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Search for methylation-sensitive amplification polymorphisms in mutant figs

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ABSTRACT. Fig (*Ficus carica*) breeding programs that use conventional approaches to develop new cultivars are rare, owing to limited genetic variability and the difficulty in obtaining plants via gamete fusion. Cytosine methylation in plants leads to gene repression, thereby affecting transcription without changing the DNA sequence. Previous studies using random amplification of polymorphic DNA and amplified fragment length polymorphism markers revealed no polymorphisms among select fig mutants that originated from gamma-irradiated buds. Therefore, we conducted methylation-sensitive amplified polymorphism analysis to verify the existence of variability due to epigenetic DNA methylation among these mutant selections compared to the main cultivar 'Roxo-de-Valinhos'. Samples of

genomic DNA were double-digested with either *Hpa*II (methylation sensitive) or *Msp*I (methylation insensitive) and with *Eco*RI. Fourteen primer combinations were tested, and on an average, non-methylated CCGG, symmetrically methylated CmCGG, and hemimethylated hmCCGG sites accounted for 87.9, 10.1, and 2.0%, respectively. MSAP analysis was effective in detecting differentially methylated sites in the genomic DNA of fig mutants, and methylation may be responsible for the phenotypic variation between treatments. Further analyses such as polymorphic DNA sequencing are necessary to validate these differences, standardize the regions of methylation, and analyze reads using bioinformatic tools.

Key words: DNA methylation; Molecular marker; Mutation analysis; Plant breeding; Epigenetic inheritance

INTRODUCTION

Brazil is the major producer of figs (*Ficus carica*) in South America, and three States are noteworthy for their fig production: Rio Grande do Sul for fig production for industrial purposes, São Paulo for *in natura* fig production, and Minas Gerais for fig production for both industrial and *in natura* purposes. Fig tree cultivation in Brazil is based exclusively on a single cultivar, 'Roxo-de-Valinhos', which is characterized by high vigor and productivity (Pereira and Nachtigal, 1999).

Fig tree improvement programs that use conventional procedures to obtain new cultivars are rare in many countries, especially owing to 1) the low genetic variability associated with fig trees and 2) the difficulty in obtaining plants via gamete fusion, as the wasp *Blastophaga psenes*, which is responsible for the natural pollination of fig trees, is not found in Brazil because of the weather conditions (Ferreira et al., 2009).

Epigenetic variation describes molecular events responsible for the modulation of gene expression without changes in DNA sequences (Bird, 2007). These variations may be induced in response to stimuli and may persist after the removal of a stimulus, being inherited via vegetative propagation without permanent changes in the genotype (Borém, 1997). The main known cause of epigenetic change is methylation, which is the addition of methyl groups to cytosine bases in DNA located before and near guanine bases (Haines et al., 2001; Dodge et al., 2002). According to Bernstein et al. (2007), this type of epigenetic variation has been associated with the regulation of gene expression, genome defense, cellular differentiation, chromatin inactivation, and genomic imprinting.

The methylation-sensitive amplified polymorphism (MSAP)-polymerase chain reaction (methylation-sensitive arbitrarily primed polymerase chain reaction) technique developed by Reyna-López et al. (1997) is an adaptation of amplified fragment length polymorphism (AFLP) analysis and has proven to be a powerful tool for analyzing DNA methylation. The MSAP technique has been applied to study CpG methylation in the genomes of rice (Ashikawa, 2001) and banana (Baurens et al., 2003); to characterize methylation changes associated with micropropagated banana (Peraza-Echeverria et al., 2001) and apple (Li et al., 2002); to analyze the somaclonal variation of palm oil (Matthes et al., 2001); to analyze wheat vernal-

ization (Sherman and Talbert, 2002); to analyze the degree of cytosine methylation during the germination of sweet pepper seeds (Portis et al., 2004); to investigate the resistance of rice to bacterial blight (Sha et al., 2005); to investigate genes differentially methylated in tomato after tomato yellow leaf curl Sardinia virus infection (Mason et al., 2008); and to examine various stages of development in *Arabidopsis thaliana* (Ruiz-Garcia et al., 2005) and among *A. lyrata* and its parent species (Beaulieu et al., 2009).

Joyce and Cassells (2002) determined the cytosine methylation status of DNA in *in vitro*-developed microplants with various leaf morphologies by using AFLP marker analysis with methylation-sensitive restriction enzymes to test the hypothesis that DNA methylation could be used to characterize differences between treatments. While attempting to improve fig trees, Rodrigues et al. (2012a) used cuttings of the cultivar 'Roxo-de-Valinhos' irradiated with gamma rays at a dose of 30 Gy to show that plants could be selected based on unique morphological characteristics that differed from those of controls. The selected characteristics included elongated fruit shape; elongated peduncle, which facilitates harvesting and increases the shelf life of fruits; large fruit size; and large fruit with a closed ostiole, which reduces the incidence of agricultural pest infestation and prevents fruit depreciation.

However, polymorphisms in these irradiated selections were not identified in fig samples evaluated using random amplification of polymorphic DNA and AFLP analyses, that is, genetic modification was absent, suggesting that epigenetic changes caused by gamma irradiation occurred between treatments (Rodrigues et al., 2012b). Given these findings, the aim of this study was to verify the existence of variability related to epigenetic DNA methylation of mutant fig selections by comparing them with the primary commercial cultivar 'Roxo-de-Valinhos' using MSAP analysis.

MATERIAL AND METHODS

The experiment was conducted at Faculdade de Medicina de Ribeirão Preto (São Paulo, Brazil) in partnership with Universidade de Ribeirão Preto using the leaves of 5 fig selections. The specimens consisted of cuttings irradiated with gamma rays at the Energy Nuclear Center in Agriculture (Piracicaba, Brazil). Five irradiated plants considered to be mutants were used (Rodrigues et al., 2012a); these selections were compared to one another and to the cultivar 'Roxo-de-Valinhos', which served as a control.

Young leaves without spots or perforations were collected and washed under running water, and their veins were removed. The extraction of total genomic DNA from plant tissues was performed according to a modified cetyltrimethylammonium bromide technique.

To detect MSAP, we performed two digestions simultaneously for each sample of genomic DNA. In the 1st reaction, 250 ng genomic DNA was digested using the One-Phor-All Buffer (Amersham Pharmacia Biotech) containing 5 U *Eco*RI restriction enzyme (New England Biolabs®, Inc.) and 5 U *Msp*I, a methylation-insensitive restriction enzyme (New England Biolabs®, Inc.), in a final volume of 20 µL. The 2nd digestion was conducted using the methylation-sensitive restriction enzyme *Hpa*II (New England Biolabs®, Inc.) instead of the *Msp*I isoschizomer. The DNA fragments were digested and ligated to *Eco*RI (5'-CTCGTAGACTGCGTACC-3'/3'-CATCTGACGCATGGTTAA-5') and *Hpa*II/*Msp*I adapters (5'-GATCATGAGTCCTGCT-3'/3'-AGTACTCAGGACGAGC-5') using T4 DNA ligase (New England Biolabs®, Inc.). The binding reaction was performed at 23°C for 3 h.

After pre-amplification using the pre-selective primers E+1 (5'-GACTGCGTACCAAT TC+A-3') and HM+1 (5'-ATCATGAGTCCTGCTCGG+C-3'), the primers E+3 (5'-GACTG CGTACCAATTC+ANN-3') and HM+3 (5'-ATCATGAGTCCTGCTCGG+CNN-3'), where NN can be AC, AG, CA, CT, CC, CG, GC, or GG, were used for selective amplification following the protocols of Reyna-López et al. (1997). The selective amplification reactions were performed in a thermocycler with the following program: 12 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 1 min; 22 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min; and a final extension at 72°C for 2 min. The 14 primer combinations used are shown in Table 1.

Table 1. Combinations of primers used for MSAP analysis.

Combination	Primer *Eco*RI + A	Primer HM + C	Combination	Primer *Eco*RI + A	Primer HM + C
1	E + AG	HM + CAT	8	E + ATT	HM + CG
2	E + AT	HM + CAG	9	E + AGT	HM + CAT
3	E + AGT	HM + CTC	10	E + AGT	HM + CTA
4	E + AG	HM + CTA	11	E + AC	HM + CAG
5	E + AA	HM + CGT	12	E + AT	HM + CTA
6	E + ATC	HM + CAG	13	E + AG	HM + CTT
7	E + AC	HM + CTT	14	E + AA	HM + CG

The selective amplification products were separated on 6% polyacrylamide gels, and the bands were visualized with silver nitrate. Visualization of the samples after electrophoresis was performed with visual reading followed by photo-documentation of the plates.

In the plate analysis, methylation of the internal cytosine (CmCGG 5'-3') was indicated when bands present in the *Eco*RI + *Msp*I reaction were absent in the *Eco*RI + *Hpa*II reaction (Figures 1-3, black arrows). When bands present in the *Eco*RI + *Hpa*II reaction were absent in the *Eco*RI + *Msp*I reaction (see Figures 1-3, red arrows), the external cytosine of the DNA chain was methylated (5'-mCCGG-3'), which was considered hemimethylation (McClelland et al., 1994).

RESULTS

In the present study, MSAP analyses were performed to investigate the existence of regions with methylation patterns that differed between polymorphisms in mutant fig plants, thus characterizing their epigenetic distinction. Profiles of DNA amplified through MSAP analysis using 14 primer combinations were generated from the genomic DNA of selected fig mutants, which were originally derived from cuttings of the 'Roxo-de-Valinhos' cultivar after their buds were irradiated with gamma rays.

A total of 553 amplified fragments were obtained, 56 of which displayed different methylation patterns between treatments. Of the 14 pairs of primers tested, 100% exhibited polymorphic bands after digestion with the 2 enzymes tested, demonstrating that differential methylation occurred in the genomic material tested as shown in Figures 1-7. Unmethylated CCGG (bands common to both enzymes), symmetrically methylated CmCGG (bands present in DNA digested with *Msp*I but not with *Hpa*II), and hemimethylated hmCCGG (bands present in DNA digested with *Hpa*II but not with *Msp*I) comprised 88, 10, and 2% of the total amplification products, respectively.

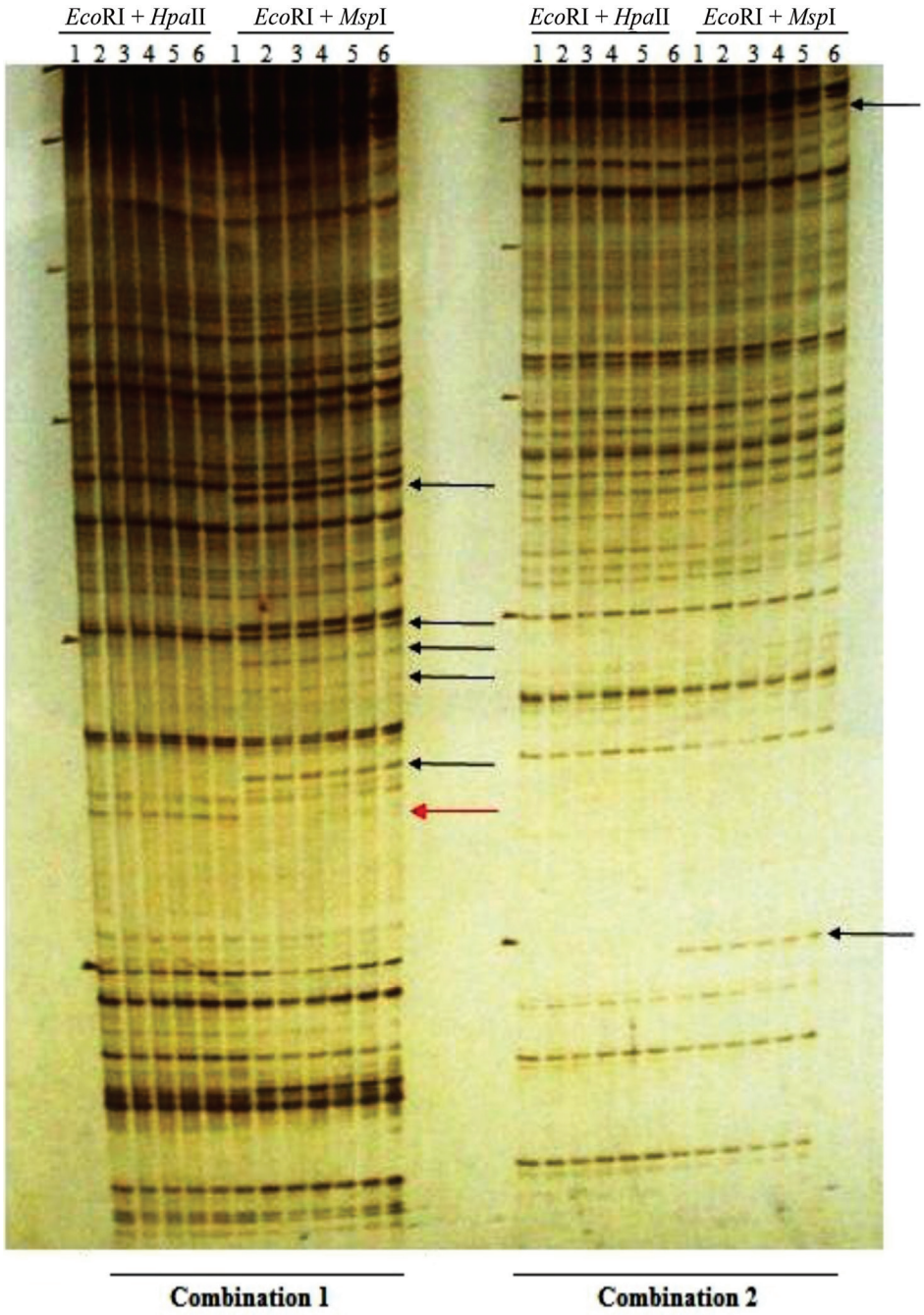

Figure 1. Amplification products using primer combinations 1 and 2 for fig mutants (*Ficus carica* L.) irradiated with gamma rays and the control 'Roxo-de-Valinhos' on 6% polyacrylamide gels. Numbers 1-6 refer to the following genotypes: *lane 1* = 'Roxo-de-Valinhos'; *lane 2* = PI 440; *lane 3* = PI 433; *lane 4* = PI 189; *lane 5* = PI 214; *lane 6* = PI 301.

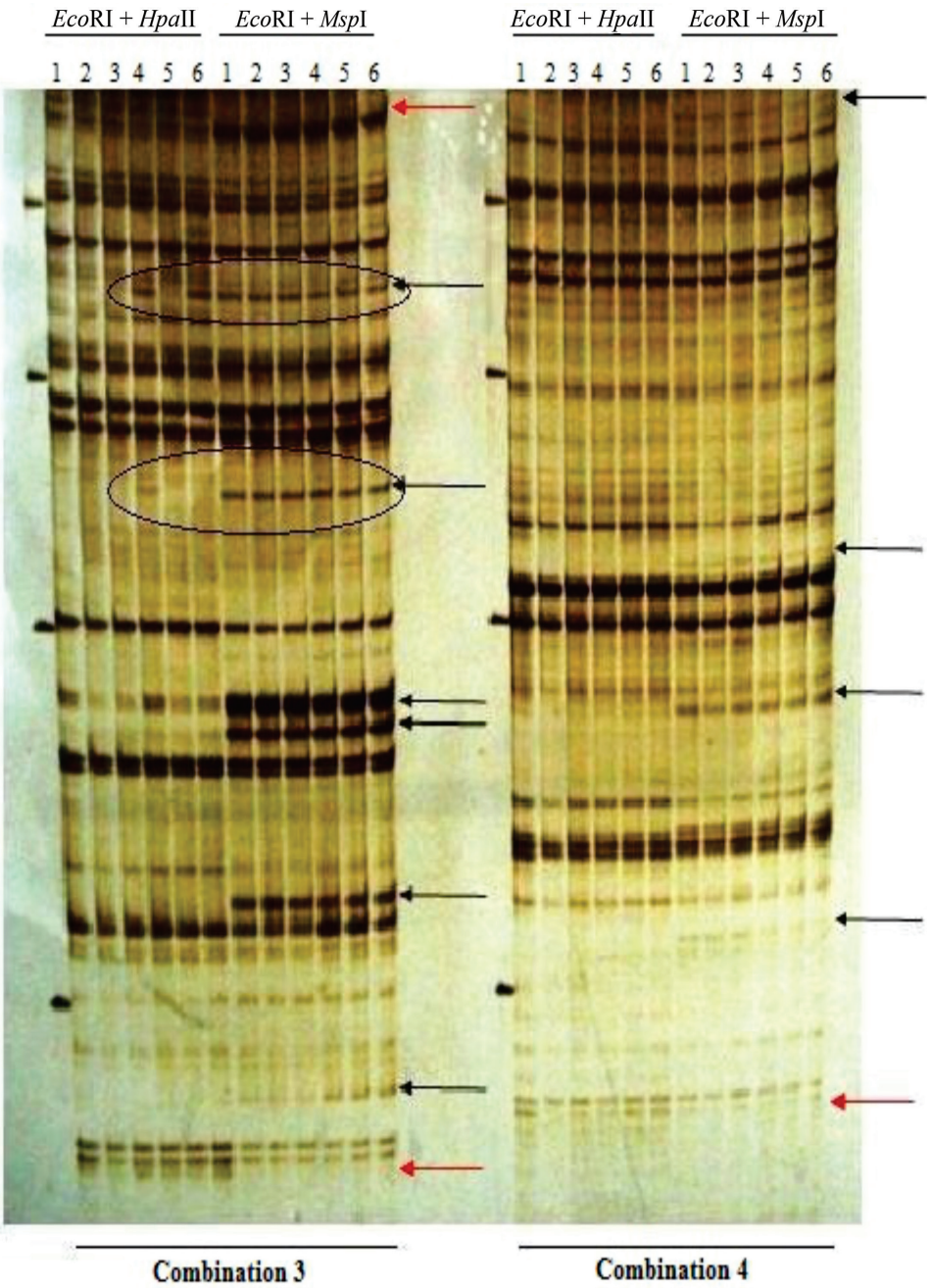

Figure 2. Amplification products using primer combinations 3 and 4 for fig mutants (*Ficus carica* L.) irradiated with gamma rays and the control 'Roxo-de-Valinhos' on 6% polyacrylamide gels. Numbers 1-6 refer to the following genotypes: *lane 1* = 'Roxo-de-Valinhos'; *lane 2* = PI 440; *lane 3* = PI 433; *lane 4* = PI 189; *lane 5* = PI 214; *lane 6* = PI 301.

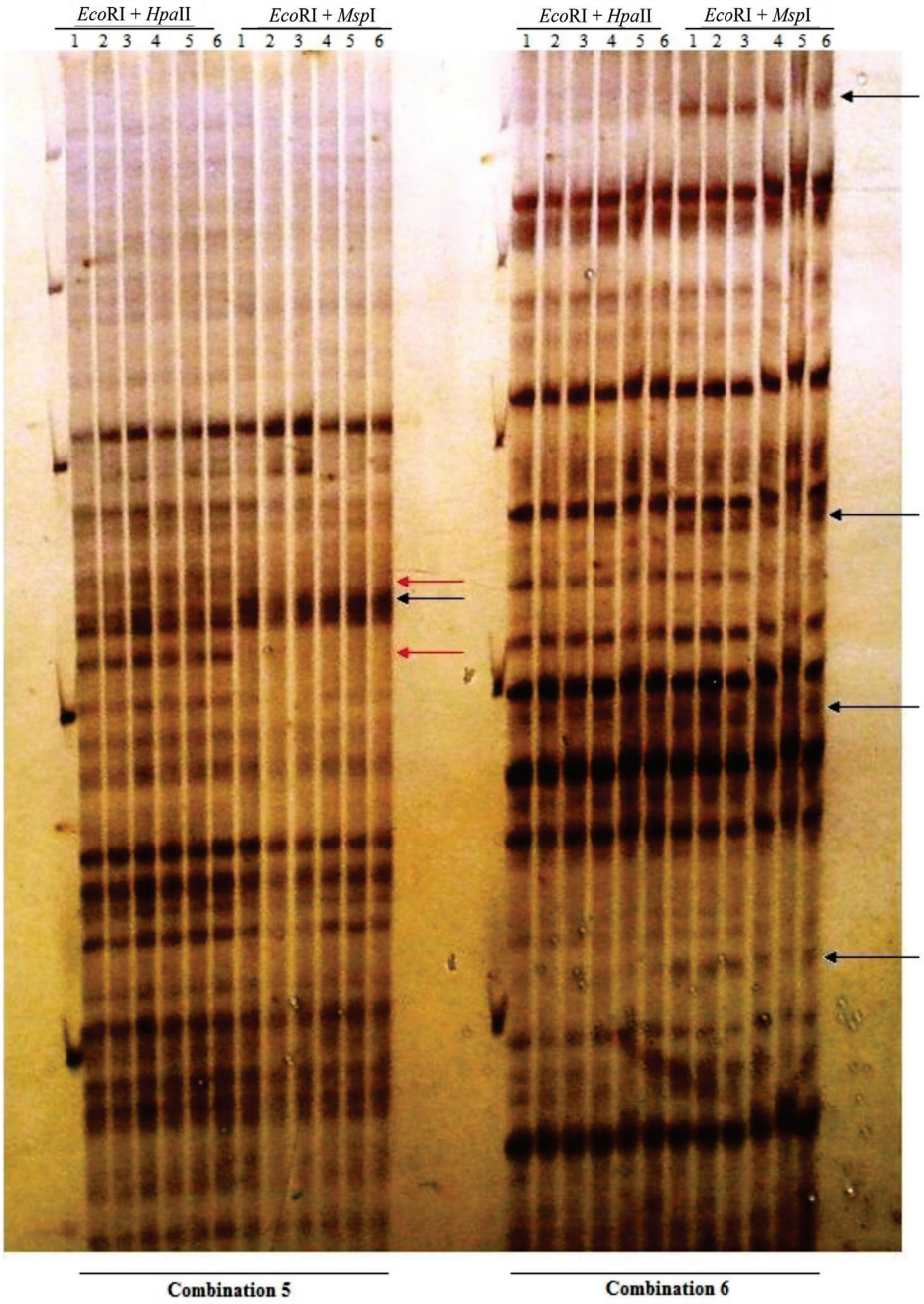

Figure 3. Amplification products using primer combinations 5 and 6 for fig mutants (*Ficus carica* L.) irradiated with gamma rays and the control 'Roxo-de-Valinhos' on 6% polyacrylamide gels. Numbers 1-6 refer to the following genotypes: *lane 1* = 'Roxo-de-Valinhos'; *lane 2* = PI 440; *lane 3* = PI 433; *lane 4* = PI 189; *lane 5* = PI 214; *lane 6* = PI 301.

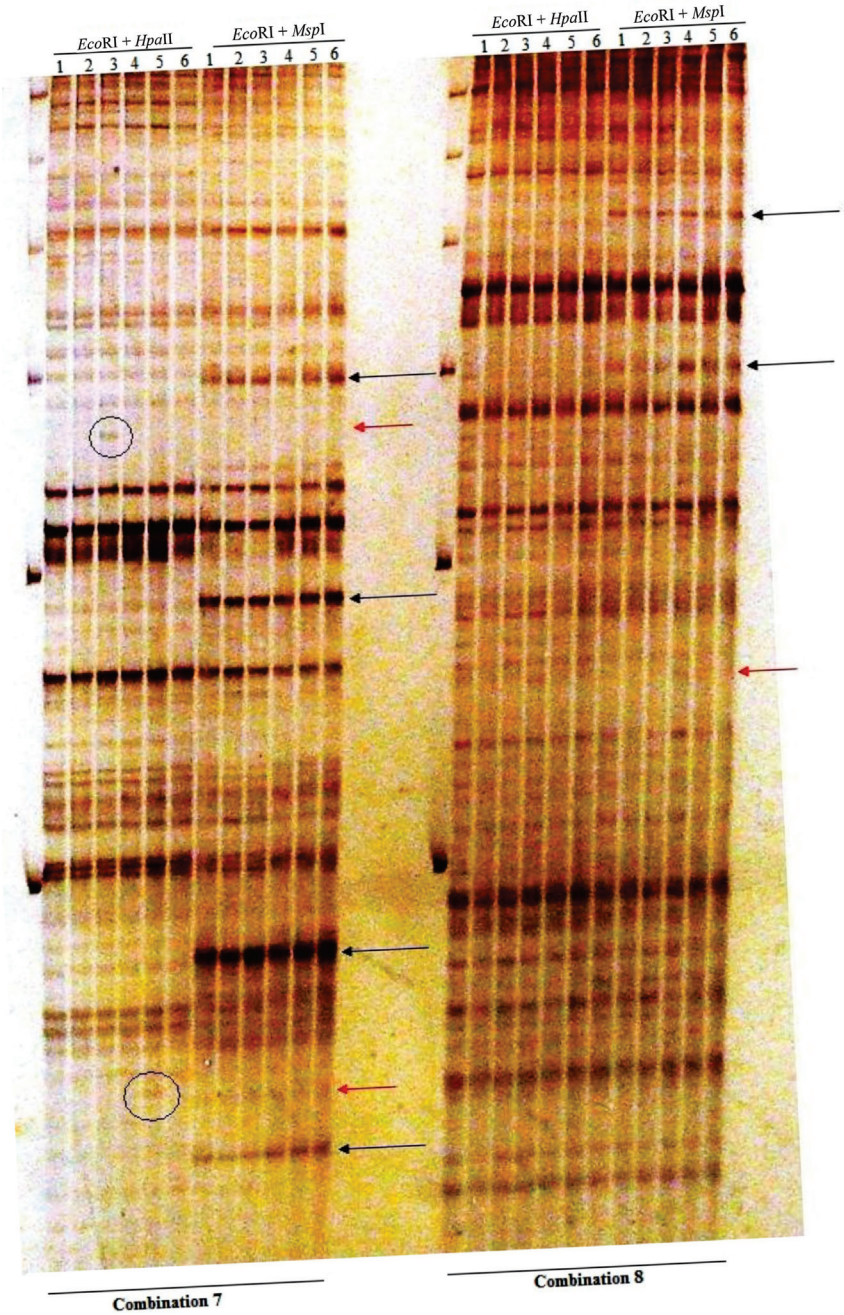

Figure 4. Amplification products using primer combinations 7 and 8 for fig mutants (*Ficus carica* L.) irradiated with gamma rays and the control 'Roxo-de-Valinhos' on 6% polyacrylamide gels. Numbers 1-6 refer to the following genotypes: *lane 1* = 'Roxo-de-Valinhos'; *lane 2* = PI 440; *lane 3* = PI 433; *lane 4* = PI 189; *lane 5* = PI 214; *lane 6* = PI 301.

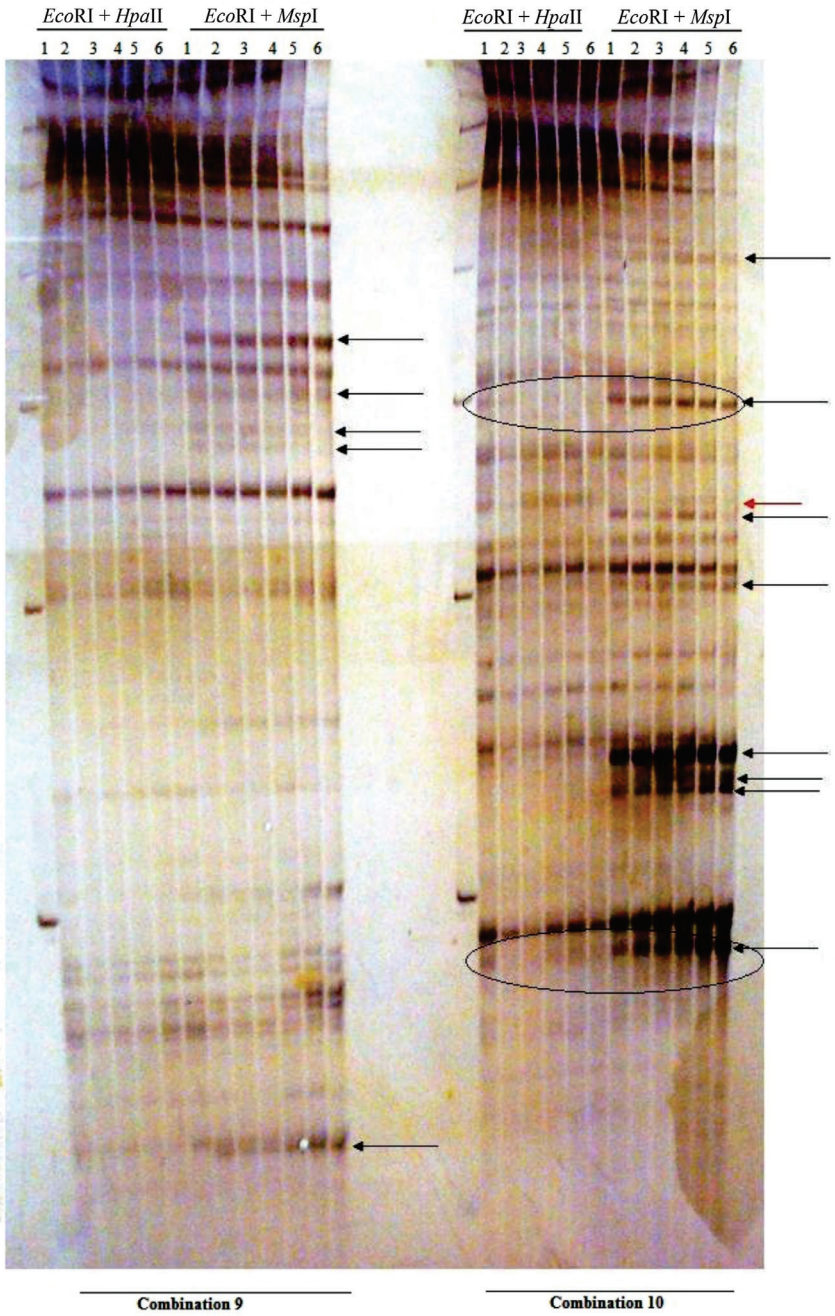

Figure 5. Amplification products using primer combinations 9 and 10 for fig mutants (*Ficus carica* L.) irradiated with gamma rays and the control 'Roxo-de-Valinhos' on 6% polyacrylamide gels. Numbers 1-6 refer to the following genotypes: *lane 1* = 'Roxo-de-Valinhos'; *lane 2* = PI 440; *lane 3* = PI 433; *lane 4* = PI 189; *lane 5* = PI 214; *lane 6* = PI 301.

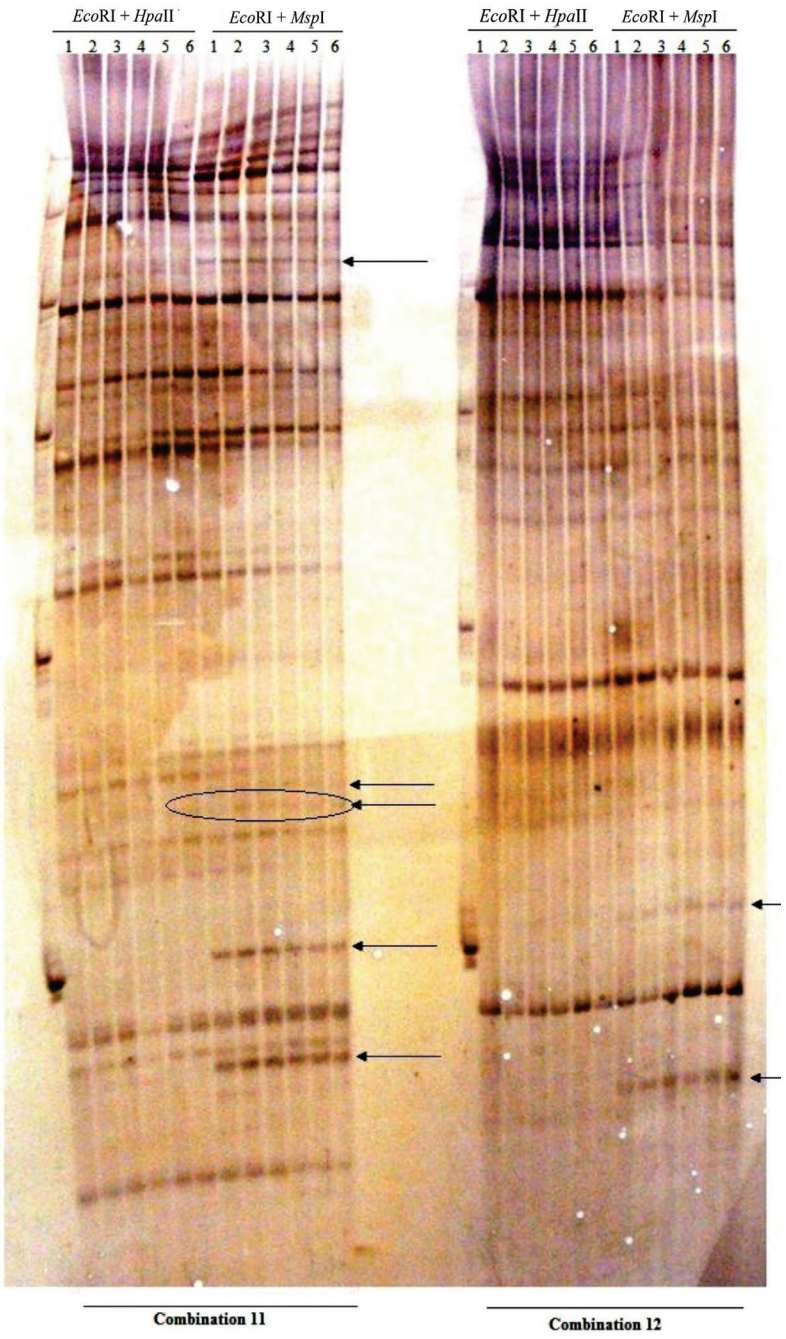

Figure 6. Amplification products using primer combinations 11 and 12 for fig mutants (*Ficus carica* L.) irradiated with gamma rays and the control 'Roxo-de-Valinhos' on 6% polyacrylamide gels. Numbers 1-6 refer to the following genotypes: *lane 1* = 'Roxo-de-Valinhos'; *lane 2* = PI 440; *lane 3* = PI 433; *lane 4* = PI 189; *lane 5* = PI 214; *lane 6* = PI 301.

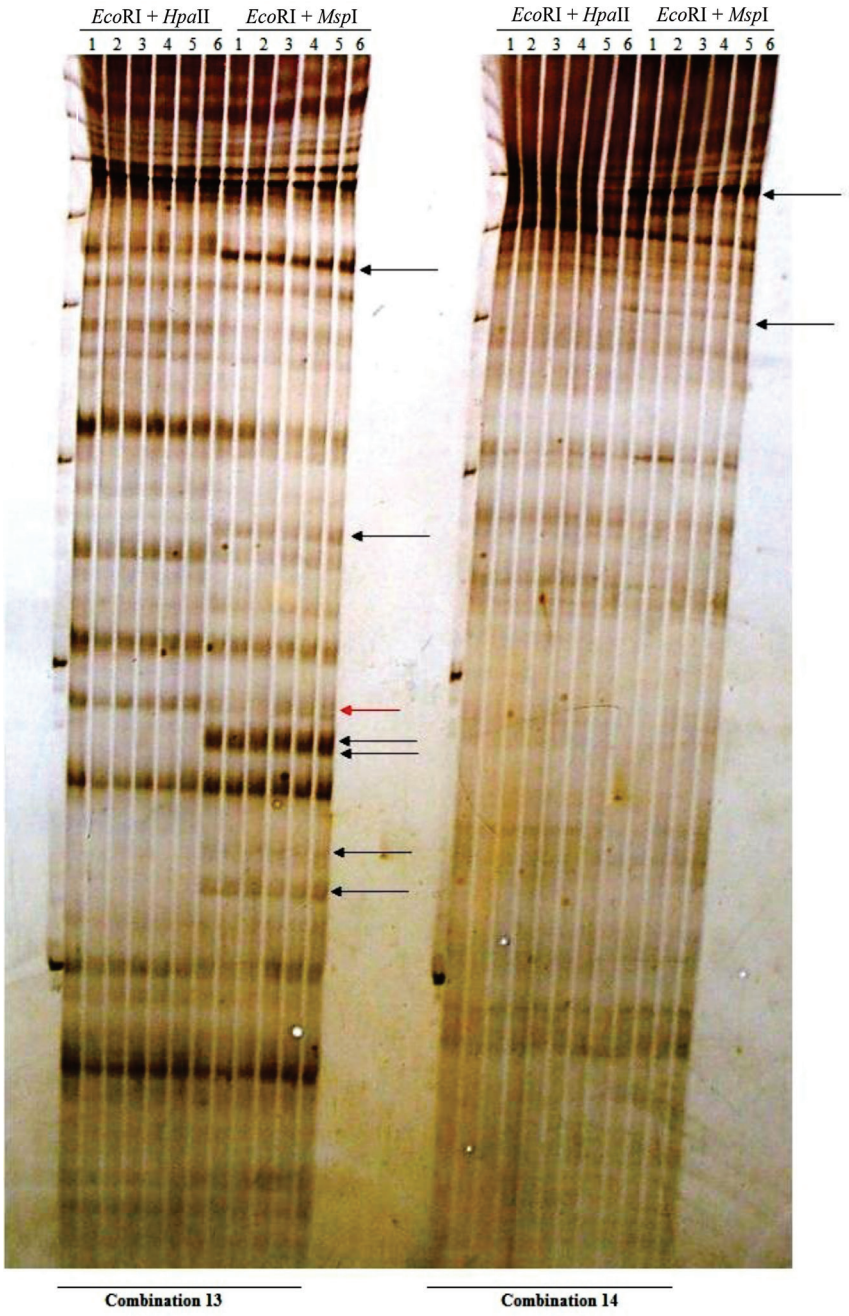

Figure 7. Amplification products using primer combinations 13 and 14 for fig mutants (*Ficus carica* L.) irradiated with gamma rays and the control 'Roxo-de-Valinhos' on 6% polyacrylamide gels. Numbers 1-6 refer to the following genotypes: *lane 1* = 'Roxo-de-Valinhos'; *lane 2* = PI 440; *lane 3* = PI 433; *lane 4* = PI 189; *lane 5* = PI 214; *lane 6* = PI 301.

Similar proportions of symmetrical methylated CCGG sites have been observed using approaches described by Ashikawa (2001) for rice (16.3%), Peraza-Echeverria et al. (2001) for micropropagated banana plants (23%), and Jaligot et al. (2004) for palm specimens. Jaligot et al. (2004) observed symmetrically methylated and hemimethylated sites in 14.7 and 4.1% of palm specimens, respectively.

Regarding the polymorphisms among treatments, a variation in the methylation pattern, which occurred with primer combinations 3, 7, 10, and 11, was observed, as displayed in focus by circles in Figures 2,4,5, and 6. This variation suggests that the individuals studied are epigenetically different from one another, as each treatment resulted in a distinct variety of fig, particularly with regards to treatment 4, which was superior to the 'Roxo-de-Valinhos' cultivar in all phenotypic characteristics evaluated (Rodrigues et al., 2009).

However, 'Roxo-de-Valinhos', which served as a control, was also methylated at all methylation sites using every combination except 10. The presence of the methylated sites in the control and some unmethylated individuals suggests a process of methylation in the genomic material referred to as DNA demethylation. This process is an inverse process to methylation and is reversible.

Accordingly, with combination 7, a single hemimethylated polymorphic band was observed in 2 sites, and these sites may be the positions of methylation associated with the regulation of gene expression in the subjects studied. Clark et al. (1997) showed that, in mammalian cells, the methylation of external cytosine blocked the binding of certain elements with their corresponding sites and inhibited their function. Therefore, these bands of interest should be investigated in further studies.

DISCUSSION

DNA methylation is an important modification that affects gene expression through epigenetic regulation involving phenotypic variation and some important agronomic characteristics (Manning et al., 2006; Hauben et al., 2009; Martin et al., 2009). In plant DNA, cytosine methylation leads to gene repression, altering genetic transcription without changing the DNA sequence; consequently, this phenomenon is one of the mechanisms responsible for phenotypic plasticity (Hepburn et al., 1987; Quemada et al., 1987).

Such gene silencing can occur in two ways: directly, whereby DNA methylation alters the binding sites of transcription factors, and indirectly, whereby binding proteins associate with methylated DNA and recruit co-repressors to establish environmental repression of chromatin (Klose and Bird, 2006).

DNA demethylation can occur both passively via the lack of methylation during several cycles of replication and actively in the absence of replication (Zhu, 2009; Ponferrada-Marín et al., 2010). Active demethylation has emerged as an important mechanism in plant genomes for modulating methylation patterns. Active demethylation may be carried out by either breaking thermodynamically unfavorable carbon-carbon bond links to pyrimidine methyl groups or through a repair process, leading to the replacement of the base m5C with C and change in gene expression (Kress et al., 2001; Bird, 2002).

The methylation of cytosine in DNA is generalized in eukaryotes; however, enormous variation occurs in its abundance and genomic distribution and points to *de novo* methylation and an inversion process of active demethylation of specific sequences (Furner and Matzke,

2011). Protection against *de novo* methylation by proteins or chromatin ensures that DNA methylation never reaches a DNA sequence domain. Unmethylated domains can also exist in an active form and remove DNA modification. Working with nuclear proteins involved in the maintenance of genome stability, Baker et al. (2007) concluded that this nuclear proteins expression is directly linked with the active demethylation of DNA, resulting in the activation of epigenetically silenced genes. Recent studies of methylation in seed development in *Arabidopsis* mutants have reported evidence of loss of methylated CG sites in the endosperm (Gehring et al., 2009; Hsieh et al., 2009). Collectively, these studies suggest that mutation is involved in active demethylation of the maternal genome, giving rise to the endosperm and increasing small interfering RNA production in this tissue (Mosher et al., 2009; Mosher, 2010).

Concluding, the MSAP technique was effective for detecting differentially methylated sites in the genomic material studied, revealing their genetic/epigenetic divergence. Methylation at some sites in the control suggested that the demethylation of genomic polymorphic materials may be responsible for phenotypic variation among different treatments. Bisulfite sequencing should be performed to verify whether these methylation events occur in regulatory genes and therefore to clarify whether methylation and active demethylation are involved in gene regulation.

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