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A protocol for regenerating genetically stable coffee (*Coffea arabica* L.) plantlets through direct somatic embryogenesis

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Abstract

Coffee is an important beverage crop owing to its economic benefits at individual and national levels. The most important part of coffee *in vitro* micropropagation is the generation of genetically homogenous and uniform plantlets with all desirable characters of the mother plant. Somaclonal variation may lead to generation of off-types planting material with undesirable character(s) due to loss of genetic fidelity. The present experiment investigated genetic stability and somaclonal variation of 54 somatic embryo-regenerated *Coffea arabica* 'Ruiru 11' sibs 93, 100, 121 and 137 and 2 clonal mother plants of each sib. The Sibs were characterized using 13 SSR molecular markers. The molecular data was organized into a matrix and genetic similarity calculated with Jaccard's distances using XLSTAT statistical software and a profile plot constructed using Unweighted Pair Group Method with Arithmetic average cluster analysis to evaluate the genetic fidelity amongst the regenerated plantlets. All banding profiles from somatic embryogenesis regenerated plantlets were monomorphic and similar to those of the mother plants with a similarity value of 1. The profile plot revealed 100% similarity between the somatic embryo-regenerated plantlets and the clonal mother plants. These results confirmed that the somatic embryo-regenerated progenies were uniform according to the SSR markers. The somatic embryogenesis process had a mechanism for selecting competent cells and the regenerated plantlets were genetically stable. Therefore, the protocol for regenerating somatic embryos is recommended for use in mass propagation of the disease-resistant somaclones for distribution to farmers to use in expansion of the acreage planted with coffee.

Keywords: Coffee; Ruiru 11; Somatic Embryogenesis; Genetic Stability

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1. Introduction

Plants regenerated in tissue culture are expected to have identical genetic make-up to the mother plants and to retain intrinsic characteristics (Campos et al., 2017). One of the most crucial concerns facing *in vitro* propagation is loss of genotypic integrity of the micropropagated plants as compared to the mother plant (Rani et al., 2000). Genotypic instability is commonly observed in plants derived from tissue culture, which is at least partly due to *in vitro* induced stress (Burg et al., 2007; Krishna et al., 2016), hence genetic stability of *in vitro* propagated plants should be assessed early (Marum et al., 2009). Plant tissue culture protocols leading to somaclonal variation have been considered as some of the possible sources of inducing genetic variability in plants (Sheidai et al., 2010). Such variation arises as a manifestation of epigenetic influence or changes in the genome of differentiating vegetative cells, but they are expected to generate stable plants, carrying interesting heritable traits (Soniya et al., 2001; Landey, 2014).

The term 'somaclonal variation' refers to the phenotypic and genotypic variations of both qualitative and quantitative traits that occur in plants regenerated from cell and tissue cultures (Jain, 2001). Tissue culture induced variation has usually been based on phenotypic differences in regenerated plants and their progeny. However, genomic changes appear to be the basis for the phenotypic alterations (Phillips et al., 1994). With respect to the origin and causes of somaclonal variation in micropropagated plants, regeneration induction systems, explant sources, culture media, conditions, cycles, and genotype effects, are some of the factors that induce variation (Bairu et al., 2006; Zayova et al., 2010; Bairu et al., 2011; Leva et al., 2012). For somatic embryogenesis method to be fully exploited for clonal propagation, the genetic integrity of resultant plants must be evaluated. This paper presents the first report demonstrating genetic stability of coffee 'Ruiru 11' plantlets regenerated through direct somatic embryogenesis.

2. Materials and methods

The experiment was conducted in the Kenya Agricultural and Livestock Research Organization-Coffee Research Institute (CRI) Laboratories at Ruiru, Kenya. The site is situated 1.05°S and 36.45°E at an elevation of 1608 m above sea level (Jaetzold et al., 2007). Plants regenerated from a particular Ruiru 11 sib were considered as a population (Biswas et al., 2009). Thus, four populations of regenerated Ruiru 11 sibs 93, 100, 121 and 137 were used. The mother plants were seed-grown Ruiru 11 sibs from clonal garden maintained at CRI.

Third leaf pair explants excised from field grown mother plants of sibs 93, 100, 121 and 137 by were sterilized and cultured in half-strength Murashige and Skoog (MS) (1962) basal salts medium, supplemented with 1 ml/L Thidiazuron, 0.2 g/L thiamine, 0.1 g/L nicotinic acid, 0.1 g/L pyridoxine, 20 g/L sucrose, 100 mg/L myo-inositol, 100 mg/L cysteine and 3 g/L gelrite. The pH was adjusted to 5.7 using 1 M NaOH before autoclaving for 15 minutes at 121°C and 100 KPa. The cultures were cultured in continuous darkness. Induced somatic embryos were transferred to germination medium comprising full strength MS (1962) basal salts supplemented with 0.2 g/L thiamine, 0.1 g/L nicotinic acid, 0.1 g/L pyridoxine, 20 g/L sucrose, 100 mg/L myo-inositol and 3 g/L gelrite and incubated at 25 ± 2°C and 16 h light. Germinated shoots were

transferred to rooting medium comprising half strength MS (1962) basal salts supplemented with 1 ml/L naphthalene acetic acid, vitamins, 100 mg/L myo-inositol, 20 g/L sucrose and 3 g/L gelrite. The germinated shoots were incubated at 25 ± 2°C and 16 h light.

The rooted plantlets were removed from the rooting medium using forceps, agar cleaned off under running tap water and soaked in 10% Ridomil fungicide for 1-2 hours. The potting mixture had 3:2:1v/v top soil, sand and manure. The pots were allowed to soak water until the mixture saturated. The plantlets were removed from the fungicide and planted in the potting mixture. The weaning pots were covered completely, opened gradually to reduce humidity, and opened fully after one month. The plants were transferred individually to similar potting mixture and maintained in the greenhouse (CRF, 2011) for further analysis.

Leaf samples were picked from the second and third nodes on tips of 54 somatic embryo-regenerated Ruiru 11 sibs 93, 100, 121 and 137, and 2 clonal mother plants of each sib maintained in the Coffee Research Institute's greenhouse and clonal garden, respectively. The leaf samples were kept in cool boxes and taken to the laboratory for DNA extraction. The leaf samples were surface-sterilized with 70% ethanol and allowed to dry for 15 minutes. About 250 mg of the fresh leaf sample was placed in a motor to which 30 ml of liquid nitrogen was added and ground to powder with a pestle. The genomic DNA was isolated as described by Diniz et al., (2005) with change of extraction buffer to be MixedAlkylTrimethylAmmonium Bromine instead of CetylTrimethylAmmonium Bromide Approximately 500 µl of lysis buffer followed by 500 µl extraction buffer were added to each ground leaf sample and allowed to thaw. The sample was transferred to a 2 ml tube and incubated at 62°C for 30 minutes. About 1 ml of chloroform, isoamyl alcohol (24:1), was added, agitated and centrifuged at 14000 rpm for 5 minutes. The supernatant was transferred to a new 1.5 ml tube, an equal volume of chilled iso-propanol added and agitated. The DNA was left to precipitate overnight at 4°C, then centrifuged at 14000 rpm for 5 minutes. The supernatant was decanted and the pellet washed twice with 70% ethanol and allowed to dry at room temperature for 15 to 30 minutes. The DNA was suspended in 200 µl 1X TE (Tri EDTA pH 8.0) buffer, about 20 µl of RNase (10 mg/ml) added and incubated in a water bath maintained at 37°C for 30 minutes before centrifuging for 10 minutes at 14000 rpm. The supernatant was decanted and the DNA pellet was washed twice using 200 µl 70% ethanol, allowed to dry at room temperature and re-suspended in 1X TE overnight at 4°C. Quantification of the DNA was done by diluting 2 µl of the DNA sample with 5 µl double distilled water and 3 µl of 6x loading dye, loading in 1x agarose gel and electrophoresing along a standard at 50 W for 45 min. The samples were diluted with double distilled water to a concentration of 10 ng/µl.

A 25 µl aliquot containing 1X one-Taq PCR buffer was taken, added to 75 µM of each dNTP's, 1 U one-Taq DNA polymerase (all from Biolabs), 0.4 µM of each primer, namely SAT 11, 32, 172, 207, 227, 229, 235, 240, 254, 255, 262, or 283 and M24 (Fisher Scientific) (Table 1), 2.5 mM $MgCl_2$, 100 ng DNA template and made to volume with double distilled water.

The mixture, in a 200 µl PCR tube, was subjected to PCR in a pre-programmed Flexi-gene thermocycler, comprising initial denaturation at 94°C for 5 minutes, followed by 5 cycles of denaturation at 94°C for 45 seconds, primer annealing at 60°C to 56°C (reducing by 1°C every cycle) for 90 seconds, elongation at 72°C for 120 seconds and 35 cycles of denaturation at 94°C for 30 seconds, primer annealing at 54°C for 60

seconds and elongation at 72°C for 90 seconds, final extension at 72°C of 10 min and held at 4°C to complete the primer extension reaction. The PCR products were analyzed on 2.3% agarose with power supply (Consult) set at 1000V, 100A, 100W for 3 hours. The gel was stained with 10 mg/ml ethidium bromide for 30 minutes and rinsed in distilled water for 30 minutes before visualizing in UV Trans-illuminator and photographing using a digital camera. For SSR analysis, the presence of the band was scored as 1, whereas absence was scored as 0. The scores were converted into 0/1 binomial matrix. Genetic similarity was calculated as Jaccard's distances using XLSTAT software and a profile plot constructed using Unweighted Pair Group Method with Arithmetic average cluster analysis.

Table 1. SSR Primer sequence

SN	Primer	Forward primer (5'>3')	Reverse primer (5'>3')
1.	SAT 11	ACCCGAAAGAAAGAACCAA	CCACACAACTCTCCTCATTC
2.	SAT 32	AACTCTCCATTCCCGCATTC	CTGGGTTTTCTGTGTTCTCG
3.	SAT 172	ACGCAGGTGGTAGAAGAATG	TCAAAGCAGTAGTAGCGGATG
4.	SAT 207	GAAGCCGTTTCAAGCC	CAATCTCTTTCCGATGCTCT
5.	SAT 227	TGCTTGGTATCCTCACATTCA	ATCCAATGGAGTGTGTTGCT
6.	SAT 229	TTCTAAGTTGTTAAACGAGACGCTTA	TTCCTCCATGCCCATATTG
7.	SAT 235	TCGTTCTGTCATTAAATCGTCAA	GCAAATCATGAAAATAGTTGGTG
8.	SAT 240	TGCACCCTTCAAGATACATTCA	GGTAAATCACCGAGCATCCA
9.	SAT 254	ATGTTCTTCGCTTCGCTAAC	AAGTGTGGGAGTGTCTGCAT
10.	SAT 255	AAAACCACACAACTCTCCTCA	GGGAAAGGGAGAAAAGCTC
11.	SAT 262	CTGCGAGGAGGAGTTAAAGATACCAC	GCCGGGAGTCTAGGGTTCTGTG
12.	SAT 283	AGCACACACCCATACTCTCTT	GTGTGTGATTGTGTGTGAGAG
13.	M24	GGCTCGAGATATCTGTTTAG	TTTAATGGGCATAGGGTCC

Source: Kathurima et al., 2012

3. Results

The 13 primers, except SAT 229 and SAT 283, produced clear and reproducible loci. The number of scorable bands for each primer was either 1 or 2 (Table 2). All band-profiles for the somatic embryogenesis-regenerated plantlets were monomorphic and similar to those of the mother plants (Plates 1 to 11). A total of 3,240 bands were scored in 216 micropropagated plantlets. A profile plot confirmed the true-to-type nature of the progenies (Figure 1). A similarity matrix (Figure 2) based on Jaccard's coefficient revealed that the pair-wise value between the mother plant and the plantlets was 1, indicating 100% similarity.

Table 2. SSR product amplification

SN	Primer	Sib 93		Sib 100		Sib 121		Sib 137		Allele size
		Na	Npa	Na	Npa	Na	Npa	Na	Npa	(bp)
1	SAT 11	1	0	1	0	1	0	1	0	150
2	SAT 32	1	0	1	0	1	0	1	0	100
3	SAT 172	1	0	1	0	1	0	1	0	220
4	SAT 207	1	0	1	0	1	0	1	0	100
5	SAT 227	1	0	1	0	1	0	1	0	180
6	SAT 235	1	0	1	0	1	0	1	0	220
7	SAT 240	2	0	2	0	2	0	2	0	210*300**

8	SAT 254	1	0	1	0	1	0	1	0	210
9	SAT 255	1	0	1	0	1	0	1	0	150
10	SAT 262	2	0	2	0	2	0	2	0	150*200**
11	M24	2	0	2	0	2	0	2	0	150*190**
Total		14	0	14	0	14	0	14	0	

*Key: Na- Number of alleles, Npa- Number of polymorphic alleles,*1st band, **2nd band*

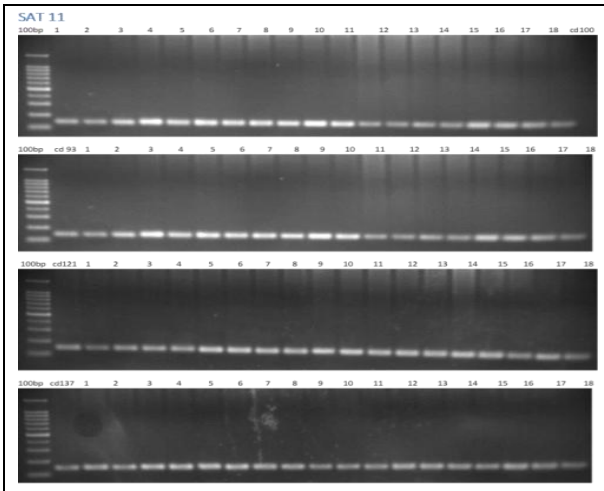

Plate 5.1. Gel electrophoresis of amplification products obtained in clonal mother plants (cd 100, cd 93, cd 121 and cd 137from top to bottom image, respectively) and their corresponding somatic embryogenesis derived plants (lanes 1 to 18) with SSR primer SAT 11.

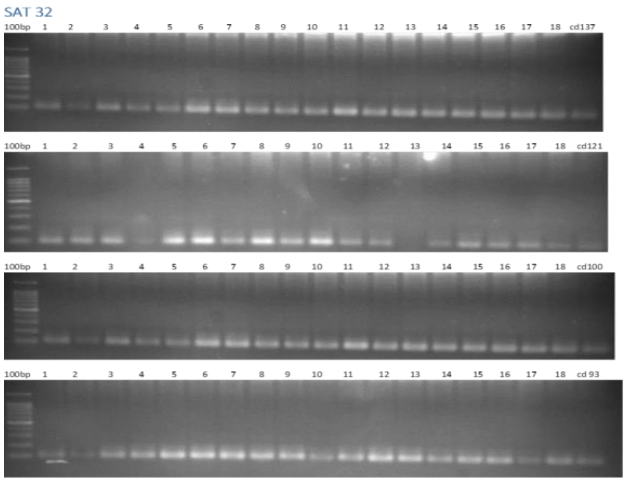

Plate 5.2. Gel electrophoresis of amplification products obtained in clonal mother plants (cd 137,cd 121, cd 100 and cd 93from top to bottom image, respectively) and their corresponding somatic embryogenesis derived plants (lanes 1 to 18) with SSR primer 32.

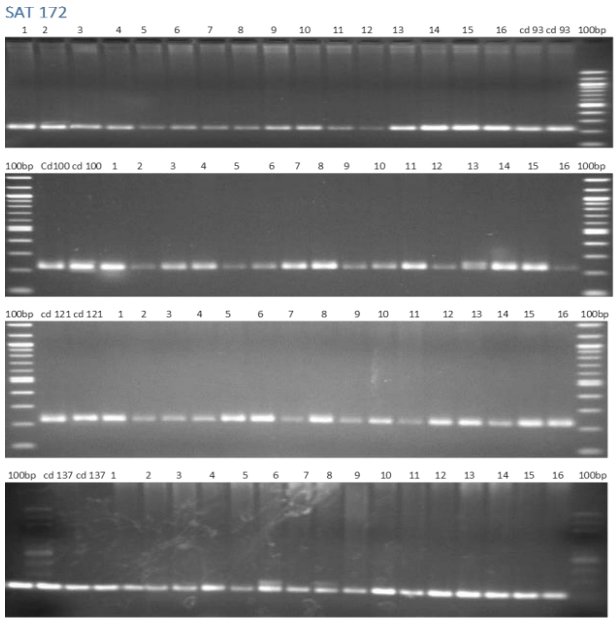

Plate 5.3. Gel electrophoresis of amplification products obtained in clonal mother plants (cd 93, cd 100, cd 121 and cd 137from top to bottom image, respectively) and somatic embryogenesis derived

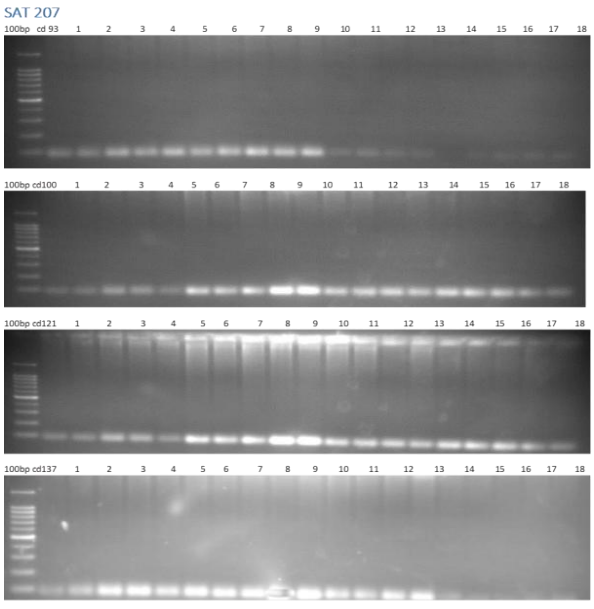

Plate 5.4. Gel electrophoresis of amplification products obtained in clonal mother plants (cd 93, cd 100, cd 121 and cd 137from top to bottom image, respectively) and somatic embryogenesis derived

plants (lanes 1 to 16) with SSR primer 172.

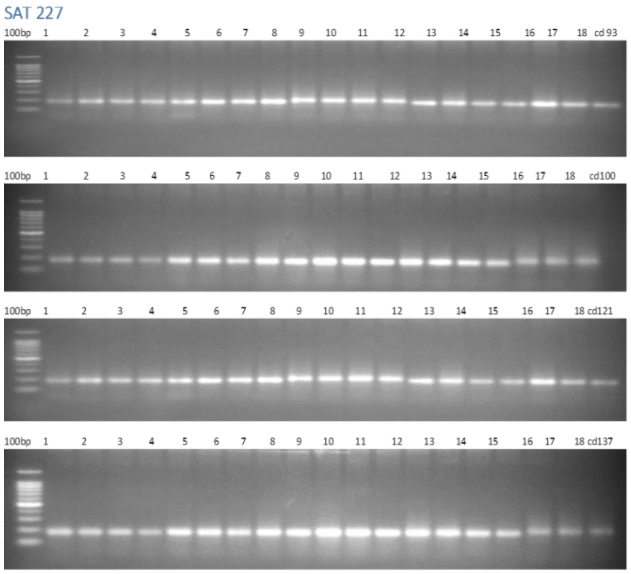

Plate 5.5. Gel electrophoresis of amplification products obtained in clonal mother plants (cd 93, cd 100, cd 121 and cd 137 from top to bottom image, respectively) and somatic embryogenesis derived plants (remaining lanes) with SSR primer 227.

plants (lanes 1 to 18) with SSR primer 207.

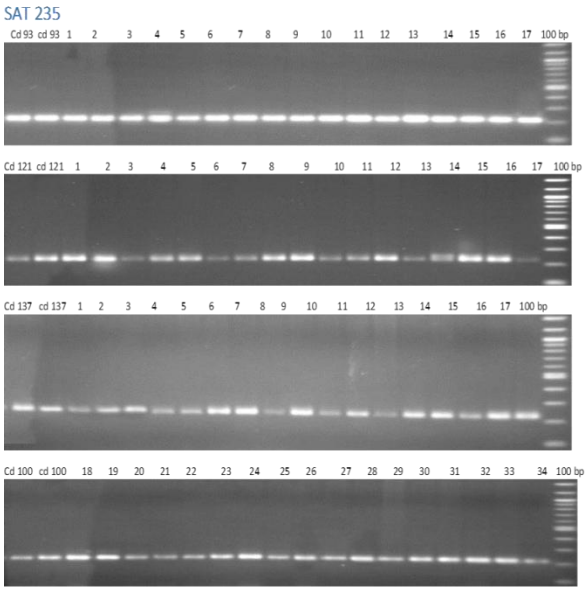

Plate 5.6. Gel electrophoresis of amplification products obtained in clonal mother plants (cd 93, cd 121, cd 137 and cd 100 from top to bottom image, respectively) and somatic embryogenesis derived plants (lanes 1 to 17 or 18 to 34) with SSR primer 235.

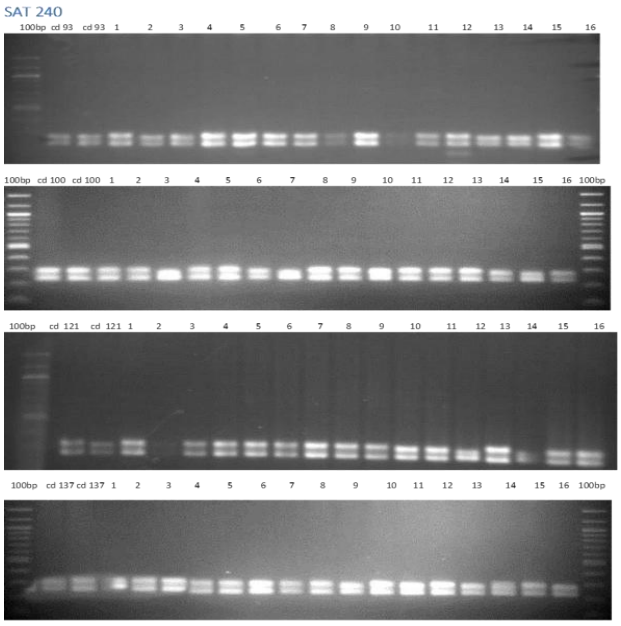

Plate 5.7. Gel electrophoresis of amplification products obtained in clonal mother plants (cd 93, cd 100, cd 121 and cd 137 from top to bottom image, respectively) and somatic embryogenesis derived plants (lanes 1 to 16) with SSR primer 240.

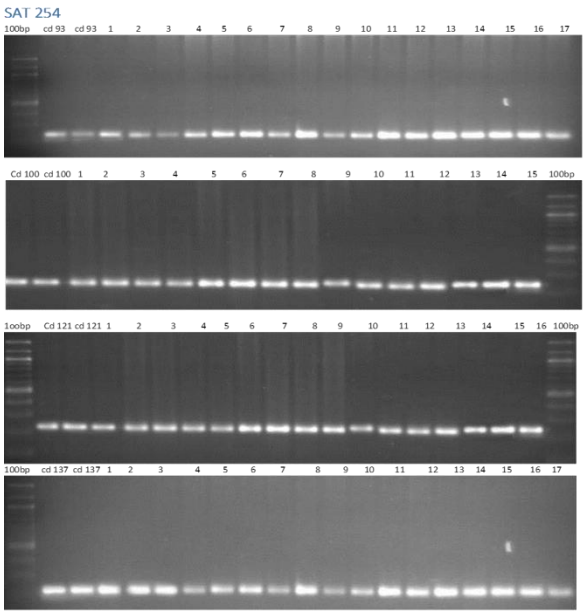

Plate 5.8. Gel electrophoresis of amplification products obtained in clonal mother plants (cd 93, cd 100, cd 121 and cd 137 from top to bottom image, respectively) and somatic embryogenesis derived plants (lanes 1 to 16) with SSR primer 254.

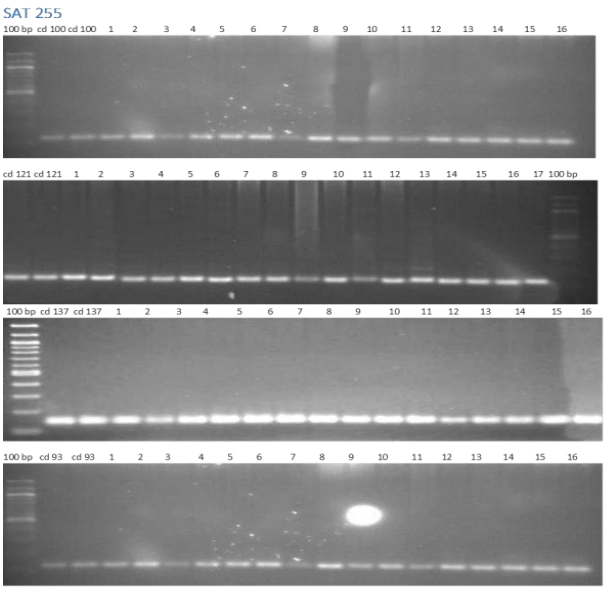

Plate 5.9. Gel electrophoresis of amplification products obtained in clonal mother plants (cd 100, cd 121, cd 137 and cd 93 from top to bottom image, respectively) and somatic embryogenesis derived plants (lanes) with SSR primer 255.

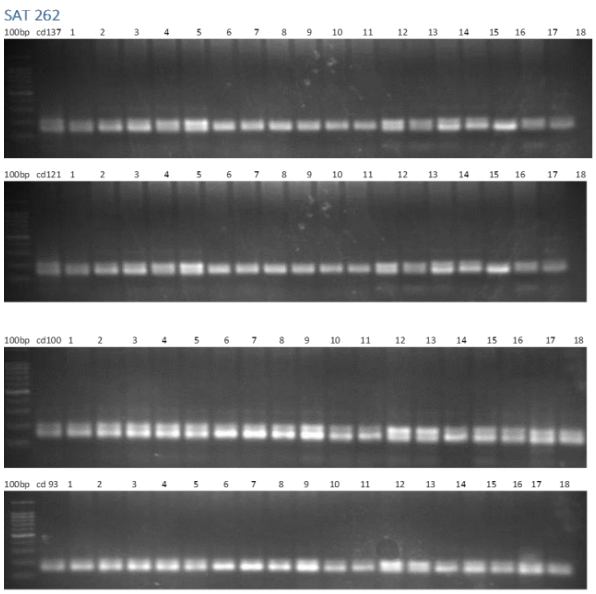

Plate 5.10. Gel electrophoresis of amplification products obtained in clonal mother plants (cd 137, cd 121, cd 100 and cd 93 from top to bottom image, respectively) and somatic embryogenesis derived plants (lanes 1 to 18) with SSR primer 262.

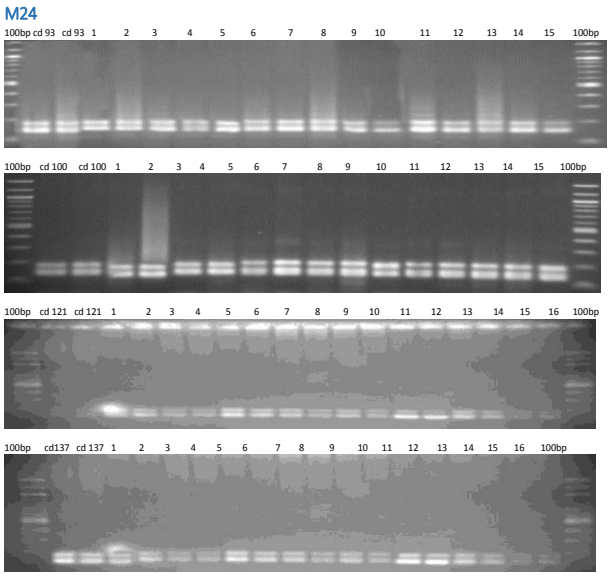

Plate 5.11: Gel electrophoresis of amplification products obtained in clonal mother plants (cd 93, cd 100, cd 121 and cd 137 from top to bottom image, respectively) and somatic embryogenesis derived plants (lanes 1 to 16) with SSR primer M24.

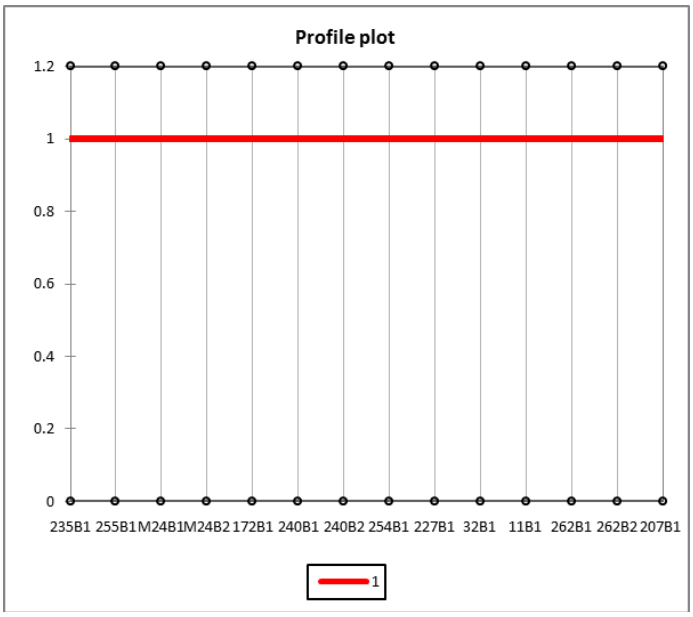

Figure 1. Profile plot showing the similarity of the somatic embryogenesis-regenerated plantlets with SSR primers.

Figure 2. Proximity similarity (Jaccard's coefficient) of mother plants and somatic embryogenesis-regenerated plantlets

4. Discussion

To establish an optimal protocol for the commercial production of high-quality somatic embryo plants, it is important to demonstrate their genetic stability. Molecular techniques are valuable tools used in the analysis of genetic fidelity of micropropagated plants. Simple Sequence Repeats (SSR) is among several molecular markers that can be used to characterize and assess genetic variability. They are highly polymorphic, multi-allelic, co-dominant, reproducible, easy to interpret and amplify by Polymerase Chain Reaction for evaluating varieties with a narrow genetic base (Bredemeijer et al., 2002).

In the case of *C. arabica*, a tetraploid species (2n=4x=44), up to four alleles per locus can be observed, if one pair of primers simultaneously amplifies both loci of ancestral chromosomes (Rovelli et al., 2000) as a result of a conserved primer site. In the present study, up to two alleles per SSR marker were observed. Arabica coffee is characterized by low genetic diversity and thus low molecular polymorphism (Anthony et al., 2001) among the somatic embryo-regenerated plantlets was expected. This is probably due to the process of autogamy and narrow genetic base, owing to homozygosis and the successive selection cycles (Moncada and McCouch, 2004).

The direct somatic embryogenesis (DSE) used to regenerate plantlets is advantageous in reducing incidences of somaclonal variation and is widely used compared to the indirect somatic embryogenesis (ISE) for regenerating seedlings from recalcitrant genotypes (Van Boxtel and Berthouly, 1996). Somatic embryogenesis is less prone to genetic alterations because it involves the expression of many genes (Vasil, 1995) and somatic embryos derived from pre-embryogenic determined cells that tend to be genetically stable (Sharp et al., 1980). Va´zquez (2001) reiterated that somatic embryogenesis is the preferred pathway for generating propagules.

In a study on somaclonal variation of callus, somatic embryo and plant regeneration in vitro of oil palm, Sanputawong and Te-chato (2011) reported that regenerated plantlets obtained from their protocol were uniform according to the SSR markers. Regeneration via embryogenesis has a better chance of obtaining genetically uniform plants than through organogenic differentiation. This is so because DNA in the initial stages of development in somatic embryogenesis contains lower levels of methylation than in the later stages (Sahijram et al., 2003). In addition, the time necessary for obtaining somatic embryos by DSE is shorter compared to ISE.

One of the most crucial concerns in micropropagation is to retain genomic integrity of the resultant plants compared to the mother plant (Rani et al., 2000). This is relevant in inherently slow-growing coffee where hardened plants are transferred to field conditions after about 1.5 years from the date of explant inoculation (Clarindo et al., 2012). Identification of somaclonal variants at an early stage of development is considered useful for quality control in plant tissue culture, transgenic plant production and introduction of variants (Soniya et al., 2001). Contrary reports on the effect of culture duration on somaclonal variation have also been made (Etienne et al., 2003; Clarindo et al., 2012). For instance, multiple shoot culture of pea maintained over a long period (24 years) remained genetically stable and was comparable to the original genotype (Smykal et al., 2007). Absence of genetic variation was also observed after a long period (17 months) of

fennel micropropagation (Bennici et al., 2004), suggesting a genotypic effect. These results could explain absence of variation after long culture duration in the present study.

5. Conclusion and recommendations

High genetic similarities were observed between the mother plants and the plants derived from somatic embryogenesis in the present study. Somatic embryo-regenerated plantlets were uniform according to the SSR markers indicating true-to type progenies. These results indicated that the somatic embryogenesis process is genetically stable, and has a mechanism for selecting competent cells. Since Ruiru 11 is a composite variety, the parents should be evaluated and prediction of embryogenic capacity of the sibs should be done. Further studies on the genetic stability of the other Ruiru 11 sibs should be done to confirm the genetic integrity of somatic embryo-regenerated plantlets. The present protocol should be adopted for mass propagation of the disease-resistant somaclones for distribution to farmers to expand coffee acreage.

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