Journal of Applied Biological Sciences
E-ISSN: 2146-0108 15(1): 12-19, 2021
Research Article

ASSESSMENT OF DNA METHYLATION LEVELS IN THE DIFFERENT ORGANS OF *SARCOPOTERIUM SPINOSUM* (L.) SPACH

Yucel Gokgoz[1], Hulya Sipahi[2,*]

[1] *Sinop University, Scientific and Technological Researches Center, Sinop, Turkey*
[2] *Sinop University, Faculty of Arts and Sciences, Department of Biology, Sinop, Turkey*

**Corresponding Author:*
E-mail:hsipahi@sinop.edu.tr

(Received 21st August 2020; accepted 30th November 2020)

ABSTRACT. DNA methylation at CCGG region plays a vital role in regulating gene expression. Cytosine methylation levels in the four organs of *Sarcopoterium spinosum* L. Spach, used as folk medicine, were detected and compared using the fluorescent Methylation-Sensitive Amplified Polymorphism (MSAP) technique. Internal cytosine full or hemimethylated (CG) at CCGG were common in all organs. The only one monomorphic region was detected only in the CG methylation type. When organs are compared binary, the leaf-stem has monomorphic regions of 14 (4.8%), leaf-fruit 4 (1.4%), leaf-root 5 (1.7%), fruit-stem 6 (2.1%), fruit-root 2 (0.7%), stem-root 5 (1.7%).These results showed that the similarity levels of CG and CHG methylations of organs are quite low. MSAP ration is the lowest in the fruit (23.9%), while the highest in stem and root (38.4%). The different methylation cases among four organs may be caused by differences in the expression of tissue-specific genes related to cell differentiation and development. However, cloning, sequencing, and uncovering the functions of tissue-specific fragments are needed to verify these results. MSAP is found a highly efficient method for large-scale detection of cytosine methylation, especially for *S. spinosum*, which is rich in cytosine methylation and do not have genome sequence knowledge.

Keywords: *Cystosine, methylation, MSAP, Sarcopoterium spinosum*

INTRODUCTION

Sarcopoterium spinosum (L.) Spach is a small barbed bush that can be used in folk as a curative against high blood sugar, kidney stone, pain, mental fears, and eye complaints [1, 2, 3, 4, 5]. This plant, which belongs to the Rosaceae family, is an important type of phrygana located in the Eastern Mediterranean Basin in Turkey, on the Aegean, Marmara, and the Black Sea coastline. This plant grows in Sinop province, which is the only place with only a warm and rainy Mediterranean climate on the entire Black Sea coastline stretching from the Eastern Black Sea to Istanbul province [6].

The genome can undergo several changes in changing environmental conditions, with the DNA sequence unchanged, but this change can be passed on to future generations by mitosis/meiosis. This phenomenon, called epigenetics, includes DNA methylation, DNA modifications, genomic stigma, histon modifications, destruction of X chromosome activity, control of mRNA expression with non-encoder RNAs, and chromatin changes [7]. After the stress, plant somatic cells remember the experience gained by epigenetic mechanisms [8]. DNA cytosine methylation in plants contributes to the provision of genomic integrity, control of gene expression, cell differentiation, development, and response to biotic-abiotic stresses. Methylation of cytosine in gene promoters plays a role in suppressing transcription by inhibiting the binding of transcription factors. In addition,

cytosine methylation regulates genomic imprinting and the activity of transposable elements [9]. Genomic imprinting with cytosine methylation is a regulatory mechanism for controlling the different expression of maternal and paternal genes in the seeds of flowering plants [10].

Environmental factors lead to the emergence of hereditary and phenotypical variations and the development of adaptation mechanisms by changing the level of DNA methylation. Cytosine-5 hypomethylation or hypermethylation regulates the expression of plant development and abiotic/biotic stress-sensitive genes [11, 12]. The loss of DNA methylation affects important characteristics of the plant such as yield, fruit ripening, seed size, flowering time, and pathogen resistance [13, 14, 15, 16]. Changes in the level of DNA methylation in tissues indicate that epigenetic control is important in forming phenotypic diversity [17]. Different levels of methylation may be associated with specific gene expression in various tissues. The total methylation levels the different tissues were found between 16 and 45% in cotton [17], rice [18], Arabidopsis [19], maize [20] and sorghum [21].

There are many approaches to assessing cytosine methylation of plant genomes. One of them is Methylation-Sensitive Amplified Polymorphisms (MSAP) derived from AFLP. In this technique, genomic DNA is cut by two restriction enzymes (*EcoRI*/*HpaII* or *EcoRI*/*MspI*). *HpaII* and *MspI* recognize the 5'-CCGG-3 sequences. These isoschizomer enzymes show different sensitivity to cytosine methylation in the 5'-CCGG-3 sequences. *HpaII* cuts this hemi-methylsequence in the outer cytosine (5'-mCCGG) (methyl only in one DNA chain), while MspI cuts the hemi- or fully methyl 5'-CMCGG. After *EcoRI*/*HpaII* and *EcoRI*/*MspI* cutting, comparison of fragment models obtained by selective amplification allows the detection of epigenetic variability. Epigenetic variability will occur with differences in the presence or absence of fragments between *EcoRI*/*HpaII* and *EcoRI*/*MspI* patterns of the same example [22].

In rice [18], Arabidopsis [19], maize [20], sorghum [21] and cotton [12, 17, 23, 24], the changes at the total DNA methylation level determined by MSAP in different tissue and development stages have been found to be an important factor in creating phenotypical diversity. In the present study, DNA cytosine methylation status in CCGG regions in the leaves, fruit, stem, root of *S. spinosum* (L.) Spach. was investigated and compared using the fluorescent MSAP technique.

MATERIALS AND METHODS

Materials

The leaf, fruit, stem, and root organs of *S. spinosum* naturally grown in Sinop Province, Boztepe Peninsula, Zeytinlik Region (42° 01' 24" N, 35° 10' 3" E) were used. Young shoots of organs were washed with pure water and kept for 5-10 minutes in 80% ethanol, and again washed three times with sterile water.

Methods

Genomic DNA is isolated by CTAB methods developed by Lade *et al.* [25] and Khan *et al.* [26]. The MSAP method is a version of the AFLP method developed by Vos *et al.* [27] modified by Reyna-Lopez *et al.* [28]. MSAP analysis developed by Baurens *et al.* [29] was used by minor modifications.

MSAP method consists of four main parts including cutting, binding reactions, pre- and selective amplification reactions, and determination of band models. The adapters and primers used were developed by Xiong *et al.* [18] (Table 1).

Table 1. *Adapters and primers sequences*

Adapters (5′→3′)
*Eco*RI adapter 1 CTCGTAGACTGCGTACC
*Eco*RI adapter 2 AATTGGTACGCAGTCTAC
*Hpa*II/*Msp*I adapter 1 GATCATGAGTCCTGCT
*Hpa*II/*Msp*I adapter 2 CGAGCAGGACTCATGA
Pre-amplification primers (5′→3′)
*Eco*RI – GACTGCGTACCAATTC
*Hpa*II/*Msp*I – ATCATGAGTCCTGCTCGG
Selective amplification primers (5′→3′)
*Eco*RI – AACGACTGCGTACCAATTCAAC
*Hpa*II/*Msp*I – TCAAATCATGAGTCCTGCTCGGTCAA
*Hpa*II/*Msp*I – TCCAATCATGAGTCCTGCTCGGTCCA

The digestion of DNA samples was carried out using *EcoRI* (Fermentas) according to manufacturer's instructions. After digestion, the mixture was inactivated at 65 °C for 20 min, and stored at 20 °C. This mixture is divided into two separate tubes for digestion with *MspI* and *HpaII* enzymes.

MspI or *HpaII* digestion was carried out by incubating at 37 ^{0}C for 6 h in a volume of 40 µl containing DNA digested with *EcoRI*, 1 µl enzyme, 4 µl Buffer Tango (10×), and 15 µl *EcoRI*. For enzyme inactivation, tubes were held for 20 m at 80 ^{0}C. After digestion, two different adapters were ligated to create *EcoRI* and *HpaII/MspI* sticky ends. The ligation of adapters was performed in mixture containing 5 µl *EcoRI* adapter, 5 µl *HpaII/MspI a*dapters, 1 µl T4 DNA Ligase, 5 µl, T4 DNA Ligase buffer (10×), and 25 µl ddH_2O. The mixture was incubated at 4 °C overnight.

Digested and ligated DNA fragments were diluted 1 to 5 (v:v) with ddH_2O for pre-amplification. Preamplified PCR reactions were done in a volume of 50 µl containing 5 µl of diluted ligation products, 5 µl *HpaII* primer (2 µM), 5 µl *EcoRI* primer (2 µM), 5 µl 10× PCR buffer, 4 µl $MgCI_2$ (2 mM), 3 µl dNTP (10 mM), 1 U Taq DNA Polymerase (1u/µl), and 22 µl ddH_2O. PCR conditions were as follows: 94 ^{0}C for 5 m, 20 cycles of 94 °C for 30 s, 56 °C for 1 m, and 72 °C for 1 min, and 72 °C for 5 min. Pre-amplification PCR mixture was diluted 1 to 20 (v:v) with ddH_2O for selective amplification. Selective amplification was carried out using *EcoRI* primer, and *HpaII/MspI* selective primer, which had three and four additional selective nucleotide bases compared to the preamplified primers (Table 1).

Selective PCR mixture included 5 mL of pre-amplification mix (diluted 1:20), 2 µl 10× PCR buffer, 1 µl dNTP (10 mM), 2 µl *EcoRI* selective primer (2 µM), 1 µl *HpaII/MspI* selective primer (2 µM), 0.4 µl $MgCI_2$ (50 mM), 1 U Taq polymerase, and 7.6 µl ddH_2O.PCR conditions were 94 ^{0}C for 5 min, followed by 12 cycles of 94 ^{0}C for 30 s, 65 ^{0}C (decreases by 0.7 ^{0}C per cycle) for 30 s, and 72 ^{0}C for 1min, 23 cycles of 94 ^{0}C for 30 s, 56 ^{0}C for 1 min, 72 ^{0}C for 1 min The final extension was at 72 ^{0}C for 5 min. DNA fragments were separated in the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The obtained electropherograms were aligned

according the signal of the DNA markers with a size range between 100–500bp, analyzed and visualized using Peak Scanner Software version 1.0. (Applied Biosystems). The fragments between 100 bp and 1000 bp with peak height >100 were scored. The fragments (peaks) present in both lanes (*HapII* and *MspI*) were scored (0); those peaks present in either *HpaII* or *MspI* were scored (1).

RESULTS AND DISCUSSION

The MSAP is a method in which genomic DNA is digested with two isoschizomer restriction enzymes (*HpaII* and *MspI*) having different sensitivity to the cytosine nucleotide in CCGG sequences, and then is done the selective amplification of digested fragments. This method is one in which AFLP is modified and differs by the use of methylation-sensitive enzymes. The variation in the materials examined in both methods is based on differences in the number and size of the fragments obtained. However, in MSAP analysis, the variation determined in terms of the presence and absence of amplified fragments between models *EcoRI*/*HpaII* and *EcoRI*/*MspI* is associated with cytosine methylation status in CCGG sequence. MSAP can measure CG, and CHG methylation content changes. In MSAP, CG methylation status means the presence of fragments in *EcoRI*/*MspI*, the absence in *EcoRI*/*HpaII* (Type II), conversely, the CHG methylation status is determined by the absence of fragments (Type III) in *EcoRI*/*MspI* and the presence of fragments in *EcoRI*/*HpaII* ([31], Table 2). Thus, the different fragment models that are selected after selective duplication reflect the methylation status and level in the CCGG region. In the case of full methylation of external cytosine or both cytosine (CC methylation), *MspI* and *HpaII* cannot cut (Type IV), and so these two methylation conditions are indistinguishable with the MSAP technique. But, CC methylation can be determined only if DNA is digested with *BsiSI*, an insensitive enzyme to methylation. In scoring the MSAP results, Type I was given 0, Type II and Type III 1. Type IV is considered missing data.

Table 2. *Methylation sensitivity and digestion model of isoschizomers*

Types	**Methylation status**		**Fragment Model**		**Score**
			EcoRI/HpaII	***EcoRI/MspI***	
Type I	5′-CCGG GGCC-5′	Non-methylated cytosines	1	1	0
Type II	5′-C**C**GG GG**C**C-5′ 5′-C**C**GG GGCC-5′	Full or hemi methylation of internal cytosine (CG)	0	1	1
Type III	5′-**C**CGG GGCC-5′	Hemi methylation of external cytosine (CHG)	1	0	1
Type IV	5′-**CC**GG GG**CC**-5	Full and hemi methylation of both cytosines	0	0	-
	5′-**C**CGG GGC**C**-5′	Full methylation of external cytosine			

*Bold and underlined cytosines are methylated.

The two types of cytosine methylation modification (Type II (CG), Type III (CHG)) in CCGG regions were defined in the leaf, stem, fruit, and root organs of *S. spinosum*,

total methylation levels were calculated and compared between organs (Table 2). After *EcoRI*/*HpaII* and *EcoRI*/*MspI* enzyme digestion, the selective amplifications were done using 2 combinations with 2 *EcoRI* primers (-AAC and -ACC) labeled FAM and HEX, and 2 unlabeled *HpaII*/*MspI* primers (-TCAA and -TCCA) (Table 1). 289 fragments were detected on the all tissues (Table 2). When examining the methylation types in the CCGG regions, it was found that the maximum number of fragments in all tissues except fruits was found in Type II (internal cytosine full or hemi methylated (CG), so full or hemi methylation of internal cytosine in the *S. spinosum* genome was more common. Methylation sensitive amplification polymorphism (MSAP) ration is the lowest in the fruit (23.9%), while the highest in stem and root (38.4%). Likewise, the methylation type II (CG) ration was found to be higher than the Type III (CHG) ration in seven tissues from two inbred sorghum genotypes and five tissues from their F_1 hybrids [21]. However, Type II (15.1-19.6%), Type III (7.2-9%), and MSAP (24.1% to 28.2%) rations were generally lower than those detected in the present results. Among the seven tissues of the sorghum, the lowest rate of CG and total (CG and CHG) methylation rate was determined in the endosperm, but this difference was not found statistically important. Similarly, the methylation analysis of tassel, bracteal leaves and ear leaf tissues in maize found that the full methylation of internal cytosine (Type II, CG) was the most common methylation model, and MSAP proportions (24.1% to 28.2%) were found to be lower than those detected in the present results [20]. The MSAP was found high on the leaves and low on the stems. Besides, the result of the sequencing and blast analysis of 9 fragments found to be differently methylated in different tissues of maize revealed that the cytosine methylation was located in repetitive sequences that do not encoding and coding. As the insignificant difference in terms of relative methylation levels between inbred and hybrids ($p>0.05$) indicated that inbred parental methylation cases are transferred to F_1 hybrids with stable meiotic inheritance. Also, different levels of methylation in organs have shown in various plants. For example, the higher methylation rates have been observed in mature leaves than seedling leaves in Arabidopsis [19], a seedling leaf than the flag leaf in rice [18], and the bracteal leaf than ear leaf in maize [20].

Table 3. *Cytosine methylation levels of the four tissues*

Tissues	Non-methylated CCGG regions		Methylated CCGG regions					
	Type I Fragments	(%)	Type II Fragments (CG)	(%)	Type III Fragments (CHG)	(%)	Total Methylated Fragments	MSAP (%)
Leaf	9	3,1	61	21,1	35	12,1	96	33,2
Fruit	28	10,4	12	4,1	57	19,7	69	23,9
Stem	16	5,5	94	32,5	17	5,9	111	38,4
Root	6	2,1	58	20,1	53	18,3	111	38,4

In terms of Type II (0, 1) and Type III (1, 0) of *EcoRI*/*HpaII* / *EcoRI*/*MspI* fragment pattern, the monomorphic state (0, 1, 0, 1, 0, 1, 0, 1 or 1, 0, 1, 0, 1, 0, 1, 0) for four organs indicates similar methylation status in tissues. In the four tissues examined, one monomorphic region was detected only in Type II. This indicated that the four organs have a very different methylation state. When organs are compared binary in terms of

Type II and Type II methylation cases, the leaf-stem has monomorphic region of 14 (4.8%), leaf-fruit 4 (1.4%), leaf-root 5 (1.7%), fruit-stem 6 (2.1%), fruit-root 2 (0.7%), stem-root 5 (1.7%). These results showed that the similarity levels of methylation of organs are quite low. Contrary to these results, the methylation study of tassels, bracteal, and ear leaf tissues of 18 red and 18 white inbred maize showed the presence of tissue-specific methylation, but 80% of methylation in these three tissues was found similar [20]. The methylation state similarity between tassel and ear leaf was determined as 19.35 – 14.32%, between tassel and bracteal leaf 33.87 – 28.57% and between bracteal leaf and ear leaf 45.16 – 53.06%. The researchers attributed the cause of this result to the fact that bracteal and ear leaves are vegetative organs and have similar tasks, while tassels are a reproductive organ with functions such as flowering, fertilization, and fruit-bearing.

It has reported that the regulatory role of DNA methylation on gene expression may be effective in eliciting differences in DNA methylation levels in various organs and developmental stages [20, 30]. The most variable methylation mapped in genes is associated with CG methylation. It has reported that some genes are expressed tissue-specific by having different methylation conditions in tissues [21, 31, 32, 33]. Hypermethylation or demethylation of CpG islands in promotors can silence or activate genes. However, there are 5-CCGG-3 sequences with methylcytosine in coding and non-coding regions, introns, and repetitive elements that contain potentially active transcripts.

CONCLUSION

In this study, cytosine methylation in the genome of four organs of *S. spinosum* has investigated using the fluorescent MSAP technique, and different MSAP fragments have identified due to different methylation cases of organs in CCGG regions. The results showed that MSAP is highly efficient for large-scale detection of cytosine methylation, and is a method that sheds light on research about plants with complex genomes, which have not genome sequencing knowledge and rich in methylation polymorphism.

Differences in MSAP models of four organs in terms of DNA methylation are likely due to differences in the expression of tissue-specific genes related to cell differentiation, development and functions in these four organs. Further studies, such as cloning, sequencing, and uncovering the functions of tissue-specific MSAP fragments, may be important in uncovering genes especially involved with the synthesis of medically important volatile compounds.

REFERENCES

[1] Yaniv, Z., Dafni, A., Friedman, J., Palevitch, D. (1987): Plants used for the treatment of diabetes in Israel. Journal of Ethnopharmacology 19(2): 145-151.

[2] Reher, G., Reznicek, G., Baumann, A. (1991): Triterpenoids from *Sarcopoterium spinosum* and Sanguisorba minor. Planta Medica 57(5): 506.

[3] Reher, G., Slijepcevic, M., Kraus, L. (1991) : Hypoglycemic activity of triterpenes and tannins from the root bark of *Sarcopoterium spinosum* and two Sanguisorba species (*S. minor* and *S. officinalis*). Planta Medica 57(8): 57-58.

[4] Henkin, Z., Resenzweig, T., Yaniv, Z. (2014): *Sarcopoterium spinosum*. In: Yaniv Z, Dudai N(editors). Medicinal and Aromatic Plants of the Middle-East, Medicinal and Aromatic Plants of the World. Dordrecht: Springer, p.151-163.

[5] Bachrach, Z. Y. (2007): Ethnobotanical studies of *Sarcopoterium spinosum* in Israel. Israel Journal of Plant Science 55(1): 111-114.

[6] Elmas, E., Kutbay, H. G. (2015)**:** Plant functional types in Mediterranean enclaves in Western Black Sea Region of Turkey. Turkish Journal of Botany 39: 30-39.

[7] Roth, T. L, Roth, E. D, Sweatt, J. D. (2011)**:** Epigenetics of Memory Processes, In: Chao W. (editor). Handbook of Epigenetics, The New Molecular and Medical Genetics, Elsevier, pp 381-390.

[8] Peng, H., Zhang, J. (2008)**:** Plant genomic DNA methylation in response to stresses: Potential applications and challenges in plant breeding. Progress in Natural Science 19: 1037-1045.

[9] Ikeda, Y., Nishimura, T. **(**2015)**:** Nuclear Functions in Plant Transcription, Signaling and Development. In: Pontes O, Jin H (editors). The Role of DNA Methylation in Transposable Element Silencing and Genomic Imprinting. New York, Springer.

[10] Finnegan, E. J, Peacock, W. J., Dennis, E. S. (2000)**:** DNA methylation, a key regulator of plant development and other processes. Current Opinion in Genetics and Development 10: 217–223.

[11] Pavet, V., Quintero, C., Cecchini, N. M., Rosa, A. L., Alvarez, M. E. (2006)**:** Arabidopsis Displays Centromeric DNA Hypomethylation and Cytological Alterations of Heterochromatin Upon Attack by *Pseudomonas syringae*. Molecular Plant Microbe Interaction 9(6): 577-87.

[12] Cao, D., Gao, X., Liu, J., Kimatu, J. N., Geng, S., Wang, X. P., Zhao, J., Shi, D. C. (2011)**:** Methylation sensitive amplified polymorphism (MSAP) reveals that alkali stress triggers more DNA hypomethylation levels in cotton (*Gossypium hirsutum* L.) roots than salt stress. African Journal of Biotechnology 82: 18971-18980.

[13] Finnegan, E. J, Genger, R. K, Kovac, K., Peacock, W. J., Dennis, E. S. (1998): DNA methylation and the promotion of flowering by vernalization. Proceedings of the National Academy of Sciences of the United States of America 95: 5824–5829.

[14] Manning, K., Tor, M., Poole, M., Hong, Y., Thompson, A. J., King G. J., Giovannoni, J. J., Seymour, G. B. (2006)**:** A naturally occurring epigenetic mutation in a gene encoding an SBP-box transcription factor inhibits tomato fruit ripening. Nature genetics 38: 948–952.

[15] Hauben, M., Haesendonckx, B., Standaert, E., Van Der Kelen, K., Azmi, A., Akpo H, Breusegem, F. V., Guisez, Y., Bots, M., Lambert, B., Laga, B., De Block, M. (2009)**:** Energy use efficiency is characterized by an epigenetic component that can be directed through artificial selection to increase yield. Proceedings of the National Academy of Sciences of the United States of America 106: 20109– 20114.

[16] Zhong, S., Fei, Z., Chen, Y. R., Zheng, Y., Huang, M., Vrebalov, J., McQuininn, R., Gapper, N., Liu, B., Xiang, J., Shao, Y., Giovannoni, J. J. (2013)**:** Single-base resolution methylomes of tomato fruit development reveal epigenome modifications associated with ripening. Nature Biotechnology 31: 154–159.

[17] Osabe, K., Clement, J. D., Bedon, F., Pettolino, F. A., Ziolkowski, L., Llewellyn, D. J., Finnegan, E. J., Wilson, I. W. (2014)**:** Genetic and DNA Methylation Changes in Cotton (*Gossypium*) Genotypes and Tissues. PLoS One 9(1): 1-11.

[18] Xiong, L. Z., Xu, C. G, Saghai Maroof, M. A., Zhang, Q. (1999). Patterns of cytosine methylation in an elite rice hybrid and its parental lines, detected by a methylation-sensitive amplification polymorphism technique. Molecular Genetics and Genomics 261: 439–446.

[19] Ruiz-Garcia, L., Cervera, M. T., Martinez-Zapater, J. M. (2005): DNA methylation increases throughout Arabidopsis development. Planta 222: 301–306.

[20] Lu, Y., Rong, T., Cao, M. (2008): Analysis of DNA methylation in different maize tissues. Journal of Genetics and Genomics 35: 41–48.

[21] Zhang, M., Xu, C., Wettstein, D., Liu, B. (2011): Tissue-Specific Differences in Cytosine Methylation and their Association with Differential Gene Expression in Sorghum bicolar. Plant physiology 156: 1955–1966.

[22] Guevara, M. Á, Nuria, M., Sáez-Laguna, E., Vélez, M. D., Cervera, M. T., Cabezas, J. A. (2017): Plant Epigenetics: Methods and Protocols, Methods in Molecular Biology. In: Kovalchuck I, (editor). Analysis of DNA Cytosine Methylation Patterns Using Methylation-Sensitive Amplification Polymorphism (MSAP). Boston: Humana Press, pp 99-112.

[23] Zhao, Y., Yu, S., Xing, C., Fan, S., Song, M. (2008): Analysis of DNA methylation in cotton hybrids and their parents. Molecular Biology 42: 169.

[24] Zhao, Y., Yu, S., Ye, W., Wang, H., Wang, J., Fang, D. (2010): Study on DNA Cytosine Methylation of Cotton (*Gossypium hirsutum* L.) Genome and Its Implication for Salt Tolerance. Agricultural Science China 9(6): 783-791.

[25] Lade, B. D., Patil, A. S, Paikrao, H. M. (2014): Efficient genomic DNA extraction protocol from medicinal rich *Passiflora foetida* containing high level of polysaccharide and polyphenol. Springer plus. 3: 457.

[26] Khan, S., Qureshi, M. I., Kamaluddin, Alam T, Abdin, M. Z. (2007): Protocol for isolation of genomic DNA from dry and fresh roots of medicinal plants suitable for RAPD and restriction digestion. African Journal Biotechnology 6(3): 175-178.

[27] Vos, P., Hogers, R., Bleeker, M., Reijans, M., Lee, T., Hornes, M., Frijters, M., Pot, J., Peleman, J., Kuiper, M., Zabeau, M. (1995): AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research 23: 4407-4414.

[28] Reyna-Lo´pez, G. E, Simpson, J., Ruiz-Herrera, J. (1997): Differences in DNA methylation patterns are detectable during the dimorphic transition of fungi by amplification of restriction polymorphisms. Molecular Genetics and Genomics 253: 703–710.

[29] Baurens, F. C, Causse, S., Legavre, T. (2008): Methylation-sensitive amplification polymorphism (MSAP) protocol to assess CpG and CpNpG methylation in the banana genome. Fruits 63: 117–123.

[30] Finnegan, E. J., Genger, R. K., Peacock, W. J., Dennis, E. S. (1998). DNA Methylation in plants. Plant Physiology 49: 223–47.

[31] Sharma, R., Mohan, S. R. K., Malik, G., Deveshwar, P., Tyagi, A. K. (2009): Rice cytosine DNA methyltransferases-gene expression profiling during reproductive development and abiotic stress. FEBS Journal 276: 6301–6311.

[32] Futscher, B. W., Oshiro, M. M., Wozniak, R. J., Holtan, N., Hanigan, C. L. (2002): Role for DNA methylation in the control of cell type specific maspin expression. Nature Genetics 31: 175–179.

[33] Vanyushin, B. F. (2005): Enzymatic DNA methylation is an epigenetic control for genetic functions of the cell. Biochemistry Moscow 70: 488–499.