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Research Article

True-to-type micropropagated plants of para rubber (*Hevea brasiliensis* Müll. Arg.) via somatic embryogenesis

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Abstract

Plant micropropagation via somatic embryogenesis is a powerful technique for rapid mass propagation, especially in para rubber (*Hevea brasiliensis* Müll. Arg.). However, somaclonal variations are the major limitation of this process. To date, DNA fingerprinting, i.e., RAPD (Randomly Amplified Polymorphic DNA), Star Codon Targeted (SCoT), and SSRs (Simple Sequence Repeats), is one of the most successful technologies to detect the genetic fidelity in the somatic embryos. The aim of present study was to induce somatic embryos from inner integument explants of para rubber cv. 'RRIM 600' at different developmental stages and subsequent acclimatization and transplantation (under greenhouse and field conditions) of the propagated seedlings. The genetic stability of the plants derived from somatic embryos was also analysed in comparison to the mother plant using RAPD, SCoT and SSRs markers. Somatic embryos derived from inner integuments of 5-week-old immature seeds after pollination were more efficient than older and younger seeds. In addition, para rubber mother plants cv. 'RRIM600' and plants derived from somatic embryogenesis demonstrated the same pattern of DNA fragments, as confirmed by three PCR-based techniques, RAPD, SCoT and SSRs, whereas these in the pattern were different from 'RRIT 226', 'PB 235', 'PB 251', 'PB 255' and 'BMP 24'. Interestingly, T2 plant was found to possess somaclonal variations when compared with mother plant. Based on the results, we confirm that the plants derived from somatic embryogenesis of para rubber cv. 'RRIM 600' were true-to-type to that of 'RRIM 600' master stock.

Keywords: DNA fingerprinting; para rubber; RAPD; SCoT; SSRs

Introduction

Para rubber or rubber tree (*Hevea brasiliensis* Müll. Arg.) is an important economic crop of Southeast Asia, i.e., Thailand, Indonesia and Malaysia. It is cultivated in ~9.7 million hectares area in the region and contributes 97% of the world's natural rubber supply (Ratnasingam *et al.*, 2015). According to the statistical

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report, global natural rubber consumption in the year 2019 was 12.119 megatons and it has been expected to increase at a rate of 16.5 megaton per year (IRSG, 2020). Maximum consumption of natural rubber has been reported in the tires of automobiles, which utilizes ~70% of the total production, and its demand has been predicted to increase by 30 megatons per year by the year 2030 (Cornish, 2017). The para rubber planting area is rapidly expanding into the Montane Mainland Southeast Asia including Grate Mekong Subregion (China, Vietnam, Thailand, Laos, Myanmar and Cambodia) (Golbon *et al.*, 2018; Yang *et al.*, 2019) and Peninsular Malaysia (Hazir *et al.*, 2020).

Para rubber cv. 'RRIM600' is a good candidate for plantation in Thailand and China with adaptation to drought, chilling and frost injuries and moderate yield (Priyadarshan *et al.*, 2005; Gonçalves *et al.*, 2011; Pethin *et al.*, 2015; Yang *et al.*, 2019). Elite clones of para rubber with high yield of latex, rapid growth, long-lifetime tapping (20-30 years) and disease-resistant traits being developed through breeding programs (Supriya *et al.*, 2019). Rootstock grafting with elite scion genotype is a common protocol to generate master stock of para rubber for small holder farmers in both Thailand (>85% of the total area; Chantuma *et al.*, 2011) and Malaysia (>93% of the total area; Hazir *et al.*, 2020), which are the major plantation areas. Recently, the limitation of cross-talking (grafting efficiency) between rootstock and scion has been found to significantly affect the latex yield and growth rate of scion (Gonçalves and Martins, 2002; Cardinal *et al.*, 2007; Yao *et al.*, 2017) and to induce DNA methylation (epigenetic changes) in heterografted plants (Uthup *et al.*, 2018). Self-rooted para rubber is an alternative way for elite clonal propagation (75% success rate from shoots to mature rooted plants) at the commercial scale to produce large number of healthy plants by cuttings (Masson and Monteuuis, 2016). Microcutting (Kalawong *et al.*, 2014) and nodal segment micropropagation (Sirisom and Te-chato, 2014) via *in vitro* culture are the other alternatives. However, the vegetatively propagated plants lack taproot system, which makes it susceptible to wind disasters (Masson *et al.*, 2013; Masson and Monteuuis, 2017). Somatic embryogenesis may overcome this problem, as it produces large number of plantlets containing tap root system, similar to the seedlings produced by seed germination (Zhao *et al.*, 2015; Rahman *et al.*, 2017; Wang *et al.*, 2017). Recently, somatic embryos have been successfully developed from inner integuments of immature seeds and uninucleate anthers of male flower (Srichuay *et al.*, 2014; Lardet *et al.*, 2008; Kouassi *et al.*, 2018; Tisarum *et al.*, 2018). However, success of somatic embryogenesis in para rubber depends on genotype, and there are risks associated with *in vitro* acclimatization, somaclonal variation and high cost of production (Masson and Monteuuis, 2016; Tisarum *et al.*, 2018).

True to type somatic embryos of para rubber, without somaclonal variation, are required for large scale production of elite clones (Wang *et al.*, 2017). Based on this requirement, genetic and epigenetic changes have been studied in para rubber using molecular markers [including randomly amplified polymorphic DNA (RAPD) and simple sequence repeats (SSRs)] and DNA methylation [including methylation-sensitive amplification polymorphism (MSAP)] (Karumamkandathil *et al.*, 2015; Wang *et al.*, 2017). RAPD and SSRs are a simple, rapid, reproducible, and costly techniques to identify the genetic similarity in both intergeneric and intrageneric diversity (Thakur *et al.*, 2016; Rohela *et al.*, 2019). SCoT (start codon targeted marker) is a novel technique to confirm homogeneity of micropropagated plantlets derived from organogenesis or embryogenesis. In addition, two or three molecular markers, confirming genetic stability, have been well established in many plant species (Bhattacharyya *et al.*, 2017a,b; Sharma *et al.*, 2019). The aim of present study was to induce somatic embryos from inner integuments of para rubber cv. 'RRIM 600' at different developmental stages and subsequent acclimatization and transplantation (under greenhouse and field conditions) of the propagated seedlings. The genetic stability of the plants derived from somatic embryos was also analysed in comparison to the mother plant using RAPD, SCoT and SSRs markers.

Materials and Methods

In vitro culture and acclimatization of plantlets

Immature seeds of *Hevea brasiliensis* cv. 'RRIM' 600 in different ages, 4, 5 and 6 weeks were collected after pollination. These were dipped in 95% ethanol and burned for surface sterilization in aseptic clean bench. Thereafter, inner integuments of each seed were dissected as initial explants and inoculated on MH-IN medium (Carron *et al.*, 1989) under dark conditions for 25 d. Number of callus formed in MH-IN medium was recorded, and callus induction percentage in each treatment was calculated. Callus derived from inner integuments were proliferated on MH-EXP medium for 25 d (Carron *et al.*, 1989) and then embryogenic callus were induced using MH-EXP for 25 d. Number of embryogenic callus in each treatment were counted. Somatic embryos derived from embryogenic callus inoculating on MH-DEN for 25 d (Carron *et al.*, 1989) were induced. Embryos and elongated embryos from single somatic embryos were induced by MH-MAT and MH-GER medium for 25 d (Carron *et al.*, 1989), respectively. Para rubber plantlets derived from somatic embryos were transferred to test tube containing MH-PL medium with activated charcoal (1.5 g L^{-1}) and kept for 25 d. The whole process of *in vitro* culture system of para rubber is demonstrated in Figure S1. *In vitro* acclimatization (Figure S2) and *ex vitro* adaptation (Figure S3) were practiced by following the method given by Tisarum *et al.* (2018) as along with 1.5 year-field trial (Figure S3).

DNA extraction

Leaf samples of three master stocks *(*mother plants*)* of para rubber cv. 'RRIM 600', nine *in vitro* acclimatized plantlets (Figure 1A), ten plants derived from somatic embryogenesis transplanted in the field for 1.5 year (Figure 1B) and an individual master stock of other cultivars, i.e., 'RRIT 226', 'RRIT 251', 'PB 235', 'PB 255' and 'BPM 24' were collected (Table 1). DNA was extracted using genomic DNA Mini Kit (Plant) (GP100, Geneaid™, Geneaid Biotech Ltd., South Korea) according to company protocol. In brief, 50 mg of leaf tissues were ground using liquid nitrogen, transferred to 1.5 mL plastic tube, mixed with 400 mL GPX1 buffer containing 5 mL RNase A (mixed by vortex) and incubated at 60 °C for 15 min (invert the tube 5 min interval). Thereafter, 100 mL GP2 buffer was added, mixed by vortex and then incubated on ice for 3 min. Solution was filtered through filter column, centrifuged at 12,000×*g* for 2 min. The filter column was discarded, and supernatant was collected and transferred to a new 1.5 mL plastic tube to which 1.5 volume GP3 buffer was added, and immediately mixed with vortex for 5s and filtered through GD column. The extracted solution was centrifuged at 12,000×*g* for 2 min, and added with 400 mL W1 buffer, then again centrifuged at 12,000×*g* for 30s, and added with 400 mL absolute ethanol (wash buffer), and again centrifuged at 12,000×*g* for 30s. After discarding the flow-through, the GD column was placed in the 1.5 mL collection tube and centrifuged at 12,000×*g* for 3 min to dry the column. Dry GD column was moved to a new 1.5 mL plastic tube, 150 mL pre-heated elution buffer (10 mM Tris-HCl, pH 8.5 at 60 °C) to the centre of the column matrix, incubated at room temperature for 5 min, centrifuged at 12,000×*g* for 1 min, and then the purified DNA was collected and incubated at -20 °C for further assay.

Table 1. List of para rubber (*Hevea brasiliensis*) plant samples, code and descriptive information

Sample	Code	Descriptive information	Symbol
1	600/1	RRIM 600 master stock 1	1
2	600/2	RRIM 600 master stock 2	2
3	600/3	RRIM 600 master stock 3	3
4	600 tissue/1	RRIM 600 *in vitro* plantlet #1	T1
5	600 tissue/2	RRIM 600 *in vitro* plantlet #2	T2
6	600 tissue/3	RRIM 600 *in vitro* plantlet #3	T3
7	600 tissue/4	RRIM 600 *in vitro* plantlet #4	T4

8	600 tissue/5	RRIM 600 *in vitro* plantlet #5	T5
9	600 tissue/6	RRIM 600 *in vitro* plantlet #6	T6
10	600 tissue/7	RRIM 600 *in vitro* plantlet #7	T7
11	600 tissue/8	RRIM 600 *in vitro* plantlet #8	T8
12	600 tissue/9	RRIM 600 *in vitro* plantlet #9	T9
13	RRIM 600/1	RRIM 600 transplanted plant in the field trial #1	R1
14	RRIM 600/2	RRIM 600 transplanted plant in the field trial #2	R2
15	RRIM 600/3	RRIM 600 transplanted plant in the field trial #3	R3
16	RRIM 600/4	RRIM 600 transplanted plant in the field trial #4	R4
17	RRIM 600/5	RRIM 600 transplanted plant in the field trial #5	R5
18	RRIM 600/6	RRIM 600 transplanted plant in the field trial #6	R6
19	RRIM 600/7	RRIM 600 transplanted plant in the field trial #7	R7
20	RRIM 600/8	RRIM 600 transplanted plant in the field trial #8	R8
21	RRIM 600/9	RRIM 600 transplanted plant in the field trial #9	R9
22	RRIM 600/11	RRIM 600 transplanted plant in the field trial #11	R11
23	RRIT 226	RRIT 226 master stock	226
24	RRIT 251	RRIT 251 master stock	251
25	PB 235	PB 235 master stock	235
26	PB 255	PB 255 master stock	255
27	PBM 24	PBM 24master stock	24

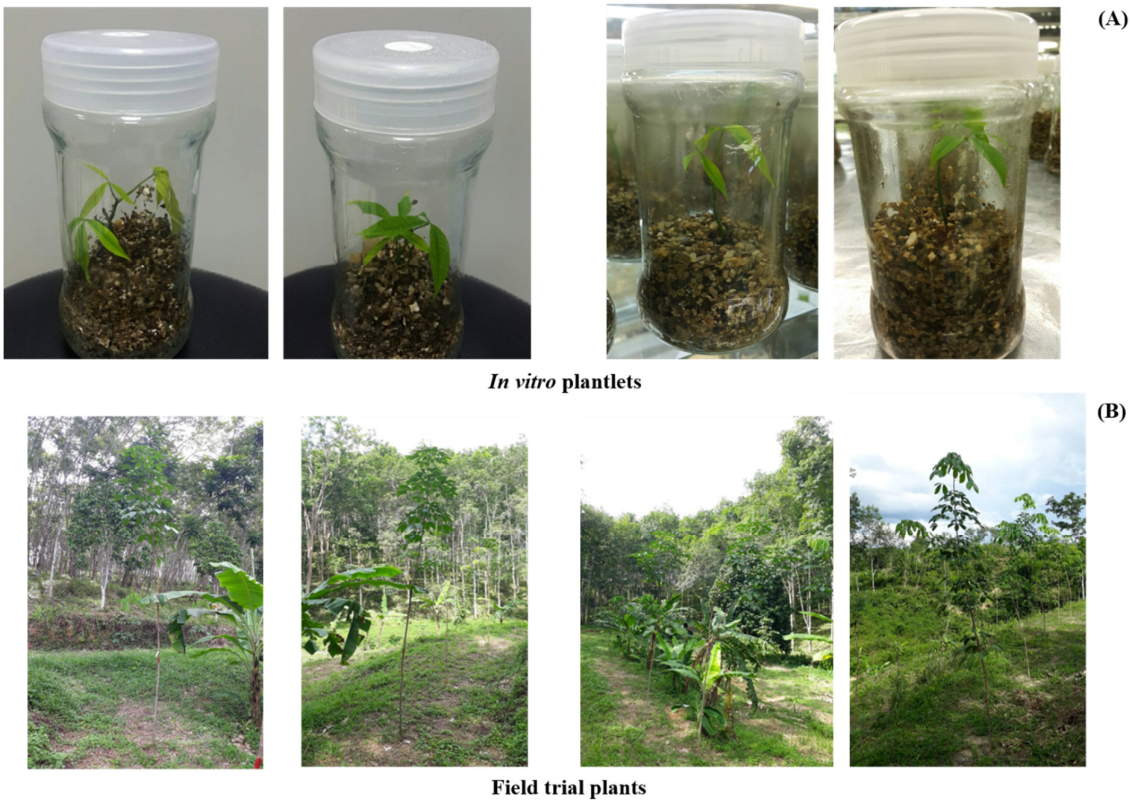

Figure 1. *In vitro* plantlets in acclimatization stage before transplanting into soil (A) and the field trial para-rubber (*Hevea brasiliensis*) plants (1.5-year-old) derived from somatic embryogenesis

RAPD fidelity assay

Extracted DNA (50 ng mL^{-1}) from each sample (3.2 mL) was mixed with 1.2 mL deoxynucleotide (2 mM dNTPs mixture of dATP, dGTP, dCTP and dTTP; N0477s, New England Biolab Inc., MA, USA), 2 mL primers (5 mM) (30 RAPD primers; Table S1), 2 mL 10X PCR buffer, 0.16 mL Taq DNA polymerase (5 U mL^{-1}; M0273s, New England Biolab Inc., MA, USA) and 11.44 mL deionized water (total volume 20 mL). Mixture was amplified using PCR equipment (Mastercycler® nexus GX2, Eppendorf, NY, USA), denaturing at 95 °C for 30s, annealing at 50 °C for 1 min, and extension at 68 °C for 90s with 40 cycles and extension at 68 °C for 10 min in the final cycle. Agarose gel electrophoresis (1.8%) with 1X TBE buffer (Amresco® 0478-40L Buffer, AMRESCO, Inc., OH, USA) was run along with 2 log DNA Ladder (M3200s, New England Biolab Inc., MA, USA), strained with ethidium bromide for 5 min, washed by water for 3 min and visualized using Gel Documentation System (Bio-Rad Laboratories, Inc., CA, USA) (Figure S4). The presence (1) or absence (0) of band of target product DNA was scored for generating genetic similarity dendrogram according to Jaccard (1901) by UPGMA (unweight pair group method arithmetic average) using NTSYSpc 2.1 software.

SCoT fidelity assay

Extracted DNA (50 ng mL^{-1}) from each sample (3.2 mL) was mixed with 1.2 mL deoxynucleotide (2 mM dNTPs mixture of dATP, dGTP, dCTP and dTTP; N0477s, New England Biolab Inc., MA, USA), 2 mL primers (5 mM) (15 SCoT primers; Table S2), 2 mL 10X PCR buffer, 0.16 mL Taq DNA polymerase (5 U mL^{-1}; M0273s, New England Biolab Inc., MA, USA) and 11.44 mL deionized water (total volume 20 mL). Mixture was amplified using PCR equipment (Mastercycler® nexus GX2, Eppendorf, NY, USA), denaturing at 94 °C for 1 min, annealing at 50 °C for 1 min, and extension at 68 °C for 2 min with 35 cycles and extension at 68 °C for 5 min in the final cycle. Agarose gel electrophoresis (1.8%) with 1X TBE buffer (Amresco® 0478-40L Buffer, AMRESCO, Inc., OH, USA) was run along with 2 log DNA Ladder (M3200s, New England Biolab Inc., MA, USA), strained with ethidium bromide for 5 min, washed by water for 3 min and visualized using Gel Documentation System (Bio-Rad Laboratories, Inc., CA, USA) (Figure S5). The presence (1) and absence (0) of band of target product DNA was scored for generating genetic similarity dendrogram according to Jaccard (1901) by UPGMA (unweight pair group method arithmetic average) using NTSYSpc 2.1 software.

SSRs fidelity assay

Extracted DNA (50 ng mL^{-1}) from each sample (3.2 mL) was mixed with 1.2 mL deoxynucleotide (2 mM dNTPs mixture of dATP, dGTP, dCTP and dTTP; N0477s, New England Biolab Inc., MA, USA), 2 mL primers (5 mM) (20 SSRs primers; Table S3), 2 mL 10X PCR buffer, 0.16 mL Taq DNA polymerase (5 U mL^{-1}; M0273s, New England Biolab Inc., MA, USA) and 11.44 mL deionized water (total volume 20 mL). Mixture was amplified using PCR equipment (Mastercycler® nexus GX2, Eppendorf, NY, USA), denaturing at 94 °C for 30s, annealing at 52-57 °C for 30s, and extension at 72 °C for 1 min with 35 cycles and extension at 72 °C for 10 min in the final cycle. Polyacrylamide gel electrophoresis (6%) with TBE buffer (Amresco® 0478-40L Buffer, AMRESCO, Inc., OH, USA) was run along with DNA marker, strained with silver nitrate, washed by water for 3 min and visualized using Gel Documentation System (Bio-Rad Laboratories, Inc., CA, USA) (Figure S6). The presence (1) and absence (0) of band of target product DNA was scored for generating genetic similarity dendrogram according to Jaccard (1901) by UPGMA (unweight pair group method arithmetic average) using NTSYSpc 2.1 software.

Experimental design and statistical analysis

The experiment was arranged as completely randomized design (CRD) with 4 biological replications. Data were subjected to Analysis of variance (ANOVA) and mean values obtained from three treatments were compared using Tukey's HSD at $p \leq 0.01$ using SPSS software (version 11.5, SPSS for Window®).

Results

Somatic embryogenesis derived from inner integuments of immature seeds

Callus induction percentage of the inner integuments of 5-week-old immature seeds was maximum (52.9%), with 160 explants per inoculum, on MH-IN as compared to younger (4-week-old; 38.9%) and older (6-week-old; 28.3%) seeds (Table 2 and Figure 2A). Highest embryogenic callus induction was also observed from the inner integuments of 5-week-old immature seeds (42.75%) with 130 embryogenic callus on MH-EXP, compared to younger (27.0%) and older (12%) seeds (Table 2 and Figure 2B). Moreover, somatic embryogenesis, embryos and plantlets derived from embryogenic callus were maximized at 33.3%, 42.0% and 36.8%, respectively when exposed to the culture medium, MH-DEN, MH-MAT and MH-PL, respectively (Table 2 and Figure 2 C-D). The plantlets were acclimatized for 3 months in the greenhouse conditions and thereafter, for 1.5 year under field conditions. The leaf samples collected from the plantlets derived from somatic embryogenesis were tagged as T1-T9 (Table 1 and Figure 1) and those collected from acclimatized plantlets were tagged as R1-R9 and R11 (Figure 1). Leaf samples were also collected to assay genetic fidelity of RRIM 600 original mother plant, 'RRIT 226', 'RRIT 251', 'PB 235', 'PB 255' and 'BPM 24'.

Table 2. Callus induction, embryogenic callus, number of somatic embryos and plantlet regeneration in para rubber (*Hevea brasiliensis*) cv. 'RRIM 600' inner integument cultured on modified MH medium

Immature seeds (weeks)	Callus induction (%)	Embryogenic callus (%)	Somatic embryos (%)	Embryos (%)	Plantlet regeneration (%)
4	38.90±4.98ab	27.00±2.58b	15.00±1.29b	14.25±1.22b	20.75±0.63b
5	52.90±3.67a	42.75±1.80a	33.25±1.14a	42.00±1.69a	36.75±2.32a
6	28.25±2.08b	12.00±1.29c	7.26±1.19c	6.33±0.85b	10.75±1.71c
Significant level	**	**	**	**	**

Different letters in each column show significant difference at $p \leq 0.01$ (**) according to Tukey's HSD.
Data presented as mean ± SE.

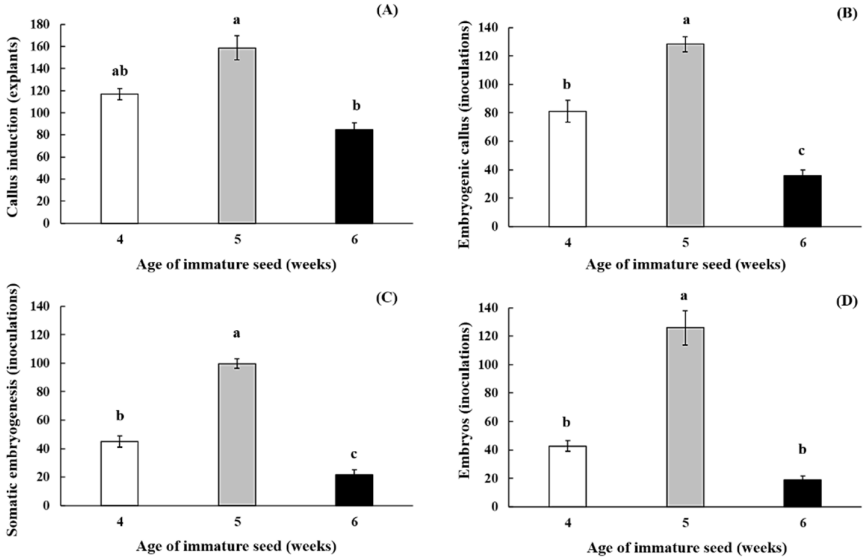

Figure 2. A response of immature inner integuments in different ages of seed development in para-rubber (*Hevea brasiliensis*) for callus induction (A), embryogenic callus (B), somatic embryogenesis (C) and embryos induction (D) on modified MH medium

Data presented as mean ± SE. Different letters in each bar show significant difference at $p \leq 0.01$ (**) according to Tukey's HSD.

Genetic fidelity evaluation using RAPD, SCoT and SSRs

Based on 30 RAPD markers, 406 bands (254 polymorphic bands and 152 monomorphic bands) were counted and used to draw a genetic similarity dendrogram using UPGMA. Similarity in DNA fingerprinting using RAPD in each plant (derived from plantlets produced under *in vitro* conditions, plants acclimatized under greenhouse conditions and plants transplanted under field conditions) was demonstrated (Table S4). Acclimatized plants #1 (T1) and #2 (T2) had low similarity (< 0.840) to 'RRIM 600' original, #1, #2 and #3 (Table S4). In addition, the similarity among plants derived from somatic embryogenesis and from 'RRIT 226', 'RRIT 251', 'PB 235', 'PB 255' and 'BPM 24' was quite less (Table S4). In cluster dendrogram, genetic stability in plantlets derived from somatic embryogenesis was in a similar pattern to that of 'RRIM 600' original, #1, #2 and #3 (Cluster I), whereas T1 and T2 were categorized in Cluster II. Moreover, 'RRIT 226' (Cluster III), 'RRIT 251' (Cluster IV), 'PB 235', 'PB 255' (Cluster V) and 'BPM 24' (Cluster VI) were classified under different groups (Figure 3).

In 15 SCoT markers, 186 bands (51 polymorphic bands and 135 monomorphic bands) were detected and analysed for genetic similarity dendrogram using UPGMA. Acclimatized plants #1 (T1) and #2 (T2) showed a small difference in pattern (< 0.930) when compared with 'RRIM 600' original, #1, #2 and #3 (Table S5). Therefore, the pattern of SCoT bands among plants derived from somatic embryogenesis was similar to that of RRIM 600 original and different from that of 'RRIT 226', 'RRIT 251', 'PB 235', 'PB 255' and 'BPM 24' (Table S5). Based on UPGMA cluster analysis, genetic fetidity in plantlets derived from somatic embryogenesis showed a similar pattern to 'RRIM 600' original, #1, #2 and #3 (Cluster I), whereas T1 and T2 were categorized in Cluster II. Moreover, 'RRIT 226' (Cluster III), 'PB 235', 'PB 255' (Cluster IV), 'RRIT 251 '(Cluster V), and 'BPM 24' (Cluster VI) were identified as other clusters (Figure 4).

In our study, 20 SSR markers were used for genetic diversity assay (Table S6). Within the plantlets derived from somatic embryogenesis, low genetic diversity was noticed (<4%) except in T2, which was different from RRIM 600 original (by >20%). Based on UPGMA cluster analysis, genetic stability in plantlets derived from somatic embryogenesis was evaluated in the same group with 'RRIM 600' original, #1, #2 and #3 (Cluster I), except T2 being categorized in Cluster III. Moreover, 'RRIT 226' (Cluster II), 'PB 235' (Cluster IV), 'PB 255' (Cluster V), 'RRIT 251' and 'BPM 24' (Cluster VI) were classified under separate categories (Figure 5).

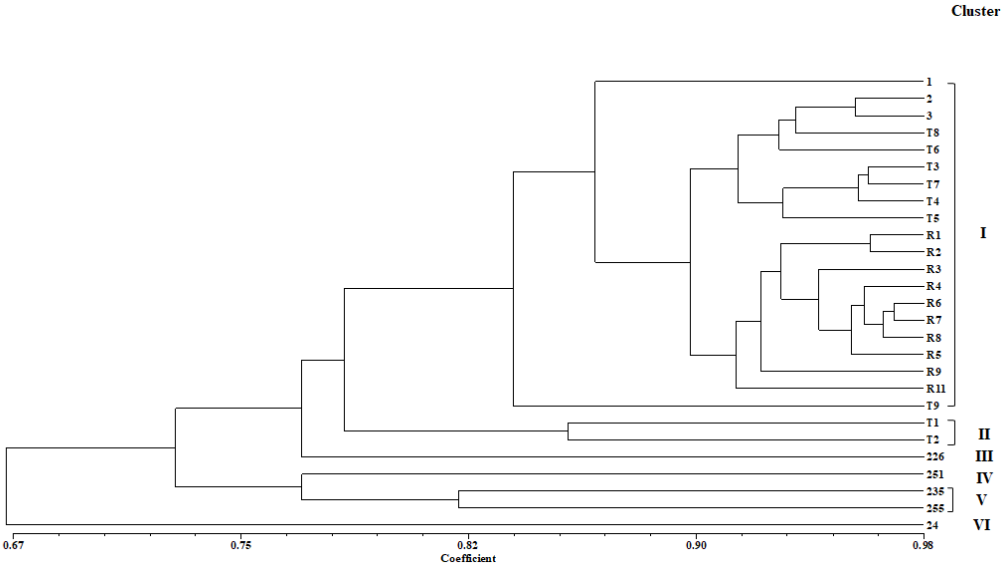

Figure 3. UPGMA (unweighted pair group method using arithmetic average) cluster analysis of para rubber (*Hevea brasiliensis*) based on RAPD method (30 primers) using NTSYSpc 2.1

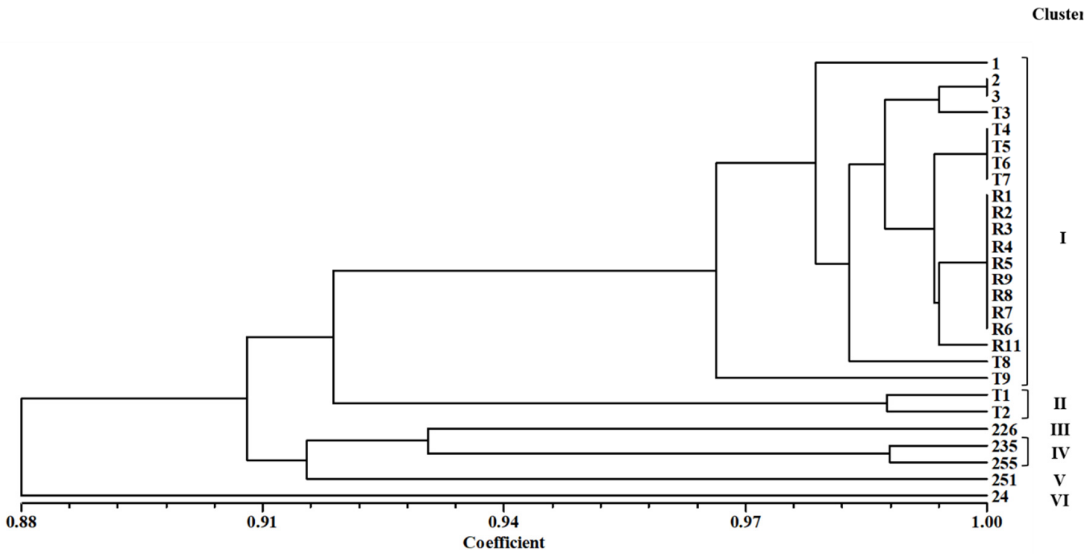

Figure 4. UPGMA (unweighted pair group method using arithmetic average) cluster analysis of para rubber (*Hevea brasiliensis*) based on SCoT method (15 primers) using NTSYSpc 2.1

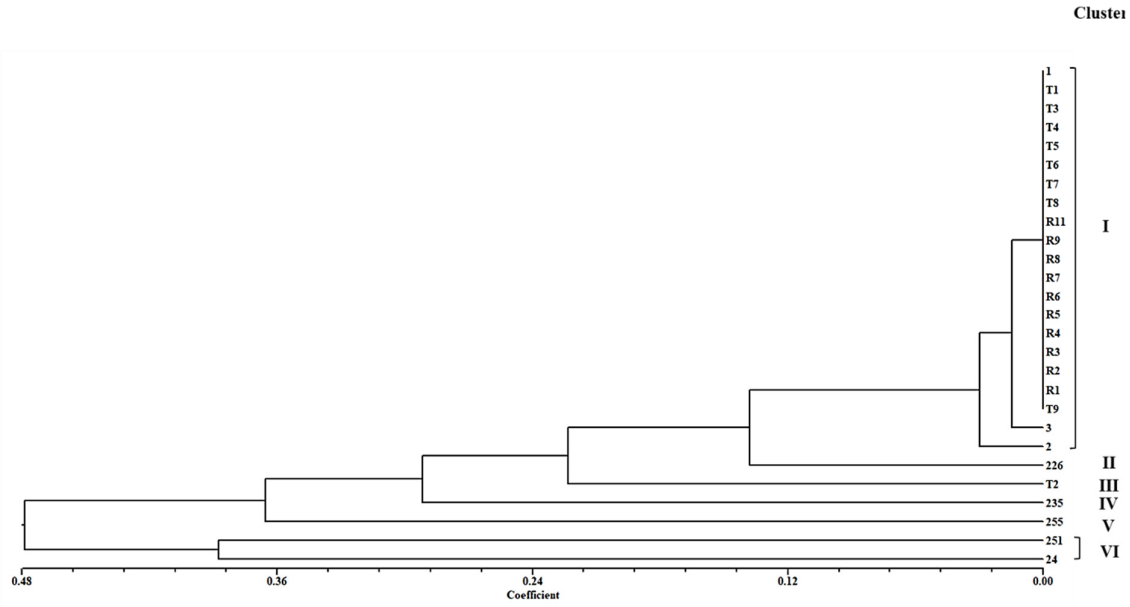

Figure 5. UPGMA (unweighted pair group method using arithmetic average) cluster analysis of para rubber (*Hevea brasiliensis*) based on SSRs microsatellite method (20 primers) using NTSYSpc 2.1

Discussion

Inner integuments of immature seeds (9-12-week-old after anthesis) of para rubber cvs. 'BT86', 'BT96' and 'EM96' have been successfully inoculated as initial explants on the MH-IN for callus induction, embryogenic callus induction, formation of somatic embryos and secondary somatic embryogenesis (Lardet *et al.*, 2008). In para rubber cv. 'PB260', inner integuments of 10-week-old immature fruits was initially inoculated as explants for callus induction [MS + 9 mM 2,4-dichlorophynoxy acetic acid (2,4-D) + 3.375 mM

Kinetin] and subsequently transferred to embryogenic callus induction and somatic embryogenesis (Modeste *et al.*, 2013). Sucrose is an alternative factor to regulate callus induction (111 mM) and embryogenic callus induction (234 mM) in para rubber cv. 'PB 260' (Blanc *et al.*, 2002; Modeste *et al.*, 2012). The other sources of explants, i.e., protoplast (cell suspension) (Sushamakumari *et al.*, 2000) and uninucleate anther (immature male flower bud) (Srichuay *et al.*, 2014; Zhao *et al.*, 2015; Wang *et al.*, 2017) have been successfully inoculated for somatic embryogenesis. Somaclonal variations depend on i) presence of strong auxins [2,4-D and 3,4-dichlorophenoxyacetic acid (3,4-D)] in inoculated medium for embryogenic callus and somatic embryogenesis induction (Modeste *et al.*, 2013), ii) genotypes/clones (Srichuay *et al.*, 2014), and iii) secondary somatic embryogenesis (subculture cycles) that ensures genetic fidelity in each plant derived from somatic embryogenesis (Wang *et al.*, 2017).

In para rubber, two RAPD makers, OPAA-07 and OPJ-19 were used to detect genetic viability of plant derived from zygotic polyembryony (Karumamkandathil *et al.*, 2015). In African hyacinth (*Ledebouria revoluta*), 17 RAPD markers were used to confirm the genetic stability in plants derived from somatic embryogenesis (Haque and Ghosh, 2016). Ten RAPD markers were used to validate the genetic stability in plants derived from indirect regeneration of *Rauwolfia tetraphylla* L. (Rohela *et al.*, 2019). Six selected lines of *Echinacea purpurea* derived from somatic embryogenesis were evaluated for their genetic stability using 10 RAPD markers (Lema-Rumińska *et al.*, 2019). In contrast, about 20-30% genetic variation using only one OPD-20 RAPD primer was detected in rough lemon (*Citrus jambhiri* Lush.) plantlets derived from somatic embryogenesis (Savita *et al.*, 2015). In *Withania coagulans*, 12 RAPD markers were used to confirm genetic stability in shoot regenerated plants derived from indirect organogenesis of leaf explants (Rathore *et al.*, 2016). Genetic homogeneity in micropropagated plants of *Pitosporumerio carpum* Royle was detected by 10 RAPD markers and the plantlets were clustered in the same group as that of donor mother plant (97% similarity) (Thakur *et al.*, 2016). Micropropagation via direct and indirect organogenesis in *Rumex nepalensis* has shown a less degree of variation within the clones when analyzed using 7 RAPD makers compared to 12 SCoT makers (Bhattacharyya *et al.*, 2017a). Moreover, RAPD marker is suggested to be suitable for identifying the diversity in genetic resources of para rubber (Venkatachalam *et al.*, 2004; Nakkanong *et al.*, 2008; Liyanage *et al.*, 2014).

This is the first report to apply SCoT markers for assessing genetic fidelity in para rubber micropropagation. In a previous study, high degree of genetic variation in plantlets of *Rumex nepalensis*, micropropagated via direct and indirect shoot organogenesis was detected by 12 SCoT primers, whereas it was undetected using 7 RAPD markers (Bhattacharyya *et al.*, 2017a). Genetic stability in 9 randomly propagated plants of *Bauhinia racemosa* using 18 SCoT makers was reported as true-to-type (Sharma *et al.*, 2019). Similarly, micropropagated plants free of somaclonal variations (100% similarity with mother plant), derived via indirect organogenesis were confirmed using SCoT markers in *Helicteres isora* (Muthukumar *et al.*, 2016), *Chrysanthemum morifolium* and *C. coccineum* (Nasri *et al.*, 2018) and *Saccharum officinarum* (Sathish *et al.*, 2018). Similarity value ranged from 0.89-1.00 in 9 individual plants derived from nodal segment micropropagation of *Pittosporum eriocarpum* tested using 10 SCoT primers (Thakur *et al.*, 2016). Moreover, genetic fidelity of plantlets derived from indirect organogenesis of *Rauwolfia tetraphylla* was confirmed using 10 SCoT primers as true-to-type without any polymorphism (Rohela *et al.*, 2019). Genetic similarity between somatic embryos and mother plants of *Coffea arabica* detected using 12 SCoT makers was found to be 0.987 (Bychappa *et al.*, 2019). In medicinal orchid (*Ansellia africana*), 16 reproducible SCoT primers were applied to identify the genetic stability in first, second, and third generation of subculture which ranged from 0.95 to 1.00 as per Jaccard's similarity matrix and were presented in the same cluster as that of mother plants as per UPGA cluster analysis (Bhattacharyya *et al.*, 2017b). Genetic stability of plantlets of *Morus alba* cv. 'Chinese', derived from indirect organogenesis was confirmed to be similar to the mother plant using 10 SCoT primers (Rohela *et al.*, 2020). In *Nardostachys jatamansi*, genetic variations in micropropagated plants was found to be dependent on the pathway of genesis of new plantlets as detected by 10 SCoT primers, with direct shoot organogenesis showing a variation of 4.38% from that of mother plant and indirect shoot organogenesis (showing a variation of 7.14% from that of mother plant) (Bose *et al.*, 2016). Moreover, genetic stability of

shoots proliferated from nodal segments of *Alhagi maurorum* was validated using 13 SCoT primers (Agarwal *et al.*, 2015).

In para rubber clone CATAS7-33-97, variation of regenerated plants (2.61% of mother plant) derived from secondary embryogenesis using 12 EST-SSR markers was observed (Wang *et al.*, 2017). Somaclonal variation in callus culture of para rubber was detected by hmct1 and hmc5 SSR primers, whereas these ignored the variations in somatic embryos from somatic embryogenesis (Srichuay and Te-chato, 2015). In addition, *in vitro* plantlets derived from nodal culture of para rubber were confirmed as true-to-type without somaclonal variation by 3 SSR primers (Sirisom and Te-chato, 2014). In guava, genetic homogeneity in plants derived from somatic embryogenesis was validated by 6 SSR markers (Rai *et al.*, 2012). Absence of somaclonal variations in the sesame plantlets, derived via indirect organogenesis was validated using 10 SSR markers (Anandan *et al.*, 2018). In contrast, somaclonal variations in somatic embryogenesis-regenerated Spanish grapevine (*Vitis vinifera*) cv. 'Torrontés' were detected in a mutant allele (231) instead of the normal allele (237) at the locus VVMD5 by SSR microsatellite analysis (Prado *et al.*, 2010). Moreover, somaclonal variations in olive plants derived from somatic embryogenesis was slightly higher in T1 lines (youngest lines; identified by lowest similarity coefficients) when compared with T2 and T4 based on SSR makers (Bradï *et al.*, 2019).

Conclusions

In the present study, somatic embryogenesis was successfully carried out from the inner integuments of 5-week-old immature para rubber fruits. The plantlets were subsequently acclimatized under in-vitro, greenhouse and field conditions. Genetic fidelity was confirmed using RAPD, SCoT and SSRs markers. It was concluded that the somaclonal variation is present only in one plant (T2) as detected by RAPD, SCoT and SSR markers (PCR-based rapid detection). Based on this investigation, a thorough research on the genetic variations in T2 plants compared to wild type 'RRIM 600' is suggested.

Authors' Contributions

Conceptualization, supervision, validation, visualization, writing - original draft, writing - review and editing: SC and WP; Methodology, data curation and analysis, RT, JM and TS.

All authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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