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ORIGINAL ARTICLE

Genetic and epigenetic uniformity of polyembryony derived multiple seedlings of *Hevea brasiliensis*

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Abstract *Hevea brasiliensis* Muell. Arg (Para rubber tree) is a tropical tree species of Amazonian origin widely cultivated in several parts of the world for natural rubber, a highly priced commodity inevitable for the world rubber industry. Large, tree to tree variation in growth and latex yield among individual plants of high yielding *Hevea* clones is a common phenomenon observed in mature rubber plantations. The genetic heterogeneity of the seedlings which are used as rootstocks for propagation through budgrafting is considered as a major factor responsible for this variation. In order to minimize this variation, attempts were made to develop highly uniform rootstock material via an in vitro technique by inducing zygotic polyembryony in *Hevea*. Immature open pollinated fruits of a high yielding clone RRII 105 were cultured by *half ovulo* embryo culture technique. Multiple embryos were induced from the 8–10-week-old zygote with a novel combination of gibberellic acid (GA_3), kinetin, and zeatin. Plantlets were successfully generated from the multiple embryos and raised in the field post hardening. Screening using genetic and epigenetic molecular markers revealed that the multiple seedlings developed are highly uniform and are of single zygotic origin. Development of plants having genetic and epigenetic uniformity suggests that this technique is ideal for raising uniform rootstock material in *Hevea* which may significantly reduce intraclonal variations. Moreover, these plants could serve as ideal material for physiological and molecular investigations towards the understanding of stock–scion interaction process in rubber.

Keywords Polyembryony · *Half ovulo* embryo culture · DNA methylation · Stock–scion interactions · *Hevea brasiliensis*

Abbreviations

CW	Coconut water
MSAP	Methylation-sensitive amplification polymorphism
BP	Banana powder
SV	Somaclonal variation
ME	Malt extract
CTAB	Cetyl trimethylammonium bromide
CH	Casein hydrolysate

Introduction

Hevea brasiliensis (Willd. ex A, Juss.) Muell. Arg. (Para rubber tree), a tropical tree species belonging to the family *Euphorbiaceae*, supplies the major chunk of natural rubber required for the world rubber industry. It is preferred over alternative sources, like Guayule, Russian dandelion, Panama rubber etc., due to the factors like abundance and high quality of the latex obtained, cheap harvesting and downstream processing methods and less labor intensiveness. In rubber, breeding is done with the major objective of increased latex yield, a character strongly associated with the scion. For maintaining this important trait commercial planting materials are raised through budgrafting technique where the desired scion is grafted on to assorted rootstocks grown from cross-pollinated seeds. Despite this vegetative mode of propagation, large tree to tree variation in growth and yield among bud grafted *Hevea* trees still exists (Combe 1975; Omokhafe 2004). Though factors like soil heterogeneity and GXE

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interactions are partly responsible for these variations, it is mainly attributed to the genetically divergent nature of the rootstocks used for propagation (Senanayake and Wijewantha 1968; Nayanakantha and Seneviratne 2007). As established by earlier studies, interaction of scion with the rootstock induces a series of responses widely known as stock–scion interactions leading to changes in the physiology, gene expression, and protein function parameters in plants (Koepke and Dhingra 2013). The extent of these interactions largely depends on the genetic divergence of the stock and scion materials used. Since the genetic base of each and every rootstock is different in rubber, it will be reflected in the stock–scion interaction process also, ultimately resulting in plant to plant variations for various characters. Studies using different graft combinations in deciduous orchard species revealed that scion–rootstock interaction influences water relations, leaf gas exchange, mineral uptake, plant size, blossoming, timing of fruit set, fruit quality, and yield efficiency (Gonçalves et al. 2005). Leaf peroxidase activity levels and tree stem growth in avocados have also indicated clear stock–scion interactions (Bower and Nel 1981). In the case of *H. brasiliensis*, the influence of rootstocks on the growth and yield of the scion was well demonstrated by Ng et al. (1981). Subsequent studies with respect to growth and rubber yield in *Hevea* by Cardinal et al. (2007) show that selection of rootstock material is vital for obtaining compatibility values for dry rubber yield. Evidence for this type of interactions at the protein level was provided by Almansa et al. (2002) in lemon tree leaves where they proved that the superoxide dismutase (SOD) activity in the leaves was rootstock controlled and varied more by rootstock than the salt levels tested when different rootstocks were used. Similarly, in rubber, Sobhana et al. (2001) identified clear isozyme polymorphism between different trees of the same clone, due to the variable rootstocks indicating the influence of stock–scion interactions at the protein level. All the above studies clearly indicate that rootstock plays a major role in intraclonal variation.

The key mode by which the rootstock controls growth and properties like protein activity in scion is through the modulation of scion gene expression using intermediary agents like signalling molecules, hormones, and epigenetic factors like small RNAs and DNA methylation. The signals emanating from the rootstock by the above modes have their impact on target genes resulting in the reprogramming of their expression profiles based on the site of action (Koepke and Dhingra 2013). This type of gene level influence of stock on scion in terms of expression was identified in model plants by Li et al. (2012). They reported that the expression of *PIN1*, a gene known to be involved in polar auxin transport, was far decreased in the trees when a dwarfing genotype was used as an interstock. Global gene expression studies using apple scions on different types of rootstock by Jensen et al. (2003) also demonstrated significant variations in expressions of several transcripts having potential roles in physiological differences in photosynthesis, tree size, stress tolerance, and flowering. Alternatively, RNA from the rootstock was also found being transported across the graft union and into the phloem of the scion by in situ hybridization technique. This indicates the possibility of epigenetic modifications like RNA-directed DNA methylation in the scion which may result in divergent phenotypic characters (Kanehira et al. 2010; Zhang et al. 2012).

All the above studies indicate that the rootstocks have significant impact on the scion and its phenotypic properties which may vary from plant to plant based on their genetic divergence. An alternative to minimize such variations arising during the propagation of rubber is to make use of uniform rootstocks. In rubber, tissue culture is the only viable method to generate large number of own rooted homogenous clonal material having genetic uniformity which can be used as rootstocks. However, plant regeneration through somatic embryogenesis is a major challenge in *Hevea* due to the recalcitrance of clonal explants, which limits the production of own rooted plants on a commercial scale. Moreover, the uniformity of the plants generated also cannot be assured as the chances of epigenetic somaclonal variants arising out of callus-mediated somatic embryogenesis is relatively high. A possible method to overcome these limitations is to explore more juvenile explants like zygote having higher potential to develop into a complete plant. Since evolvement of zygote to embryo under in vitro conditions does not require complicated intermediary steps as required in normal somatic embryogenesis process, the exposure to chemicals and reagents is significantly reduced thereby minimizing the chances of somaclonal variations arising due to epigenetic factors like DNA methylation.

The present study was conducted with the aim of developing uniform, true to type seedlings of *H. brasiliensis* by inducing zygotic cleavage polyembryony in immature fruits. We adopted a technique called *half ovulo* embryo culture where the ovule is dissected and cultured in a medium with an optimum combination of selected growth regulatory factors. Multiple embryos in significant numbers with better survival rate were successfully developed from a single zygote and were established in poly bags. Genetic and epigenetic uniformity of these plants were confirmed, and our results strongly suggest that after scaling up, this technique could serve as a viable option to generate large number of true to type *H. brasiliensis* rootstock material for various research and breeding activities in rubber.

Materials and methods

Explant inoculation

Immature open pollinated fruits (clone RRII 105) of different maturity (1–12 weeks old) were collected from the field. The fruits were washed thoroughly and immersed in 80 % alcohol for 10 min. The fruits were then allowed to dry on sterile Petri plates. Developing immature seeds were dissected from the fruits, and three types of inoculations were made. (1) The ovules were cultured intact on culture medium. (2) The ovules were cut transversely into two halves (half ovules), and the micropylar half was cultured in culture medium. (3) The ovules were cut into two halves longitudinally, and both the portions were cultured in the medium. The different types of inoculation methods followed are shown in Fig. 1. Fifty ovules (developing seeds) were kept in each treatment, and observations were recorded after 1 month.

Culture medium

Induction of multiple embryos

Nitsch basal medium reported earlier for the rescue of immature embryos (Rekha et al. 2010a, b) was used as the basal medium for all experiments in the present study. For the induction of multiple embryos, the basal medium was supplemented with different combinations of growth regulators. Combinations of gibberellic acid (GA_3) (1–5 mg/l) and kinetin (0.5–4 mg/l) were tried initially. Fifty ovules were kept per treatment, and each experiment was replicated five times. Observations on the number of embryos obtained from each ovule were recorded. Data were subjected to statistical analysis using ANOVA for CRD.

For the refinement of the media, different levels of sucrose (20–150 g/l) were tried in the basal medium along with best combination of GA_3 and kinetin. Observations on the number of ovules showing polyembryony and the number of embryos obtained per ovule were recorded. Data were subjected to square root transformation and analyzed. After optimizing the sucrose level, the effect of zeatin on induction of multiple embryos was studied with a view to improve the technique further. A concentration range of 0.1–0.5 mg/l of zeatin was tried along with the optimized concentrations of GA_3, kinetin, and sucrose. A mixture of organic supplements such as coconut water (CW) 20 %, banana powder (BP) 200 mg/l, and malt extract (ME) 100 mg/l was also added in the medium. All the cultures were kept in dark incubation until embryo maturation. Fifty ovules were kept in each combination, and observations on the number of ovules showing polyembryony and the

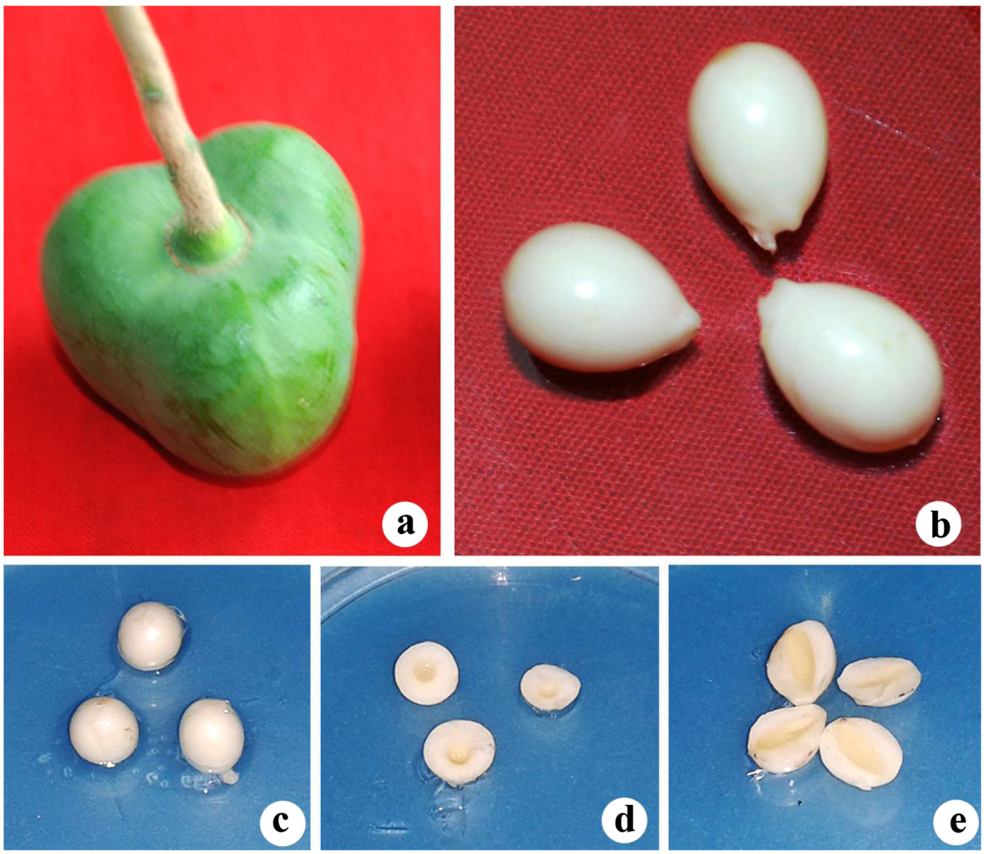

Fig. 1 Plate showing *H. brasiliensis* fruit, seed, and methods of inoculation. **a** Nine-week-old fruit of *H. brasiliensis*. **b** Immature seeds dissected from the surface-sterilized fruit. **c** Intact ovules in the culture medium. **d** Half ovules from transversely cut ovules with their micropylar end touching the culture medium. **e** Longitudinal halves of ovules in culture medium

number of embryos obtained per ovule were recorded. Data were subjected to square root transformation and analyzed as mentioned above.

Embryo maturation

Embryonic mass was separated from the half ovules and transferred to the culture medium for proliferation. For proliferation, the same media with 0.2 % charcoal was used. Embryos were separated and cultured individually for maturation as different batches consisting of 12 embryos each.

Hormone-free half strength MS medium fortified with organic supplements such as CW (20 %), BP (200 mg/l), and ME (100 mg/l) and casein hydrolysate (CH) (100 mg/l) was used for maturation. In the maturation medium, also 0.2 % charcoal was added. The cultures were dark incubated.

Germination and plant regeneration

For germination, the individual matured embryos were transferred to separate tubes having half strength Murashige and Skoog (MS) medium along with 0.3 mg/l benzyl adenine (BA) and 0.3 mg/l GA_3 and organic supplements as mentioned above. The pH of all the media prepared were adjusted to 5.7 with 1 N KOH and solidified with 0.2 % phytagel. Germination of the matured embryos was continued for 4 to 6 weeks in tubes. For germination, the embryos were incubated under 16-h photoperiod (all the chemical ingredients used except CW were from Sigma-Aldrich, India).

Acclimatization

The well-developed plantlets were transferred to small polythene bags filled with sand, soil, and soil rite mixture and kept in environmentally controlled growth chamber for 2–3 weeks, for hardening. Acclimatized plantlets were transferred to big poly bags and maintained in shade house till field planting. Three sets of plantlets derived from three independent ovules were established in the field.

Molecular marker analysis

DNA extraction and RAPD procedures

Genomic DNA was isolated from four 3-year-old plants from the first batch of polyembryony derived plants (1, 2, 3, and 4) of single zygotic origin derived from the clone RRII 105. Tender healthy leaves were used for DNA isolation following a modified CTAB method of Doyle and Doyle (1990). The DNA concentration and purity were determined spectrophotometrically. Integrity of DNA samples was also checked on 0.7 % agarose gel (Sigma). Working solutions of DNA stock for PCR were adjusted to 10 ng/μl and stored at 4 °C.

Two arbitrary decamer primers, OPAA7 and OPJ19 primers (Operon Technologies, Inc., USA), were used for this study. Amplifications were performed in a DNA Thermal Cycler (Gene Amp 9600 PCR system, Perklin Elmer, USA) in a reaction mix containing 50 ng of DNA template, 20 pmol of primer, 2 mM $MgCl_2$, 200 μM dNTPs, 0.7 units of Taq polymerase with 1X PCR buffer (Promega), in a final volume of 25 μl for each reaction. The PCR program consisted of an initial denaturation at 94 °C for 5 min, followed by 40 cycles of 30 s at 94 °C, 1 min at 36 °C and 2 min at 72 °C with a final extension for 10 min at 72 °C. The amplified products were analyzed by electrophoresis in 1.4 % agarose (Sigma-Aldrich, India) gels, stained with ethidium bromide (0.5 μg/ml of TAE buffer), and photographed using a Gel Documentation system (Eagle Eye II, Stratagene).

Genotyping with microsatellite markers

Three selected microsatellite loci, hmac13, hmac14, and hmac17, showing heterozygosity in the clone RRII 105 were used for genotyping the four plants derived through zygotic/cleavage polyembryony for discriminating them from their maternal plant RRII 105. The microsatellite primer pairs were the following: hmac13F:CATCCCTGCATTCCCCTAAT/hmac13R:ATGGGATGGGACCGTAACAA, hmac14F:CTCCACTTGTGCAAGCGTTC/-hmac14R:GCAATCAAACGTGCCAAGAA and hmac17F:CGCTGTGCCTTGTTGATCTT/hmac17R: CACTGGCACGTGAATCCCTA. PCR reaction was carried out in a 10-μl final volume containing 20 ng of genomic DNA, 0.2 μM each of the forward and reverse primers, 200 μM dNTPs and 0.7 units of AmpliTaq Gold polymerase along with the buffer supplied by Applied Biosystems. The temperature cycle profile involved an initial denaturation step of 5 min at 95 °C followed by a touchdown PCR program. Temperature profiles of the touchdown PCR for 7 cycles were as follows: 94 °C for 30 s, 63 °C for 1 min, Δ↓ 1 °C for 7 cycles, 72 °C for 1 min. This was followed by a normal cycling of 94 °C for 30 s, 56 °C for 1 min, 72 °C for 1 min for 23 cycles, and a final extension at 72 °C for 10 min. After completion of PCR, the reactions were stopped by the addition of 10-μl formamide loading buffer. Amplification products were run on a 6 % denaturing polyacrylamide gel containing 7 M urea using 0.6X TBE buffer at a constant power of 55 W. The gels were then silver stained following the procedure of Roy et al. (2012).

Methylation-sensitive amplification polymorphism (MSAP)

In this modified amplified fragment length polymorphism (AFLP) protocol by Xu et al. (2000), the frequent cutting endonuclease *Mse*I was replaced by the two isoschizomeric restriction enzymes *Hpa*II and *Msp*I having different

sensitivity to the methylation state of the symmetric sequence CCGG. To detect MSAP, two reactions were set up at the same time. In the first reaction, 1 μg of genomic DNA of the four polyembryony derived plants was digested with 10 U of *Eco*RI plus 10 U of *Msp*I in a final volume of 50 μl containing 50 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, and 50 mM NaCl by incubating overnight at 37 °C. The second digestion reaction was carried out as above with the exception that *Hpa*II was used in place of *Msp*I. The digested fragments were then ligated to the adaptor by adding 10 U T4 DNA ligase, 5 pmol *Eco*RI adaptor (5′-CTCGTAGACTGCGTACC-3′/3′-CATC TGACGCATTGGTTAA-5′), and 50 pmol *Msp*I-*Hpa*II adaptor (5′-GACGATGAGTCTAGAA-3′/3′-CTACTCAGATCT TGC-5′) and incubated overnight at 16 °C. The reactions were stopped by incubating at 65 °C for 10 min. Preamplification reactions were performed by using 5 μl of the ligated product with 75 ng *Eco*RI primer with one selective base (E+A primer: 5′-GACTGCGTACCATTCA-3′), 75 ng *Msp*I-*Hpa*II primer with one selective base (Met+T primer: 5′-ACGATG AGTCTAGAACGGT-3′) in a volume of 50-μl polymerase chain reaction (PCR) mixture. The reaction conditions were 20 cycles of 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 2 min. Preamplified mixtures were diluted 1:50 from their original volume with sterile Milli Q water. Selective amplifications were conducted in a volume of 20 μl, containing 5 μl of the diluted preamplification product, 30 ng of *Eco*RI primer with two selective bases (E+AG/E+GT/E+GC/E+GA/E+ GG), 10 ng *Msp*I-*Hpa*II primer with two selective bases (Met+TAC/Met+TAG). Primer combinations used are listed in Table 1. The reaction mixtures were amplified for 12 cycles at 94 °C for 30 s, 65 °C for 1 min, Δ↓ 1 °C for 12 cycles, 72 °C for 1 min. This was followed by a normal cycling of 94 °C for 30 s, 56 °C for 1 min, 72 °C for 1 min for 35 cycles, and a final extension at 72 °C for 10 min. The selectively amplified products were mixed with an equal volume of formamide gel loading buffer and denatured at 95 °C for 5 min. Three microliters of each product was electrophoresed on 6 % (*w*/*v*) denaturing polyacrylamide gel containing 7 M urea and 1× TBE. Gels were run at 1,200 V for 4 h and stained by the silver staining method.

Table 1 Combination of primers used for MSAP analysis

Combination	Primer EcoR1+ A	Primer Met+T
1	EC-7 E+AAG	Met−+TAC
2	EC-7 E+AAG	Met−+TAG
3	C17 E+AGT	Met−+TAC
4	C17 E+AGT	Met−+TAG
5	C18 E+AGC	Met−+TAG
6	C19 E+AGA	Met−+TAC
7	C19 E+AGA	Met−+TAG
8	C20 E+AGG	Met−+TAC
9	C20 E+AGG	Met−+TAG

Results

Effect of inoculation method and explant maturity

Three different inoculation strategies were attempted to identify the right method of polyembryony induction. Among the three, the one with intact ovule dried up within few days. Of the two types of cuts, *half ovulo* culture method with transversely cut ovules having their micropylar end touching the medium generated multiple embryos (Fig. 2a–c). The number of embryos per ovule from the initial cultures ranged from 2 to 12 which could be raised to >60 in subsequent experiments by manipulating sucrose level and growth regulator concentrations in the medium. The multiple embryos obtained were successfully recovered from the culture medium. In the case of the longitudinal cut halves, no growth was observed.

Among the fruits of different maturity, polyembryony was obtained only from 8- to 10-week-old fruits by *half ovulo* culture method (Fig. 2a–c). Fruits of 5–8-week maturity and those older than 10 weeks yielded single viable embryo by *half ovulo* culture method. Fruits of age less than 5 weeks yielded no embryogenesis by all the three methods mentioned above. Therefore, further experiments were continued adopting the *half ovulo* culture technique using 8–10-week-old fruits as explant.

Effect of culture medium

The concentration of carbohydrate source and growth regulators in the culture media plays a crucial role in the in vitro induction of polyembryony in any plant species. Therefore, attempts were made to identify the optimum concentration of these components for polyembryony induction in *H. brasiliensis*

Polyembryony could be successfully induced in *Hevea* with an optimized growth regulator combination of GA_3 and kinetin. Table 2 shows the effect of GA_3 and kinetin on the induction of polyembryony. The number of embryos per ovule increased with increase in concentration of growth regulators. Treatment T_3 with a combination of 3 mg/l GA_3 and 2 mg/l kinetin yielded significantly high number of embryos compared to other combinations. Further increase in growth regulator concentrations resulted in a reduction in the number of embryos.

Among the different levels of sucrose tried, maximum number of embryos from a single zygote could be obtained when the medium was supplemented with 100 g/l sucrose. The effect of sucrose on induction of multiple embryos is represented in Table 3. The number of embryos/ovule

Fig. 2 Plate showing multiple embryo induction, germination, and acclimatized rubber plants. **a**, **b**, **c** Different developmental stages of multiple embryos inside the ovule. **d** Four-week-old germinating individual embryos, isolated from the proliferated embryonic mass. **e** One-year-old uniform polyembryony derived seedlings in polybags. **f** Three-year-old polyembryony derived plants in the field

increased gradually from 4 to 31 with subsequent increase in sucrose concentration up to 100 g/l. Moreover, 8 % of the cultured ovules showed induction of multiple embryos. Concentration of sucrose beyond 100 g/l resulted in a reduction in the number of embryos obtained.

Table 2 Effect of growth regulators on the induction of multiple embryos in *H. brasiliensis*

Treatments	GA_3 (mg/l)	Kin (mg/l)	Number of embryos obtained per ovule
T1	1	0.5	2.66
T2	2	1	4.16
T3	**3**	**2**	**12.83**
T4	4	3	1.33
T5	5	4	1.16
		CD (0.5)	0.86

Fifty ovules were inoculated in each combination, and the number of embryos generated per ovule was recorded.

The results of the experiment with different concentrations of zeatin with the best combination of sucrose and other growth regulators are shown in Table 4. A gradual increase in the number of ovules showing polyembryony and the number of embryos per ovule was noticed with increase in concentration of zeatin up to 0.3 mg/l. Further increase in concentration resulted in low number of embryos as well as the number of polyembryonic ovules. From the given data, it is evident that the above combination of GA_3 and kinetin with a novel combination of 0.3 mg/l zeatin in presence of 100 g/l sucrose enhanced the number of embryos to almost five times than that obtained in the initial culture. It was observed that 0.3 mg/l zeatin, supplied along with the above concentrations of GA_3 and kinetin at a higher sucrose level, has a positive

Table 3 Influence of sucrose on the induction of multiple embryos from cultured fruits of *H. brasiliensis*

Sucrose (g/l)	Percentage of ovules with multiple embryo induction[a]	Highest number of embryos obtained per ovule
20	4.4 (2.08)	5.2 (2.27)
50	5.4 (2.31)	6.6 (2.56)
75	6.4 (2.52)	13.2 (3.53)
100	**8.0 (2.83)**	**31.4 (60)**
150	7.4(2.70)	5.8 (2.40)
CD (0.5)	0.33	0.28

[a] Fifty ovules were kept in each combination, and the percentage of ovules showing multiple embryos as well as the highest number of embryos obtained per ovule in each combination were recorded. Data were subjected to square root transformation, and transformed values are given in parenthesis

influence on increasing the number of induced embryos significantly. With the above combination, the number of embryos from a single ovule could be enhanced up to 60 with the addition of zeatin in the culture medium which is noteworthy and not reported elsewhere. Since all the other organic supplements used were taken as per the standardizations done previously from our own lab, their impact was not studied separately in the present study (Rekha et al. 2010a, b; Jayashree et al. 2003; Venkatachalam et al. 2007).

Incubation conditions

Based on the culture responses observed, dark incubation was found to be ideal till embryo maturation stage. Light inhibited the development of embryos, and no embryos under light incubation could be matured and regenerated into plantlets. Matured embryos were kept in 16-h photoperiod for germination. When the embryos were light incubated during their early stages of development, they turned green and remain stunted without further growth and enlargement.

Table 4 Effect of different levels of zeatin on the induction of multiple embryos

With 3 mg/l GA_3 and 2 mg/l kinetin	Zeatin (mg/l)	Percentage of ovule showing multiple embryo induction	Highest number of embryos obtained
T1	0.1	4.8 (2.18)	12.2 (3.48)
T2	0.2	5.8 (2.40)	14.3 (3.85)
T3	**0.3**	12.4 (3.52)	**60.4(7.77)**
T4	0.4	8.2 (2.86)	20.2 (4.49)
T5	0.5	4.8 (2.18)	15.2 (19.49)

Fifty ovules were kept per treatment, and the percentage of ovules showing multiple embryos as well as the highest number of embryos per ovule in each combination was recorded. Values were subjected to square root transformation, and transformed values are given in parenthesis.

Embryo development, plant regeneration, and acclimatization

The embryos were separated individually from the embryonic clumps and cultured for maturation in the maturation medium devoid of growth regulators. The embryos enlarged in size, and the cotyledons turned ivory colored and opaque. Maturation of the embryos needed 4 to 6 weeks under dark incubation. After 4–6 weeks, they were transferred to germination medium. Plantlets were raised from the multiple embryos followed by acclimatization in growth chamber (Fig. 2d–f). Three sets of plantlets derived from three independent ovules were successfully established in the field.

Molecular marker analysis

RAPD and microsatellite analysis were performed to assess the genetic variability/similarity of the four plants derived through zygotic polyembryony and to establish their genetic relatedness with their maternal parent RRII 105. The RAPD profiles of the poly embryony derived plants were found to be different from that of their female parent RRII 105, for both the primers tested. At the same time, no variations were noted among the four polyembryony derived plants for both the primers tested (Fig. 3). Microsatellite analysis using three heterozygous marker yielded similar results as that of the RAPD results mentioned above. All the three markers showed similar allelic segregation in the four polyembryony derived plants whereas the maternal parent RRII 105 exhibited different allelic combination. As expected, one allele from the maternal parent was shared among all the four polyembryony derived plants whereas the second maternal allele was absent in all the polyembryony derived plants. Instead, a unique

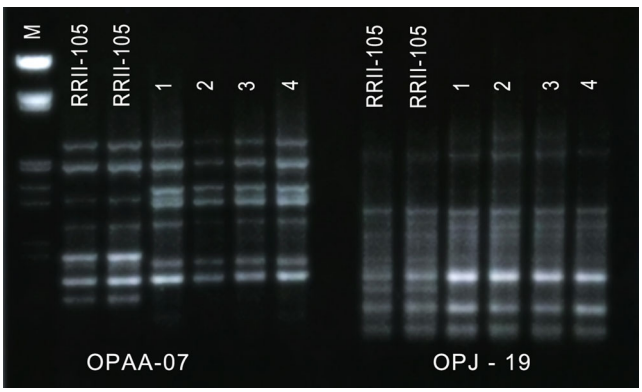

Fig. 3 RAPD profiles of polyembryony derived plants. The banding pattern of the maternal parent RRII-105 is different from that of the four polyembryony derived plants for both the primers (OPAA-07 and OPJ-19). No variations could be detected among the four plants

second allele was observed in all the four plants, which may be derived from their unknown male parent (Fig. 4).

Epigenetic marker analysis

Epigenetic similarity/dissimilarity among the four plants was determined by assessing their global genomic DNA methylation pattern using the MSAP technique. All the nine primer combinations analyzed showed clear variation in the banding pattern between the *Msp*I and *Hpa*II digests. The presence of more number of bands in the *Msp*I digest set compared to *Hpa*II set indicates the presence of methylated regions in *Hevea* genome since the methylation-insensitive *Msp*I will cleave the DNA, irrespective of the methylation status of the recognition site generating more number of fragments while the methylation-sensitive *Hpa*II cleaves at nonmethylated sites only. Notable variations were not observed among the four plants in the *Msp*I as well as *HpaII* digest sets for all the primer combinations analyzed (Fig. 5).

Discussion

Heterogeneity in phenotypic characters is a common phenomenon observed by breeders in all plant species where bud grafting technique is adopted as the propagation technique. Though these variations are attributed to several internal and external factors, genetic heterogeneity of the rootstocks is considered as one of the major contributor. It is presumed that

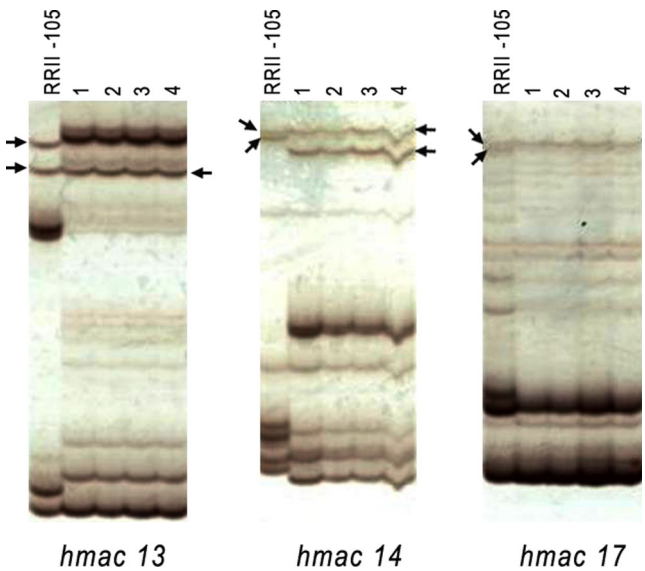

Fig. 4 Microsatellite profiling of polyembryony derived plants. Allelic segregation can be seen in all the four polyembryony derived plants proving their zygotic origin. The maternal parent RRII 105 shows a different allelic combination. A unique allele derived from the paternal parent is also visible which shows their hybrid nature (*arrowheads* indicate alleles)

a series of responses called stock–scion interactions are triggered as a result of the grafting procedure when a genetically diverse stock and scion are joined together to form a single plantlet. The impact of these interactions on several aspects of plant growth and development is an area under extensive investigation for the past many years (Rogers and Beakbane 1957; Webster 2004; Martinez-Ballesta et al. 2010; Koepke and Dhingra 2013). In the case of an out-breeding tree species like rubber, the impact of these interactions is well apparent by the intraclonal variations observed in latex yield (Cardinal et al. 2007). Since rubber is an economically important crop where the major agronomic trait of interest is latex yield from the bark, plant to plant intraclonal variation may have huge impact on the productivity of plantations. Moreover, the above interactions may greatly affect the consistency of newly developed high yielding clones as grafting is the sole method of their propagation. As mentioned earlier, the genetically divergent rootstocks raised out of open pollinated seeds may be a major contributing factor for the above incongruity. Therefore, it is imperative to develop strategies to generate true to type rootstocks for the preservation cum maintenance of important characters like yield and to bring about consistency in latex production in rubber.

Though conventional clonal propagation methods like cutting and layering are widely being practiced in plants like avocado, apple, walnut etc., the success rate of these techniques is very stumpy in *Hevea* due to various physiological aspects. At this juncture, in vitro propagation technique seems to be the best viable option. But, the occurrence of somaclonal variants arising out of conventional in vitro plant propagation techniques is another bottleneck in maintaining cent percent uniformity among such plants. In this respect, earlier studies showed that culture condition, callus phase, duration of the nonmorphogenic stage, and the growth regulators are some of the important factors influencing the induction of somaclonal variations in the in vitro generated plants (Shepard et al. 1980; Hartman et al. 1984; Evans et al. 1987). Later studies further established that the use of explants from highly differentiated tissues such as roots, leaves, and stems resulted in a higher frequency of variants when compared to more juvenile explants like axillary buds, shoot tips, and meristems (Duncan 1997). One of the major reason for the low-frequency occurrence of SV in juvenile tissues is the high genetic homogeneity and the lack of preexisting genetic variation within the explant tissue. As conventional micropropagation techniques using somatic explants require the artificial induction of embryogenic competence in cells which are not naturally embryogenic, the influence of the above factors will be more in somatic embryogenesis process thereby increasing the possibility of generating more somaclonal variants (Dodeman et al. 1997). An alternative method is the zygotic embryogenesis technique where the above effects will be negligible because the explant zygote is intrinsically embryogenic. In short, the zygote shows

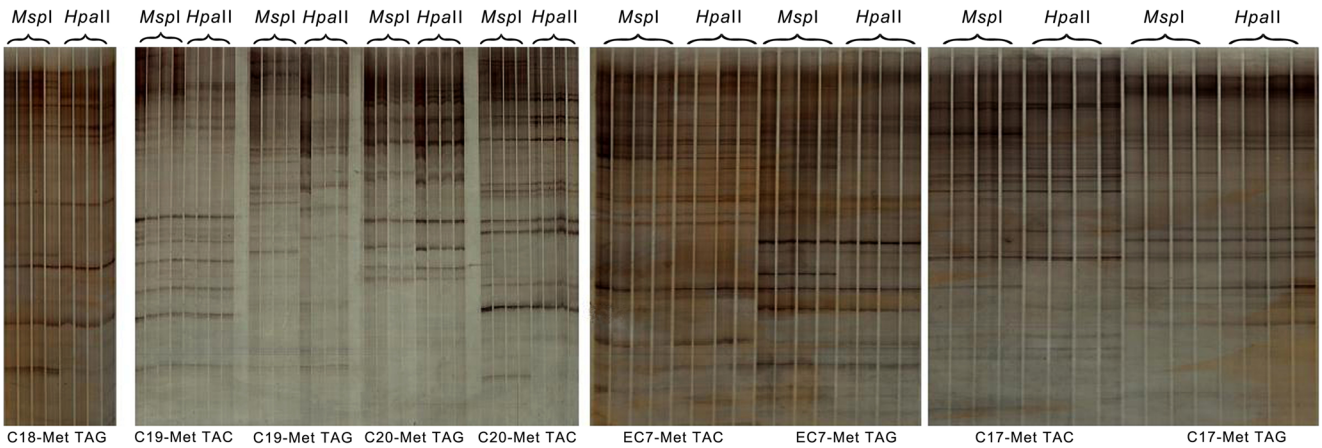

Fig. 5 Epigenetic profiling by MSAP analysis. Methylation-sensitive AFLP results using nine different primer combinations. Variations in banding pattern between the *Msp*I set and *Hpa*II set indicate the presence of methylated regions in *Hevea* genome. Lack of variations among the four plants within the same set indicates the absence of genetic and epigenetic (methylation) variation in polyembryony derived plants

some structural and functional characteristics which are intimately linked with the formation of the first embryonic developmental stage with many of the genes required for the induction process already expressed. Due to this embryonic potentiality of the cells, their fate is already committed and does not need to be redirected toward a new developmental path. Considering the above facts, it is assumed that the chance induction of somaclonal variation is more in the somatic embryogenesis process than that in zygotic embryogenesis. Therefore, instead of adopting conventional micropopagation strategies using recaltricant somatic explants so far used in rubber, an alternate method using a more juvenile explant like zygote with modifications in the inoculation method and media composition was attempted in the present study to develop true to type multiple seedlings in rubber.

Effect of method of inoculation

Embryo culture is a popular invitro technique for embryo rescue being practiced for more than half a century to save the hybrid products of fertilization from degeneration. It was in practice since early 1940s to understand the physiochemical requirements for embryonic development, overcome seed dormancy, reduce breeding cycle, seed viability analysis, supply material for micropropagation, and rescue immature hybrid embryos from incompatible crosses (Hu and Wang 1986). *In ovulo* embryo culture is a modification of embryo culture where the embryo is cultured while still inside the ovule so that the damage to embryo during the excision process is avoided. Since the embryo which is inside the ovule being the only functional live tissue left, the chances of its growth getting triggered in the presence of ambient nutritional and hormonal supply are very high. A modified version of the *in ovulo* embryo culture called half *ovulo* culture was adopted in the present study to develop true to type multiple embryos from a single zygote avoiding the chances of apomixis. Although this type of *in ovulo* embryo culture has been successfully carried out in commercially important plants like cotton and Peruvian lily to rescue hybrid embryos, they have not yet successfully established in any tree species except *H. brasiliensis* where it was used for rescuing immature embryos (Stewart and Hsu 1978; Buitendijk et al. 1995; Rekha et al. 2010a). Therefore, the present study forms the first attempt of its kind to make use of this technique in tree species to induce polyembryony for generating true to type plants.

Among the different culture techniques tried in the present study to induce polyembryony, *half ovulo* embryo culture proved to be successful in inducing polyembryony. The removal of the upper portion of the ovule by the transverse cut aided in the triggering of growth due to proper aeration as well as direct contact of nutrients to the embryo tissue. On the contrary, there exists no direct contact between the embryo and the culture medium in the case of intact ovule where the embryo is embedded inside the ovule (Reed 2005). According to Batygina (1979, 1989) the disturbances of integration and organization of the embryo and surrounding structures, as well as morphogenetic and morphophysiological correlations, are some of the reasons for the transition of embryos into a vegetative phase leading to the development of multiple embryos. The externally supplied growth regulators may further stimulate this process. Contrary to the culture response of *half ovulo* culture, the ovule will try to develop into full maturity when intact ovules were cultured. However, the media supplements given may not be sufficient enough to support the growth of the whole ovule resulting in the gradual death of the ovular tissue. Furthermore, senescence signal from the ovular tissue may also reach the embryo leading to its death. This may be the reasons for the failure of intact ovule culturing

method. In the case of the longitudinal halves, damage to the embryo may be the reason for the absence of embryo growth as the chances of injury during longitudinal cut are more than that in transverse cut which is evident from Fig. 1d, e.

Effect of age of the fruits

Age of the fruit influencing culture response is an established phenomenon in many plant species. As reported by Sharma et al. (1996), successful production of plants from the cultured embryos largely depends upon the maturation stage and the composition of the medium. In *Hevea*, the growth of the embryo, seed, and the fruit follows a definite pattern. The fruit walls enlarges first and after attaining the maximum size, the seeds starts growing and only during last stage of development, the zygote will develop into an embryo which attains maturity only after 14th week of pollination. Previous in vitro regeneration studies in *Hevea* based on this information proved that the fruit age significantly influenced plant regeneration from rescued immature embryos and the percentage of plant recovery increased with the age of the fruits (Rekha et al. 2010a). The present study revealed that fruits of the age 8–10 weeks were ideal for the induction of multiple embryos as they are the most actively dividing stage of the pre-embryonic cells. Based on the observations made from the current study, it is assumed that 8–10 weeks may be the optimum maturity for initiation of zygotic division which leads to cleavage of the zygote yielding multiple embryos.

Effect of culture media

Carbohydrate type and concentration have been found to play important role in different stages of the embryogenesis process (Reed 2005; Gerdakaneh 2009). Of the different carbohydrate sources available, sucrose is considered as the standard carbon and energy source in majority of media used in plant tissue culture. The significance of carbohydrate source and its concentration for induction of embryonic callus, embryo development, and regeneration is well established by various studies in different plant species (Karami et al. 2006; Vengadesan 2002; Gerdakaneh et al. 2009). But, no studies have been reported so far regarding the possibilities of using different concentrations of sucrose for inducing multiple embryos. In the present study, a high concentration of sucrose (100 g/l) was found to be ideal for increasing the number of embryos developed from a single zygote of *H. brasiliensis*. After the cleavage of zygotic embryo, the enhanced availability of carbohydrates along with growth hormones may have influenced the multiplication of cells and contributed toward embryo proliferation. The above results indicate that the concentration of carbon source strongly influenced the induction of polyembryony in *Hevea*.

Growth regulating hormones are critical factors which influence the induction and further development of multiple embryos. Previous studies in *Vitis* states that growth hormones play a significant role in the induction of polyembryony (Tsolova and Atanassov 1994). Additionally, experiments on *Eranthis hiemalis* by Haccius (1955) proved that in the undifferentiated embryos, differentiation was transiently arrested in the presence of 2,4-phenoxyacetic acid, without compromising on the high mitotic activity in the tissues. Moreover, upon removal of the inhibitory agent, differentiation was resumed followed by initiation of the development of an adventive embryo to form a twin. They also reported that treatment of such embryos with citrate phosphate buffer at pH 3.5–4.5 suppressed the meristematic activity of the embryo cells, while the suspensor cells remained viable and started active division to form normal or abnormal embryos. Similar experiments with 2,4-D in plants like Datura, *Triticum*, and *Zea* resulted in the formation of twins by splitting of the embryo (Sanders 1950; Ferguson et al. 1979; Erdelska and Vidovencova 1992). Polyembryony was also reported to be induced in fertilized ovules of *Ribes rubrum* when cultured on a medium containing kinetin where few of the embryoids either developed directly into plantlets or formed embryonic masses (Zatykó et al. 1975). In the fertilized ovules of black current cultivar, polyembryony was induced in Millers medium. After transferring to a medium containing GA_3 but free from auxins and cytokinins, most of the embryoids developed directly into plantlets while the rest proliferated further and formed embryonic masses (Zatykó 1981). Similarly a 4–5 times increase in the vitamin and hormone level in the induction medium also may increase the percentage of polyembryogenic clusters significantly in *Vitis* as per the reports of Tsolova and Atanassov (1994). Additionally, elevated levels of ascorbic acid have also proven to promote polyembryony in tobacco (Chen and Gallie 2012). All the above studies support our assumption that the novel combination of GA_3, kinetin, and zeatin described in the present study was responsible for the significant increase in the number of embryos obtained by multiple embryo induction in rubber.

Effect of incubation conditions

Significance of light on shoot/bud initiation and embryo maturation under in vitro conditions is a well-studied area (Biondi and Thorpe 1982; Villalobos et al. 1984). The significance of dark incubation of the cultures till embryo maturation for successful plant regeneration is also well established. For example, in Floribunda rose, higher rate of germination was reported in cultures incubated for 2 weeks in dark and subsequently transferred to light at 16-h photoperiod for 2 weeks (Mohapatra and Rout 2005). Similar results were reported in *Hevea* by Das et al. (2003) where they have established that dark incubation of cultures for 2 weeks was optimum for multiple shoot induction from zygotic embryos. The failure of *Hevea* embryos to regenerate into plantlets under light

incubation in the present study is justified based on the above reports and further compliment the argument that dark incubation is ideal for embryo maturation and plant regeneration in *Hevea*.

Plant regeneration

Zygotic embryos are known to possess higher regenerative capacity compared to somatic embryos irrespective of the culture medium used. High survival rate of the zygotic embryos from our study compared to earlier somatic embryogenesis studies is well in agreement with this statement. In the present study a medium reported earlier by Rekha et al. (2010a) was used for the regeneration of plants from the individual embryos isolated from the polyembryonic mass. Similarly, the acclimatization process was also easy for these plants since they showed better rooting than usually observed in somatic embryo derived plants. The survival during hardening process was also high compared to somatic embryo derived plants. The successful establishment of plants in the field indicates that the use of most juvenile zygote as explant resulted in minimum causalities during the hardening stage of seedlings.

Molecular genetic analysis

Polyembryony can be defined as the development of two or more embryos within an ovule. There are different types of polyembryony such as formation of adventive embryos as a result of fusion of two or more ovules, development of several embryo sacs in the same ovule, those originating from a single zygote, and those resulting from proembryo division (Braun 1860). The identification of zygotic and nucellar seedlings arising out of any of the above modes by traditional means at an early stage is a major obstacle faced by breeders in most plant species. But, molecular genetic markers has served as a very reliable technique to prove the uniformity and single zygotic origin of the multiple embryos developed from a single ovule (Williams et al. 1990; Abirami et al. 2008; Ercan et al. 2013).

The present study made use of molecular genetic markers like microsatellites and RAPDs to prove the efficiency of the *half ovulo* embryo culture in developing true to type multiple embryos in rubber. The above markers were also used to distinguish the monozygotic multiple embryos, from their maternal parent and somatic embryos developed from the parental tissue. The RAPD profiles proved beyond doubt that the four polyembryony derived plants were different from their maternal parent RRII 105. This variation aroused due to the hybrid origin of the four progenies arising from a cross between RRII 105 and an unknown male parent. At the same time, the similar allelic segregation pattern displayed by all polyembryony derived plants may be attributed to their single zygotic origin. Similarly, microsatellite analysis using markers showing heterozygosity in parental clone RRII 105 also established the single zygotic origin of all the four plants, which was the product of a cross-pollination as evidenced through the allelic profiles of the locus *hmac* 13, 14, and 17. The presence of a unique allele derived from their unknown paternal parent further ascertained the hybrid nature of these plants (Fig. 4).

Epigenetic marker analysis

The term "somaclonal variation" (SV) describes the tissue culture-induced stable genetic, epigenetic, or phenotypic variation in clonally propagated plant populations (Larkin and Scowcroft 1981). Somaclonal variation is considered to be one of the main bottlenecks in the development of micropropagation procedures, especially in view of large-scale commercial operations where strict maintenance of genetic and agronomic traits from selected individuals is required. The rates of SV in regenerated plants arise at greater frequencies than attributable to spontaneous mutations (Duncan 1997). SV that specifically affects phenotypic traits varies in their frequency depending on the species, cultivars, and micropropagation systems. For example, analysis of a progeny comprising of phenotypic variants showed that some of the variations produced by somatic embryogenesis can occur in the form of stable and heritable mutations (Karp 1994; Kaeppler et al. 2000). In maize, Kaeppler and Phillips (1993) reported stable segregation of somaclonal variant phenotypic qualities in several seed generations. Though the frequency of incidence of somaclones due to genome sequence variation is less in regenerated plants, it is very high in terms of epigenetic modifications, either in the form of transient mutations or as heritable mutations (Kaeppler et al. 2000; Miguel and Marum 2011).

Epigenetic traits are heritable changes associated with chemical modification of DNA without altering the primary DNA sequence. Cytosine methylation is one of the major epigenetic modification in plants leading to SV during in vitro plant propagation procedure (Kaeppler et al. 2000; Kaeppler and Phillips 1993). DNA methylation in plants commonly occurs at cytosine (5-methylcytosine, m5C) bases in all sequence contexts: the symmetric CG and CHG (in which H could be A, T, or C) and the asymmetric CHH contexts. Depending on the site as well as type of tissue it occurs, DNA methylation can mediate the transmission of an active or silent gene either for short-term during mitosis or for long-term across generations during meiosis (Saze 2008). Therefore, phenotypic changes induced by variations in DNA methylation patterns may either be transient or heritable in nature depending on the type of tissue they occur. DNA methylation variations in callus-derived somatic embryos are reported from many plant species, and they are often found to

be heritable. For example, the existence of zones susceptible to methylation variations were recently shown in somatic embryogenesis-derived plants (emblings) in grapevine and barley (Schellenbaum et al. 2008; Bednarek et al. 2007). It is understood that methylation variations are induced in the somatic embryonic tissue mainly by the chemical environment confronted by it during the in vitro culturing process. During in vitro regeneration via somatic embryogenesis, a significant alteration or resetting in the global methylation level of genomic DNA occurs in response to the culture conditions like explant age and type, duration and incubation conditions, growth regulator type and concentration etc. (LoSchiavo et al. 1989). For example, Etienne et al. (2012) showed that global methylation levels decreased with increasing concentration of kinetin but increased with increasing amounts of the auxin 2,4-D. The influence of the number and duration of subcultures especially in cell suspension and callus cultures on the rate of variation was also established by Bairu et al. (2006). Contrary to the normal techniques for plant regeneration by somatic embryogenesis, polyembryony derived plants of zygotic origin demands few culturing steps due to the embryonic potential of zygotic tissues. The reduction in tissue culture steps leads to less exposure of these tissues to chemical inducers and media components thereby reducing the chance of evolvement of DNA methylation-mediated epigenetic changes through this procedure.

Conventional molecular markers based on sequence polymorphisms will not be able to detect methylation changes that exist at the genomic level because DNA methylation per se will not induce any nucleotide variation. In this context, approaches like methylation-sensitive amplified polymorphism (MSAP) proved handy in the analysis of methylation pattern variations in a global level (Rodrigues 2013; Bednarek et al. 2007). In the present study, absence of genetic variability among the four polyembryony derived plants was well established by RAPD as well as microsatellite marker analysis. The lack of variation in the *Msp*I digests in all primer combinations of the MSAP further corroborates the above results as this is equivalent to a normal AFLP analysis based on sequence variation where *Msp*I is replaced by *Mse*I, a methylation-insensitive isoschizomer of *Hpa*II. Alternatively, the absence of notable variations among the four plants in the methylation-sensitive *Hpa*II digest set for all the primer combinations suggests that there exists no methylation variation among the four plants indicating their epigenetic uniformity. Concurrently, the presence of more number of bands in the *Msp*I digest set compared to *Hpa*II set is a good indication that *Hevea* genome is significantly methylated because, the additional bands arised due to the indiscriminate cleavage of DNA by the methylation-insensitive *Msp*I compared to the sensitive *Hpa*II which leaves the methylated loci uncut. These results fully agree with the earlier report by Uthup et al. (2011) regarding the abundance of methylated region in *Hevea* genome. It is interesting to note that methylation pattern polymorphisms were not detected among the polyembryony derived plants despite the presence of several methylated loci in the *Hevea* genome. The reason for this uniformity despite the heavily methylated status of *Hevea* may be attributed to the nature of explant tissue used and the culture conditions as discussed elsewhere due to which the chance of external factors influencing the induction of methylation patterns variation is minimal. Based on the results obtained from the MSAP studies, on four plants regenerated from the randomly selected embryos from the same poly-embryonic cluster, it is assumed that methylation variation which is a major factor responsible for SV is less frequent in polyembryony derived plants, and they are more or less epigenetically as well as genetically the same. Additionally, the results also support the assumption that the zygotic explants posses more genetic and epigenetic stability than the somatic tissues of rubber during plant regeneration and are ideal for generating highly uniform plants.

Conclusion

The study forms the first report of multiple embryo induction in *H. brasiliensis* from a single zygote and subsequent plant regeneration by embryo culture. Our results suggest that the *half ovulo* embryo culture technique can be utilized as an effective technique for developing uniform multiple seedlings in *H. brasiliensis*. From the present study, it is obvious that zygotic polyembryony could be successfully induced in *H. brasiliensis* following *half ovulo* embryo culture technique. Seedlings were developed from three independent ovules and were hardened and field planted. Concurrently, based on the molecular data, we arrived at the assumption that the plants developed were genetically and epigenetically true to type. The media refinement data indicates that there is scope for further improvement of the technique and after fine-tuning; it can be employed for large-scale generation of uniform plants. Further work should be geared toward the enhancement of the percentage of poly-embryonic ovules to a tangible level so that ultimately, this method can be used for developing uniform rootstocks which may aid in reducing the high rate of intraclonal variation presently seen among popular clones and for breeding experiments involving replicated evaluation trials in the clonal nursery. Additionally, the plantlets raised by this technique form excellent material for understanding the somatogenetic interactions occurring during the grafting of divergent rootstocks and its subsequent impact on the physiology, gene expression, and protein function in the scion.

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References

Abirami K, Singh SK, Singh R, Mohapatra T, Kumar AR (2008) Genetic diversity studies on polyembryonic and monoembryonic mango genotypes using molecular markers. Indian J Hortic 65:258–262

Almansa MS, Hernandez JA, Jimenez A, Botella MA, Sevilla F (2002) Effect of Salt stress on the superoxide dismutase activity in leaves of *Citrus limonum* in different rootstock-scion combinations. Biol Plantarum 45:545–549

Bairu MW, Fennell CW, Van Staden J (2006) The effect of plant growth regulators on somaclonal variation in.Cavendish banana (Musa AAA cv. 'Zelig'). Sci Hortic 108:347–351

Batygina TB (1989) New approach to the system of reproduction in flowering plant. Phytomorphology 39:311–325

Batygina TB, Freiberg TE (1979) Polyembryony in *Poa pratensis* L. (Poaceae). Botanicheskii Zhurnal 64:793–804

Bednarek PT, Orłowska R, Koebner RMD, Zimny J (2007) Quantification of tissue-culture induced variation in barley (*Hordeum vulgare* L.). BMC Plant Biol 7:10

Biondi S, Thorpe TA (1982) Growth regulator effects, metabolic changes and respiration during shoot initiation in cultured cotyledon explants of *Pinus radiata*. Bot Gaz 143:22–25

Bower JP, Nel M (1981) Avocado (*Persea americana*) (Mill.) stock-scion interactions as evidenced by peroxidase activity and stem growth. South African Avocado Growers' Association Yearbook 4:117–120

Braun A (1860) Uber polyembryony and Kelmung von Caelebogyna.EinNachting under Abhandlungen uber parthenogenesis bei pflanzen. Abhandlungen der koniglichen Akademie der Wissenschaften Bedin 1859:109–263

Buitendijk JH, Pinsonneaux N, Van Donk AC, Ramanna MS, Van Lammeren AAM (1995) Embryo rescue by half-ovule culture for the production of interspecific hybrids in Alstroemeria. Sci Hortic 64:65–75

Cardinal ABB, Goncalvis PS, Mello MAL (2007) Stock-scion interactions on growth and rubber yield of *Hevea brasiliensis*. Sci Agric (Piracicaba, Braz) 64:235–240

Chen Z, Gallie DR (2012) Induction of monozygotic twinning by ascorbic acid in tobacco. PLoS ONE 7(6):e39147. doi:10.1371/journal.pone.0039147

Combe JC (1975) Demonstration of intraclonal variability in young graft trees. Revue Generale des Caouth Plast 52:91–94

Das K, Das G, Dey SK (2003) *In vitro* culture of immature embryos of *Hevea brasiliensis*. Indian J Nat Rub Res 16:122–126

Dodeman VL, Ducreux G, Kreis M (1997) Zygotic embryogenesis versus somatic embryogenesis. J Exp Bot 48:1493–1509

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus 12:13–15

Duncan RR (1997) Tissue culture-induced variation and crop improvement. Adv Agron 58:201–240

Ercan Y, Mustafa K, Turan HD, Aydın U, Celil T (2013) Identification of zygotic and nucellar individuals produced from several citrus crosses using SSR Markers. Notulae Botanicae Horti Agrobotanici Cluj-Napoca 41:478–484

Erdelska O, Vidovencova Z (1992) Cleavage polyembryony in maize. Sex Plant Reprod 5:224–226

Etienne H, Bertrand B, Montagnon C, Bobadilla Landey R, Dechamp E et al (2012) Un exemple de transfert technologique réussi en micropropagation: la multiplication de Coffea arabica par embryogenèse somatique. Cah Agric 21:115–124

Evans DA, Sharp WR and Bravo JE (1987). Plant somaclonal variation and mutagenesis. Nestlé Res News

Ferguson SJ, Jones TG, Kell DB, Sorgato MC (1979) Biochem J 180:75–85

Gerdakaneh M, Mozafari AA, Khalighi A, Sioseh-mardah (2009) The effects of carbohydrate source and concentration on somatic embryogenesis of Strawberry (Fragaria x ananassa Duch). Am-Eurasian J Agric Environ Sci 6:76–80

Gonçalves B, Pereira MJ, Alberto S, Paula AS, Bacelar E, Carlos E, Rosa E (2005) Scion–rootstock interaction affects the physiology and fruit quality of sweet cherry. Tree Physiol 26:93–104

Haccius B (1955) Experimentally induced twinning in plants. Nature 176: 355–356

Hartman CL, McCo TJ, Knous TR (1984) Selection of alfalfa (*Medicago sativa*) cell lines and regeneration of plants resistant to the toxin(s) produced by *Fusarium oxysporum* f.sp. *medicaginis*. Plant Sci Lett 34:183–194

Hu C, Wang P (1986) Embryo culture: technique and applications. In: Evans DA, Sharp WR, Ammirato PV (eds) Handbook of plant cell culture, vol 4. Macmillan, New York, pp 43–96

Jayashree R, Rekha K, Venkatachalam P, Uratsu SL, Dendekar AM, Jayasree KP, Kala RG, Priya P, Sushamakumari S, Sobha S, Asokan MP, Sethuraj MR, Thulaseedharan A (2003) Genetic transformation and regeneration of rubber tree (*Hevea brasiliensis* Muell. Arg) transgenic plants with a constitutive version of an anti-oxidative stress superoxide dismutase gene. Plant Cell Rep 22: 201–209

Jensen PJ, Rytter J, Detwiler EA, Travis JW, McNellis TW (2003) Rootstock effects on gene expression patterns in apple tree scions. Plant Mol Biol 53:493–511

Kaeppler SM, Phillips RL (1993) DNA methylation and tissue culture-induced DNA methylation variation in plants. In Vitro Cell Dev Biol-Pl 29:125–130

Kaeppler SM, Kaeppler HF, Rhee Y (2000) Epigenetic aspects of somaclonal variation in plants. Plant Mol Biol 43:179–188

Kanehira A, Yamada K, Iwaya T, Tsuwamoto R, Kasai A, Nakazono M, Harada T (2010) Apple phloem cells contain some mRNAs transported over long distances. Tree Genet Genomes 6:635–642

Karami OA, Deljou M, Esna-ashari P, Ostat-ahmadi (2006) Effect of sucrose concentrations on somatic embryogenesis in carnation (*Dianthus caryophyllus* L.). Sci Hortic 110:340–344

Karp A (1994) Origins, causes, and uses of variation in plant tissue cultures. In: Vasil IK, Thorpe TA (eds) Plan cell and tissue culture. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 136–151

Koepke T, Dhingra A (2013) Rootstock scion somatogenetic interactions in perennial composite plants. Plant Cell Rep 32:1321–1337

Larkin PJ, Scowcroft WR (1981) Somaclonal variation: a novel source of variability from cell cultures for plant improvement. Theor Appl Genet 60:197–214

Li HL, Zhang H, Yu C, Ma L, Wang Y, Zhang XZ, Han ZH (2012) Possible roles of auxin and zeatin for initiating the dwarfing effect of M9 used as apple rootstock or interstock. Acta Physiol Plant 34: 235–244

LoSchiavo F, Pitto L, Giuliano G, Torti G, Nuti-Ronchi V et al (1989) DNA methylation of embryogenic carrot cell cultures and its

variations as caused by mutation, differentiation, hormones, and hypomethylating drugs. Theor Appl Genet 77:325–331
Martinez-Ballesta MC, Alcaraz-Lopez C, Muries B, Mota-Cadenas C, Carvajal M (2010) Physiological aspects of rootstock–scion interactions. Sci Hortic 127:112–118
Miguel C, Marum L (2011) An epigenetic view of plant cells cultured in vitro: somaclonal variation and beyond. J Exp Bot 62:3713–3725
Mohapatra A, Rout GR (2005) Study of embryo rescue in floribunda rose. Plant Cell Tiss Organ Cult 81:113–117
Nayanakantha NMC, Seneviratne P (2007) Tissue culture of rubber: past, present and future prospects. Cey J Sci (Bio Sci) 36:116–125
Ng AP, Ho CY, Sultan MO, Ooi CB, Lew HL, Yoon PK (1981) Influence of six rootstocks on growth and yield of six scion clones of *Hevea brasiliensis.* In: RRIM planters's conference, London, Proceedings: 134–151
Omokhafe KO (2004) Clonal stability of tree dryness in *Hevea brasiliensis* Muell. Arg. Genet Mol Biol 27:242–244
Reed SM (2005) Embryo rescue. In: Trigiano RN, Gray DJ (eds) Plant development and biotechnology. CRC press, LLC
Rekha K, Jayashree R, Gireesh T, Sushamakumari S, Jomini KT, Priya SA, Jayasree PK, Sobha S, Kala RG, Thulaseedharan A (2010a) Embryo rescue and plant regeneration in *Hevea brasiliensis*. Nat Rub Res 23:147–154
Rekha K, Jayashree R, Sushamakumari S, Sobha S, Gireesh T, Supriya R, Anita A, Saha T Thulaseedharan A (2010) Induction of zygotic polyembryony and development of of true-to-type seedlings of *Hevea brasiliensis*. In: International conference on "Biotechnology: A Global Scenario". Kakatiya University, India abstract: 121–122
Rodrigues MGF, Martins ABG, Bertoni BW, Figueira A, Giuliatti S (2013) Search for methylation-sensitive amplification polymorphisms in mutant figs. Genet Mol Res 12:2267–2280
Rogers WS, Beakbane AB (1957) Stock and Scion Relations. Annu Rev Plant Physiol Plant Mol Biol 8:217–236
Roy CB, Ravindran M, Saha T (2012) Efficient screening of aflp primer combinations for evaluating genetic diversity among cultivated rubber (*Hevea brasiliensis*) clones. Nat Rub Res 25:21–30
Sanders ME (1950) Development of self and hybrid *Datura* embryos in artificial culture. Am J Bot 37:6–15
Saze H (2008) Epigenetic memory transmission through mitosis and meiosis in plants. Semin Cell Dev Biol 19:527–536
Schellenbaum P, Mohler V, Wenzel G, Walter B (2008) Variation in DNA methylation patterns of grapevine somaclones (*Vitis vinifera* L). BMC Plant Biol 8:78
Senanayake YDA, Wijewantha T (1968) Synthesis of *Hevea* cultivars: a new approach. J Rub Res Inst Ceylon 44:16–24
Sharma DR, Kaur R, Kumar K (1996) Embryo rescue in plants—a review. Euphytica 89:325–337
Shepard JF, Bidney D, Shahin E (1980) Potato protoplast in crop improvement. Science 28:17–24
Sobhana P, Jayasree G, Jacob J, Sethuraj MR (2001) Physiological and biochemical aspects of stock-scion interaction in *Hevea brasiliensis*. Indian J Nat Rub Res 14:131–136
Stewart JM, Hsu CL (1978) Hybridisation of diploid and tetraploid cottons through *in ovulo* embryo culture. J Hered 69: 404–408
Tsolova' V, Atanassov A (1994) Induction of polyembryony and secondary embryogenesis in culture for embryo rescue of stenospermocarpic genotypes of *Vitis vinifera* L. Vitis 33:55–56
Uthup TK, Ravindran M, Bini K, Saha T (2011) Divergent DNA methylation patterns associated with abiotic stress in *Hevea brasiliensis*. Mol Plant 4:996–1013
Vengadesan GA, Ganapath AV, Ramesh DRP (2002) Somatic embryogenesis in cell suspension cultures of *Acacia sinuate* (Lour.) Merr. In Vitro Cell Dev Biol Plant 38:52–57
Venkatachalam P, Jayasree PK, Sushmakumari S, Jayashree R, Rekha K, Sobha S, Priya P, Kala RG, Thulaseedharan A (2007) Current perspectives on application of biotechnology to assist the genetic improvement of rubber tree (*Hevea brasiliensis* Muell. Arg.): an overview. Func Plant Sci Biotechnol 1:1–17
Villalobose VM, Oliver MJ, Yeung EC, Thorpe TA (1984) Cytokinin induced switch in development in excised cotyledon of radiata pine cultured *in vitro*. Physiol Plant 61:483–489
Webster AD (2004) Vigour mechanisms in dwarfing rootstocks for temperate fruit trees. Acta Hortic 658:29–41
Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18:6531–6535
Xu M, Li X, Korban SS (2000) AFLP-based detection of DNA methylation. Plant Mol Biol Rep 18:361–368
Zatykó JM, Simon I, Szabó CS (1975) Induction of polyembryony in cultivated ovules of red currant. Plant Sci Lett 4:281
Zatykó JM, Kis F, Simon I, Szalay F (1981) Polyembryony of currents and gooseberry induced *in vitro*. Acta Hortic 120:211–216
Zhang WN, Gong L, Ma C, Xu HY, Hu JF, Harada T, Li TZ (2012) Gibberellic acid-insensitive mRNA transport in Pyrus. Plant Mol Biol Rep 30:614–623