella undulata* in Indian arid zone. J Trop Forest 27:17–28

Kumari S, Singh N (2012) Multiplication of desert teak *Tecomella undulata* under *in vitro* conditions. J Trop Med Plant 13:137–143

Kumari S, Singh N (2014) Micropropagation of *Tecomella undulata* (Sm.) Seem and genetic fidelity testing of *in vitro* raised plants. As Pac J Mol Biol Biotechnol 22:191–198

Lakshmanan V, Venkataramareddy SR, Neelwarne B (2007) Molecular analysis of genetic stability in long-term micropropagated shoots of banana using RAPD and ISSR markers. Electron J Biotechnol 10: 106–113

Leroy XJ, Leon K, Charles G, Branchard M (2000) Cauliflower somatic embryogenesis and analysis of regenerant stability by ISSRs. Plant Cell Rep 19:1102–1107

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

Mathur N, Singh J, Bohra S, Bohra A, Mehboob VM, Vyas A (2010) Phytoremediation potential of some multipurpose tree species of Indian Thar Desert in oil contaminated soil. Adv Environ Biol 4: 131–137

Nandwani D, Mathur N, Ramawat KG (1995) *In vitro* shoot multiplication from cotyledonary node explants of *Tecomella undulata*. Gartenbauwisseschaft 60:65–68

Nandwani D, Sharma R, Ramawat KG (1996) High frequency regeneration in callus culture of tree *Tecomella undulata*. Gartenbauwisseschaft 61:147–150

Negi D, Saxena S (2010) Ascertaining clonal fidelity of tissue culture raised plants of *Bambusa balcooa* Roxb. using inter simple sequence repeat markers. New For 40:1–8

Pal M, Kalia RK, Chaudhary A, Dhawan AK (2009) Role of biotechnological tools in the production of secondary metabolites from medicinal plants—Shikonin: a case study. In: Trivedi PC (ed) Biotechnology and medicinal plants. Aavishkar publishers, Jaipur, pp. 92–135

Palombi MA, Damiano C (2002) Comparison between RAPD and SSR molecular markers in detecting genetic variation in kiwifruit (*Actinidia deliciosa* A. Chev. Plant Cell Rep 20:1061–1066

Pandey RN, Singh SP, Rastogi J, Sharma ML, Singh RK (2012) Early assessment of genetic fidelity in sugarcane (*Saccharum officinarum*) plantlets regenerated through direct organogenesis with RAPD and SSR markers. Aust J Crop Sci 6:618–624

Peschke VM, Phillips RL (1992) Genetic implications of somaclonal variation in plants. Adv Genet 30:41–75

Phillips RL, Kaeppler SM, Olhoft P (1994) Genetic stability of plant tissue cultures: breakdown of normal controls. Proc Natl Acad Sci U S A 91:5222–5226

Rani V, Raina SN (2000) Genetic fidelity of organized meristem-derived micropropagated plants: a critical reappraisal. In Vitro Cell Dev Biol Plant 36:319–330

Rathore TS, Singh RP, Shekhawat NS (1991) Clonal propagation of desert tree (*Tecomella undulata*) through tissue culture. Plant Sci 79:217–222

Rathore MS, Chikara J, Mastan SG, Rahman H, Anand KVG, Shekhawat NS (2011) Assessment of genetic stability and instability of tissue culture-propagated plantlets of *Aloe vera* L. by RAPD and ISSR markers. Applied Biochem Biotechnol 165:1356–1365

Rathore NS, Rai MK, Phulwaria M, Rathore N, Shekhawat NS (2014) Genetic stability in micropropagated *Cleome gynandra* revealed by SCoT analysis. Acta Physiol Plant 36:555–559

Rodrigues PHV, Tulmann Neto A, Cassieri Neto P, Mendes BMJ (1998) Influence of the number of subcultures on somaclonal variation in micropropagated Nanico (*Musa* spp., AAA group). Acta Hort 490: 469–473

Rohlf FJ (2000) NTSYS-pc: numerical taxonomy and multivariate analysis system. Version 2.0 Exeter Software, Setauket, New York, USA

Singh R, Rathore M, Mishra GP, Kumar M, Singh R, Ahmed Z (2009) Adventitious shoot regeneration and *Agrobacterium tumefaciens* mediated transformation in Rohida (*Tecomella undulata*). The Ind For 135:751–764

Singh SR, Dalal S, Singh R, Dhawan AK, Kalia RK (2012a) Seasonal influences on *in vitro* bud break in *Dendrocalamus hamiltonii* Arn. ex Munro nodal explants and effect of culture microenvironment on large scale shoot multiplication and plantlet regeneration. Indian J Plant Physiol 17:9–21

Singh SR, Dalal S, Singh R, Dhawan AK, Kalia RK (2012b) Micropropagation of *Dendrocalamus asper* {Schult. & Schult. F.} Backer ex K. Heyne): an exotic edible bamboo. J Plant Biochem Biotechnol 21:220–228

Singh SR, Dalal S, Singh R, Dhawan AK, Kalia RK (2013a) Ascertaining clonal fidelity of micropropagated plants of *Dendrocalamus hamiltonii* Nees et Arn. ex Munro using molecular markers. In Vitro Cellular and Developmental Biology-Plant 49:572–583

Singh SR, Singh R, Dalal S, Dhawan AK, Kalia RK (2013b) Evaluation of genetic fidelity of *in vitro* raised plants of *Dendrocalamus asper* (Schult. & Schult. F.) Backer ex K. Heyne using DNA-based markers. Acta Physiol Plant 35:419–430

Tripathi JPM, Jaimini SN (2002) Floral and reproductive biology of Rohida (*Tecomella undulata* (Sm.) seem. Indian J For 25:341–343

Tyagi H, Tomar UK (2013) Factors affecting *in vitro* shoot proliferation and rooting of mature *Tecomella undulata* (Sm.) Seem tree. Res. Plant Sci 1:38–44

Varshney A, Anis M (2012) Improvement of shoot morphogenesis *in vitro* and assessment of changes of the activity of antioxidant enzymes during acclimation of micropropagated plants of Desert Teak. Acta Physiol Plant 34:859–867

Venkatachalam L, Sreedhar RV, Bhagyalakshmi N (2007) Micropropagation in banana using high levels of cytokinins does not involve any genetic changes as revealed by RAPD and ISSR markers. Plant Growth Regul 51:193–205

Vinson CC, Sampaio I, Ciampi AY (2008) Eight variable microsatellite loci for a Neotropical tree, *Jacaranda copaia* (Aubl.) D.Don (Bignoniaceae. Mol Ecol Resour 8:1288–1290

Xiong FQ, Zhong RC, Han ZQ, Jiang J, He LQ, Zhuang WJ, Tang RH (2011) Start codon targeted polymorphism for evaluation of functional genetic variation and relationships in cultivated peanut (*Arachis hypogaea* L.) genotypes. Mol Biol Rep 38:3487–3494

Yu HY, Gao J, Luo YB, Bai WN (2011) Development of polymorphic microsatellite markers for *Incarvillea sinensis* (bignoniaceae. Am J Bot:e224–e225