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DNA methylation in sugarcane somaclonal variants assessed through methylation-sensitive amplified polymorphism

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ABSTRACT. Micropropagation is an important tool for large-scale multiplication of plant superior genotypes. However, somaclonal variation is one of the drawbacks of this process. Changes in DNA methylation have been widely reported as one of the main causes of somaclonal variations in plants. In order to investigate the occurrence of changes in the methylation pattern of sugarcane somaclonal variants, the MSAP (methylation-sensitive amplified polymorphism) technique was applied to micro-propagated plantlets sampled at the third subculture phase. The mother plant, *in vitro* normal plantlets, and *in vitro* abnormal plantlets (somaclonal variants) of four sugarcane clones were screened against 16 MSAP selective primers for *Eco*RI/*Msp*I and *Eco*RI/*Hpa*II restriction enzymes. A total of 1005 and 1200 MSAP-derived markers with polymorphism percentages of 28.36 and 40.67

were obtained for *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I restriction enzyme combinations, respectively. The genetic similarity between the mother plant and the somaclonal variants ranged from 0.877 to 0.911 (*Eco*RI/*Msp*I) and from 0.928 to 0.955 (*Eco*RI/*Hpa*II). Most of the MASPs among mother plant and micro-propagated plantlets were derived from *Eco*RI/*Msp*I restriction enzymes suggesting alteration due to gain or loss of internal cytosine methylation. A higher rate of loss of methylation (hypomethylation) than gain of methylation (hypermethylation) was observed in the abnormal *in vitro* sugarcane plantlets. Although changes in the methylation pattern were also observed in the *in vitro* normal plantlets, they were lower than those observed for the *in vitro* abnormal plantlets. The MASP technique proved to be a promising tool to early assessment of genetic fidelity of micro-propagated sugarcane plants.

Key words: Epigenetic stability; DNA methylation; Somaclonal variation; Sugarcane

INTRODUCTION

Sugarcane is among the most important economical crops, mainly in the tropics, due to its high potential for sugar, alcohol, and biomass production. The sugarcane- breeding programs are becoming dependent on tissue culture for its support, such as, growing callus for genetic transformation, large-scale multiplication of promising cultivars, among others (Joyce et al., 2014; Lal et al., 2015; Wekesa et al., 2015; Rastogi et al., 2015). The use of tissue culture to produce clonally propagated plants is very advantageous as it allows the production of pathogen-free plants in a large scale multiplication at a low cost and in a reduced working space (Lal et al., 2015). However, the major drawback of this technique is the frequent occurrence of individuals with phenotypic variation differing from the original plant in one or more traits termed somaclonal variation (Larkin and Scowcroft, 1981; Bairu et al., 2011). This phenotypic variation can be either genetic or epigenetic in origin (Shawn et al., 2000; Miguel and Marum, 2011).

The DNA methylation is an epigenetic event, in which, a methyl group is added to the DNA cytosine bases to form 5-methylcytosine (He et al., 2011) without changing the DNA sequence. This modification changes the compression levels of chromatin and therefore can affect gene expression (D'Alessio and Szyf, 2006) leading to morphological changes of an organism. The changes in the DNA methylation have been reported in different crops as the main cause of somaclonal variation (Gonzalez et al., 2013; Landey et al., 2015). According to López and Wilkinson (2015), the efficiency of the micropropagation and genetic transformation systems depends on the production of plants genetically and epigenetically faithful to the original plant material.

Several approach such as phenotypic characterization, cytological and molecular marker analyses have been used to detect somaclonal variants derived from micro-propagated plants (Bobadilla Landey et al., 2013; Landey et al., 2015; Sudarshana et al., 2015; Hsie et al., 2015; Rathore et al., 2015). The methylation-sensitive amplification polymorphism (MSAP) technique allows the evaluation of the global DNA methylation status of an organism (Yaish et al., 2014). Basically, the MSAP is a modification of the AFLP (amplified fragment length

polymorphism) protocol (Vos et al., 1995) in which one of the standard methylation insensitive enzymes such as *Eco*RI and *Mse*I, usually used in the AFLP assays are replaced by methylation-sensitive restriction endonucleases such as the isoschizomers *Hpa*II and *Msp*I in the restriction digestion step. As the methylation-sensitive isoschizomers *Msp*I and *Hpa*II differ in their sensitivity to cytosine methylation (Lei et al., 2006; Schulz et al., 2013; Yaish et al., 2014), the comparison of the differential digestion pattern generated by each enzyme allows us to identify the methylation status of the DNA. The MSAP technique has been considered efficient in the detection and characterization of somaclonal variants in several crops (Lei et al., 2006; Gao et al., 2010; Bobadilla Landey et al., 2013). Despite the economic importance of sugarcane and also the impact of tissue culture technique to this crop, reports on the methylation status of sugarcane plants derived from tissue culture have not been published yet. In the present study, the methylation status of *in vitro* sugarcane plantlets was investigated through the MSAP technique. In addition, the efficiency of the MSAP technique to detected alterations in the epigenome of *in vitro* sugarcane plantlets is discussed.

MATERIAL AND METHODS

Plant material

Four sugarcane clones identified as SVA, SVB, SVC, and SVD were supplied by a sugarcane commercial biofactory at the third subculture phase. These clones have different discard percentages and were categorized according to the *in vitro* visual frequency of somaclonal variation symptoms (abnormal *in vitro* plantlets) in two groups: high and low somaclonal variation clones (Table 1). The mother plant (used as the source tissue for regeneration) of each clone, and their micro-propagated treatments (*in vitro* normal plantlets and abnormal *in vitro* plantlets) were evaluated with the MSAP technique. The *in vitro* normal plantlets showed 100% of normal phenotype expected for *in vitro* plantlets while the abnormal *in vitro* plantlets (somaclonal variants) as having 100% of abnormal *in vitro* phenotypic appearance.

Table 1. Sugarcane clones and respective treatments (mother plant, *in vitro* normal plantlets, abnormal *in vitro* plantlets), percentage of discard and categorization according to *in vitro* visual frequency of somaclonal variation symptoms.

Clones	Treatments		Discard (%)	Category
SVA	SVA1	Mother plant		
	SVA2	*In vitro* normal plantlets		
	SVA3	Abnormal *in vitro* plantlets	9.92	High
SVB	SVB1	Mother plant		
	SVB2	Normal *in vitro* plantlets		
	SVB3	Abnormal *in vitro* plantlets	1.01	Low
SVC	SVC1	Mother plant		
	SVC2	*In vitro* normal plantlets		
	SVC3	Abnormal *in vitro* plantlets	28.5	High
SVD	SVD1	Mother plant		
	SVD2	*In vitro* normal plantlets		
	SVD3	Abnormal *in vitro* plantlets	1.19	Low

Methylation patterns (hypermethylation, hypomethylation) and respective numbers observed among the mother plant and the abnormal *in vitro* plantlets.

DNA extraction

The genomic DNA was extracted from mother plant and *in vitro* tissue culture plantlets according to the CTAB method (Aljanabi et al., 1999). For each treatment (normal and abnormal *in vitro* plantlets), a bulk of five plantlets was sampled for DNA extraction.

MSAP technique

The MSAP technique was performed in 200 ng DNA based on the AFLP protocol (Vos et al. 1995) adapted for the MSAP technique as described by Lei et al. (2006) in which *Mse*I was replaced by the methylation-sensitive restriction enzymes *Msp*I and *Hpa*II in combination with *Eco*RI (*Eco*RI/*Msp*I and *Eco*RI/*Hpa*II). The adapter ligation and pre-selective amplification reactions were conducted according to Vos et al. (1995) adopting the MSAP adapter sequences described in Lei et al. (2006). The list of the 16 selective primer combinations (*Eco*RI/*Msp*I and *Eco*RI/*Hpa*II) is shown in Table 2. The selective amplification reaction was performed in a total final volume of 10 µL containing 2 µL of the pre-amplification reaction diluted 10X, 0.1 µM of each *Eco*RI and *Msp*I/*Hpa*II selective primer, 100 µM of each dNTP, 2.0 mM $MgCl_2$, 10 mM Tris-HCl, 50 mM KCl, and 0.5 U Taq DNA polymerase. The *Eco*RI selective primers were labeled with infra-red (IR700 or IR800). Reactions were amplified on a thermocycler using a touchdown program as follows: 94°C for 30 s; annealing temperature at 65°C for 30 s in the first cycle, and an extension step at 72°C for 1 min. In the next cycle, the annealing temperature of 65°C was reduced (-0.7°C) in each cycle during 12 cycles until reach 56°C for the remaining 23 cycles. A final elongation step was performed at 72°C for 2 min. Amplification products were separated by electrophoresis on 6% denatured polyacrylamide gels in a 4300 DNA Analyzer (LiCor). The selective amplification products of two reactions (IR700/IR800) were mixed at equal volumes, diluted 5X in water and added to 2 µL of the sample buffer (LiCor, Bioscience) before denaturation at 95°C for 5 min.

Table 2. MSAP (*Eco*RI/*Hpa*II-*Msp*I) selective primer combinations.

MSAP selective primer combination	
Sequence (5'-3'): (*EcoR*I / *Hpa*II-*Msp*I)	Sequence (5'-3'): (*EcoR*I / *Hpa*II-*Msp*I)
CTGCGTACCAATTC**aac** / GATGAGTCCTGATCGG**aca**	CTGCGTACCAATTC**agc** / GATGAGTCCTGATCGG**aca**
CTGCGTACCAATTC**aac** / GATGAGTCCTGATCGG**act**	CTGCGTACCAATTC**agc** / GATGAGTCCTGATCGG**gaa**
CTGCGTACCAATTC**aac** / GATGAGTCCTGATCGG**gaa**t	CTGCGTACCAATTC**agg** / GATGAGTCCTGATCGG**acc**
CTGCGTACCAATTC**aac** / GATGAGTCCTGATCGG**tcg**	CTGCGTACCAATTC**agg** / GATGAGTCCTGATCGG**aca**
CTGCGTACCAATTC**agc** / GATGAGTCCTGATCGG**acc**	CTGCGTACCAATTC**agg** / GATGAGTCCTGATCGG**gaa**
CTGCGTACCAATTC**agc** / GATGAGTCCTGATCGG**act**	CTGCGTACCAATTC**agg** / GATGAGTCCTGATCGG**gag**
CTGCGTACCAATTC**agc** / GATGAGTCCTGATCGG**gaa**	CTGCGTACCAATTC**agg** / GATGAGTCCTGATCGG**tcg**
CTGCGTACCAATTC**agc** / GATGAGTCCTGATCGG**ttg**	CTGCGTACCAATTC**agg** / GATGAGTCCTGATCGG**ttg**

In bold: three base selective nucleotides.

Data analysis

The mother plant and respective treatments were genotyped by the presence (1) and absence (0) of markers. The pairwise genetic similarity was based on the Jaccard coefficient using the NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System) software (version 2.02) (Rohlf, 1998). To estimate the extent of the *in vitro* micro-propagated clones, deviance from the true-to-type mother plant, both monomorphic and polymorphic markers

were used to calculate the similarity coefficient, once clonal propagation is expected to produce individuals identical to the mother plant, i.e., similarity coefficient of 100%. A dendrogram was constructed based on UPGMA (unweighted pair-group method using arithmetic average), and the coefficient of variation (CV%) associated with the genetic similarity estimate was calculated with 10,000 bootstraps using the dBOOD (Coelho, 2001) software. The reliability of the dendrogram nodes was evaluated by the BOOD program (Coelho, 2000) with 10,000 bootstraps.

Change in methylation patterns

Although the isoschizomers *Msp*I and *Hpa*II recognize the same sequence 5'-CCGG-3' they display differential sensitivity to DNA methylation (Lei et al., 2006). According to Schulz et al. (2013), "*Msp*I only recognizes sites being hemi- or fully methylated at the internal cytosine (^{m}CG or ^{m}CG) while *Hpa*II only recognizes sites that are hemi-methylated at the external cytosine (^{m}CCG) when any of the two cytosines is fully methylated". The evaluation of changes in the cytosine methylation, i.e., loss of methylation (hypomethylation), gain of methylation (hypermethylation) was done by comparing side by side the MSAP profile displayed by both *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I among the mother plant and the abnormal *in vitro* plantlets and estimated in terms of percentages.

RESULTS

Methylation profile stability of the mother plant and reproducibility of the MSAP profile

To discard previous instability of the mother plant MSAP profile, two DNA samples of each mother plant from each clone were individually extracted and submitted separately to the MSAP technique. The mother plant MSAP profiles used as the source tissue for regeneration were stable (Figure 1A) allowing the use of the mother plant MSAP profile as reference pattern (control) expected for the micro-propagated treatments (*in vitro* normal plantlets and abnormal *in vitro* plantlets). In addition, the MSAP profile reproducibility was also tested by using two independent DNA samples extracted from the SVD mother plant clone. The MSAP profile obtained with the 16 selective combinations was exactly the same, discarding the possibility of artifacts (Figure 1B).

MSAP polymorphism

The 16 MSAP primer combinations produced a total of 1005 and 1200 markers, respectively, for *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I among mother plant and respective treatments (*in vitro* normal plantlets and abnormal *in vitro* plantlets). The total number of polymorphic markers ranged from 285 to 488 with polymorphism percentage of 28.36 and 40.67 for *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I, respectively (Table 3).

The *Eco*RI-AAC/*Msp*I-ACT selective combination showed the highest number 57 (11.65%) of polymorphic markers while for the *Eco*RI/*Hpa*II restriction enzyme the highest number 31 (10.87%) of polymorphic markers was obtained for the selective combination *Eco*RI-AAC/*Hpa*II-TCG (Figure 2). An average of 31 and 17 polymorphic markers was obtained with the *Eco*RI/*Msp*I and *Eco*RI/*Hpa*II selective primers, respectively.

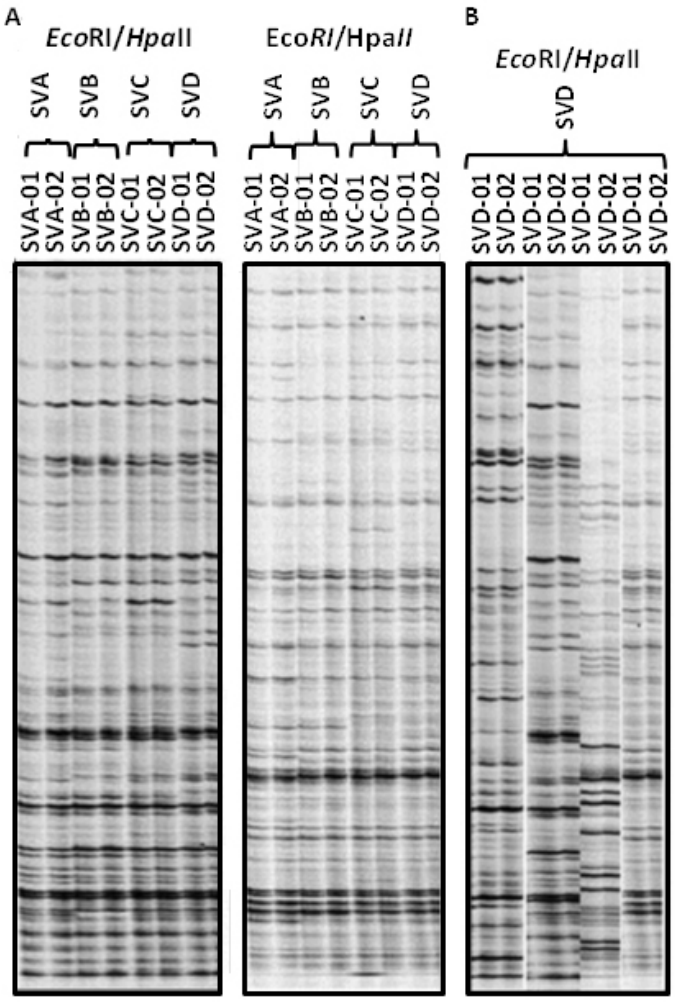

Figure 1. MSAP profile revealed in 6% denaturing polyacrylamide gels at 4300 DNA Analyser (Licor). **A.** MSAP profile stability of two sampled mother plants (SVA, SVB, SVC, SVD) for the selective combinations: *Eco*RI.AGC/*Hpa*II.ACA and *Eco*RI.AGC/*Hpa*II.ACA, respectively. **B.** SVD clone MSAP profile reproducibility test of two sampled DNAs (SVD1 and SVD2) for four selective combinations (left to right: *Eco*RI.AAC/*Hpa*II.GAA; *Eco*RI.AGC/*Hpa*II.ACA; *Eco*RI.AGC/*Hpa*II.GAA; *Eco*RI.ACG/ *Hpa*II.ACA).

Table 3. MSAP polymorphism for *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I enzyme combination.

Enzyme combination	Nsc	N	Np	%P
*Eco*RI/*Hpa*II	16	1005	285	28.36
*Eco*RI/*Msp*I	16	1200	488	40.67
Methylation pattern	Hypermethylation		Hypomethylation	
	41 (36.6%)		57 (50.9)	

Number of selective combination (Nsc), total number of markers (N), total number of polymorphic markers (Np), and percentage of polymorphism (%P).

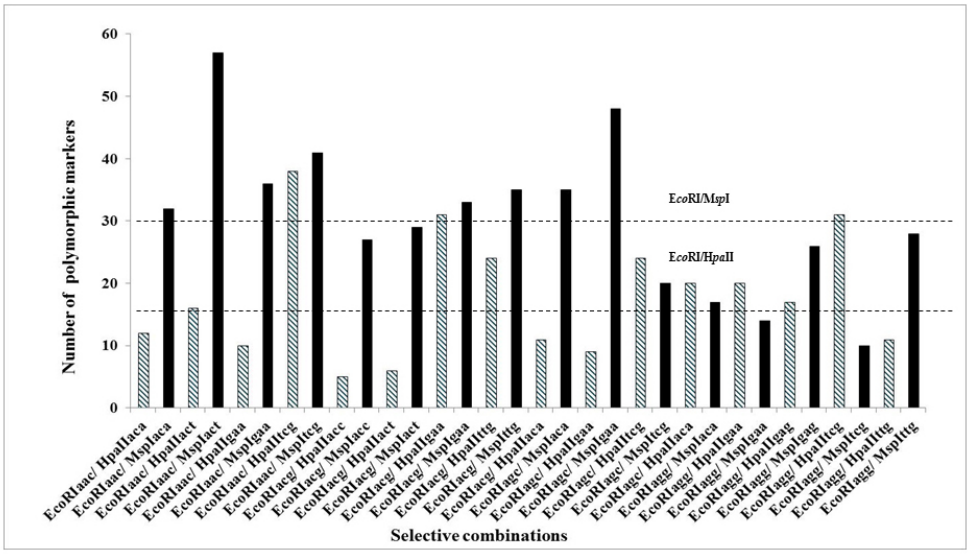

Figure 2. Number of polymorphic markers obtained with different *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I selective primer combinations. Dotted line indicates the average number of polymorphic markers for *Eco*RI/*Msp*I and *Eco*RI/*Hpa*II respectively.

Detection of methylation patterns (hypomethylation, hypermethylation) in the somaclonal variants

By comparing side by side the MSAP profile obtained by both *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I (Figure 3) between the mother plant (taken as the reference standard profile) and respective abnormal *in vitro* plantlets, it was possible to estimate the percentage of methylation. The percentage of hypomethylation observed in the abnormal *in vitro* plantlets was higher (50.9%) than the percentage of hypermethylation (36.6%) indicating a prevalence of a loss rather than a gain of methylation due to the somaclonal variation (Table 3).

In addition, among the polymorphic markers obtained between *Eco*RI/*Msp*I and *Eco*RI/*Hpa*II selective combination profile for the abnormal *in vitro* plantlets, 66.15% was derived from *Eco*RI/*Msp*I enzyme combination signalizing a high occurrence of internal cytosine methylation.

Genetic similarity among the treatments

The genetic similarity between the mother plant and their respective treatments (*in vitro* normal plantlets and abnormal *in vitro* plantlets) was estimated for *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I, separately (Table 4). The smallest genetic similarity obtained with the *Eco*RI/*Hpa*II restriction enzyme was found between the mother plant (SVC1) and the abnormal *in vitro* plantlet (SVC3) with 92.8% genetic similarity or 7.2% dissimilarity between them. The greatest genetic similarity was found between the mother plant (SVB1) and the *in vitro* normal plantlet (SVB2) with 95.6% similarity or 4.4% dissimilarity between the *in vitro* normal plantlet and the mother plant, which was the original source of the explant.

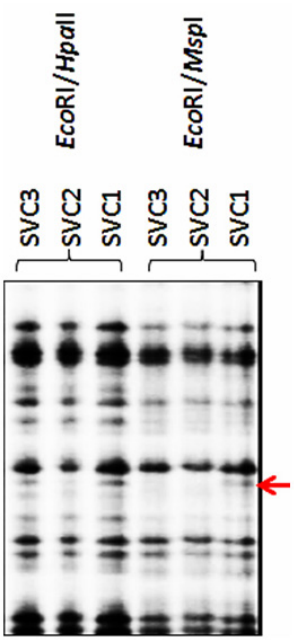

Figure 3. MSAP profile comparison (*Eco*RI.ACG/*Hpa*II.TTG and *Eco*RI.ACG/*Msp*I.TTG) among mother plant and regenerants. Arrow shows marker absence in the regenerants (SCV2 and SCV3) and present in the mother plant (SCV1) when digested by *Eco*RI/*Msp*I.

Table 4. Pairwise genetic similarity obtained for the clones evaluated.

	Clones			Clones			
Clones	SVA1	SVA2	SVA3	Clones	SVC1	SVC2	SVC3
SVA1	1	0.907	0.877	SVC1	1	0.908	0.885
SVA2	0.954	1	0.921	SVC2	0.943	1	0.944
SVA3	0.955	0.946	1	SVC3	0.928	0.966	1
Clones	SVB1	SVB2	SVB3	Clones	SVD1	SVD2	SVD3
SVB1	1	0.935	0.911	SVD1	1	0.893	0.884
SVB2	0.956	1	0.941	SVD2	0.940	1	0.948
SVB3	0.949	0.967	1	SVD3	0.950	0.940	1

Mother plant (SVA1, SVB1, SVC1, SVD1); *in vitro* normal plantlets (SVA2, SVB2, SVC2, SVD2); abnormal *in vitro* plantlets (SVA3, SVB3, SVC3, SVD3). *Eco*RI/*Msp*I (above the diagonal) and *Eco*RI/*Hpa*II (below the diagonal).

The smallest genetic similarity observed with the *Eco*RI/*Msp*I restriction enzyme was observed between the mother plant (SVA1) and the abnormal *in vitro* plantlet (SVA3) with 87.7% genetic similarity and 12.3% dissimilarity. The greatest genetic similarity was found between the mother plant (SVB1) and its respective treatment in *in vitro* normal plantlet (SVB2) with 93.5% genetic similarity and only 6.5% dissimilarity of the *in vitro* normal plantlet in relation to the mother plant. The CV%, which measures the reliability of the genetic similarity, was 1.27 and 1.19%, for the *Eco*RI/*Ms*pI and *Eco*RI/*Hpa*II combination, respectively.

According to the dendrogram (Figure 4A), it was clear that the *Eco*RI/*Msp*I enzyme combination was more efficient to distinguish the mother plant from their tissue culture

treatments than the *Eco*RI/*Hpa*II enzyme combination (Figure 4B). The *in vitro* normal plantlets and the *in vitro* somaclonal variant were more similar to each other than to the respective mother plants. The reliability of the dendrogram nodes observed in the clustering of the mother plant and respective treatments was high ranging from 76.8% (SVA1, SVA2, and SVA3 node) to 100% (SVB1, SVB2, and SVB3 node) for the *Eco*RI/*Msp*I and *Eco*RI/*Hpa*II enzyme combination, respectively.

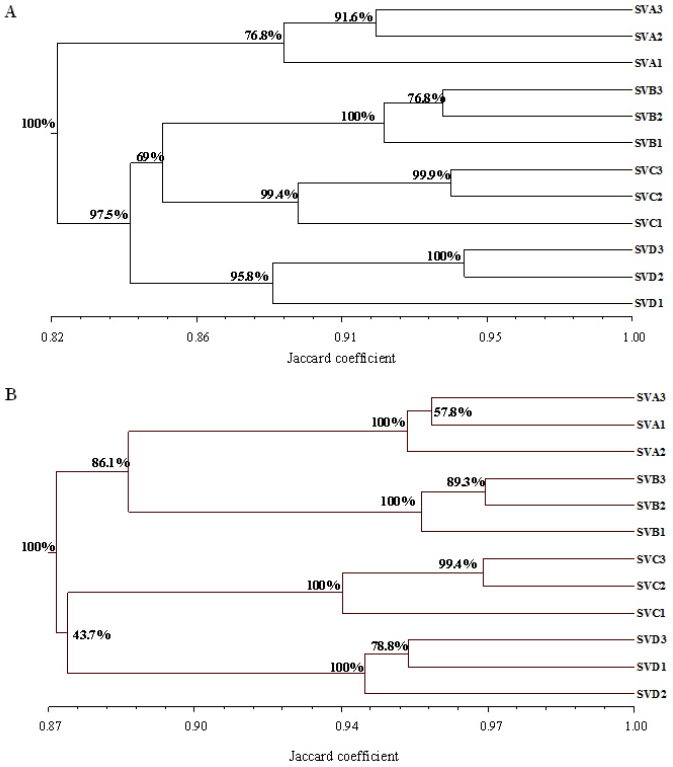

Figure 4. UPGMA dendrogram based on genetic similarity matrix (Jaccard coefficient) among mother plant (SVA1, SVB1, SVC1 and SVD1) and respective micropropagated treatments (*in vitro* normal plantlets: SVA2, SVB2, SVC2 and SVD2; abnormal *in vitro* plantlets: SVA3, SVB3, SVC3 and SVD3). **A.** *Eco*RI/M*sp*I enzyme combinations. **B.** *Eco*RI/H*pa*II enzyme combination.

DISCUSSION

In the present study, the molecular marker technology was used to characterize normal and abnormal *in vitro* plantlets sampled at the third subculture stage. These abnormal sugarcane plantlets taken as somaclonal variants are discarded in the early stages of *in vitro* clonal propagation during the multiplication process. Therefore, the molecular profile comparative analysis obtained by the MSAP technique among the mother plant (used as the source tissue for regeneration), abnormal *in vitro* plantlets (somaclonal variant), and normal *in vitro* plantlets allowed us to access changes occurred at the sugarcane epigenome during the micro-propagated process. These changes were quantified by estimating the genetic similarity

between the *in vitro* treatments and the mother plant.

The stability analysis of the mother plants used as the source tissue for regeneration reinforced the possibility that the polymorphisms obtained between mother plants and treatments can be attributed to changes occurred during the micro-propagation process. This point is important since somaclonal variation can arise from pre-existing somatic mutations in donor plants, namely genetic instability (Leva and Petruccelli, 2012). In addition, the reproducibility analysis of the MSAP technique also contributed to eliminate the possibility that the observed polymorphisms were assigned to possible technical artifacts.

In our study with sugarcane, the MSAP technique was efficient to detect polymorphism among the mother plant and respective tissue culture treatments and hence distinguish the mother plant from the micro-propagation treatments. In fact, it was noted that the genetic similarity values obtained with the *Eco*RI/*Msp*I enzyme combination were more efficient to distinguish between *in vitro* abnormal plantlets (somaclonal variants) and the respective mother plant than the *Eco*RI/*Hpa*II enzyme combination.

Our results suggest that the tissue culture process probably may lead to modifications at the epigenome of micro-propagated derived materials. According to Kaeppler et al. (2000), DNA methylation variation frequently occurs in the tissue culture process. Indeed, we observed that even in the *in vitro* normal plantlets (plantlets without symptoms of somaclonal variation) it was possible to detect differences in the MSAP molecular profile in comparison to the mother plant. Thus, not all changes associated with DNA methylation due to the tissue culture process may produce abnormal plants. These results were similar to that described by Matthes et al. (2001) in oil palm where changes in methylation were not necessarily associated with the abnormal phenotype derived from tissue culture.

In our study, it was noted a higher rate of loss methylation (hypomethylation) in the abnormal *in vitro* sugarcane plantlets than methylation gain (hypermethylation). Significant losses of methylation were also reported in rice plants regenerated by tissue culture in comparison with non-regenerated plants (Stroud et al., 2013) as also in other crops (Jaligot et al., 2000; Matthes et al., 2001). Stelpflug et al. (2014) characterized DNA methylation patterns in callus, primary regenerants, and maize regenerant-derived progenies by immunoprecipitation of methylated DNA and found that hypomethylation events were more frequently than hypermethylation after tissue culture.

In general, the detection of alterations in the MSAP molecular profile reflects changes in the states of methylation between mother plants (adult plants) and micro-propagated plantlets during the third stage of subculture. This result also reflects the power of the MSAP technique to detected changes in the epigenome of tissue culture-derived plants even subjected to a number of subcultures below the upper limit of subcultures recommended for sugarcane, which is pointed as up to 5 subcultures. In fact, no evidence of polymorphism was reported by Hsie et al. (2015) in micro-propagated sugarcane shoot tips after fifteen consecutive subcultures assessed by inter-simple sequence repeat DNA markers. Thus, based in our results, the MASP technique seems to be a promising tool to the early assessment of genetic fidelity of micro-propagated sugarcane plants with a great potential to be used as a tool in the quality control of large scale micro-propagation plants.

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