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Biochemical and Molecular Analysis of Somaclonal Variation in Regenerated Pearl Millet

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ABSTRACT

Somaclones, regenerated from Pearl millet cultivar 5141B, were evaluated for various biochemical and molecular changes. Plants (R0) regenerated through tissue culture were evaluated. Biochemical and genetic variation among the somaclones was evaluated by employing DNA- based marker techniques, methylation analysis and RAPD pattern. High frequency of variation in the protein profile was observed. Somaclonal variants analyzed were characterized either by the presence of new polypeptide or by lack of parental type polypeptides. RAPD was carried out with 9 decanucleotide primers. RAPD analysis revealed distinct identity and differentiation for each somaclone and many novel bands were obtained which were not observed in the parental. DNA methylation was evaluated with Sma I, XmaI, Hpa II and Msp I. Variation in the pattern of digestion of the somaclones by these enzymes indicated gross changes in methylation of the bases in the DNA. Also DNA content in individual plant was analyzed. It showed differences in total content indicated existence of ploidy among regenerant. Study consolidates the characterization of clones derived from cell culture. However it needs further characterization to understand changes in somaclonal variants.

Key words: *Tissue culture, Somaclone, Pearl Millet, Plant regeneration, RAPD, DNA methylation.*

INTRODUCTION

Although the ability to regenerate whole plants from cells, tissues, or organs cultured *in vitro* has been long known, the existence and occurrence of somaclonal variation among plants is a question to be answered[12, 30], and also one of the major features distinguishing plant from mammalian cells. Somaclonal variation is either genetic or epigenetic in origin, displayed among somaclones[14, 28].

A growing number of studies are focusing on the investigation of variation to evaluate stability of *in vitro* grown plants, revealing unexpectedly high frequencies of variation[3]. Changing DNA sequence is a fundamental event responsible for much of the reported somaclonal variation among regenerated crops.

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DNA sequence changes or variation include mutations involving one or a few nucleotides, deletions and insertions caused by unequal crossover, or activity of transposable elements. The precise way to determine mutations at the DNA level is to sequence the gene of interest. An alternative approach is to detect variation at DNA levels by electrophoresis of proteins and/or by PCR amplification of nucleic acid segments. Protein electrophoresis implies the existence of mutant alleles by virtue of changes in gene products. Isozyme analysis is a frequently used method of studying certain gene product changes during *in vitro* culture[16, 7, 1, 9]. Chen *et al.*[7] isolated an array of somaclones from a Phalaenopsis Blume cultivar that exhibited deformed flowers.

Epigenetics involves the alteration of phenotype without change in either the coding sequence of a gene or the upstream promoter region[24]. There are three pillars of epigenetics *i.e.,* DNA methylation, histone modifications, and effects of micro RNA (miRNA) and small interfering RNA (siRNA) [10, 24]. Among the three pillars, only DNA methylation has been thus far reported from regenerated crops. Methylation of the 5′-carbon of the cytosine aromatic ring results in genomic imprinting, alternation of gene expression, and activation of the transposable element, but without altering the primary nuclear sequence[33, 17].

Variation in DNA methylation patterns of plants cultured *in vitro* has been analyzed mainly by AFLP (amplified fragment length polymorphism)-based[31] techniques. Methylation-sensitive amplified polymorphism (MSAP) is one such technique that makes use of a pair of methylation-sensitive restriction enzymes, *Hpa* II and *Msp I,* which, being a pair of isoschizomers, recognize the same tetra nucleotide CCGG but have differential sensitivity to methylation at the inner or outer cytosine[25].

Report on potential use of somaclonal variation in Pearl millet to generate resistance to downy mildew has been documented[21]. However reports on evaluation of the mechanisms involved in somaclonal variation is scarce. In the following work attempt is made to analyze biochemical and molecular changes in regenerants.

MATERIALS AND METHODS

Plant material and Invitro culture;

Pearl millet genotype 5141 B line, highly susceptible to downy mildew pathogen obtained from ICRISAT, Hyderabad, India were used as explants to develop regenerants. The seeds were surface sterilized with 0.01 % w/v of mercuric chloride in distilled water and inoculated onto Murashige and Skoog's (MS) medium supplemented with 5ppm 2, 4-D, 30 g of sucrose and gelled with 8 g L^{-1} agar was used for callusing. The pH of the medium was adjusted to 5.6-5.8 before sterilization at 121°C and 1.2 atmospheric pressure for 15 min. All cultures were incubated under 12h photo period with a light intensity of 40W provided by cool fluorescent light. Temperature of 25± 2°C and humidity of 80 % were maintained. After 30 days, the calli were transferred for two 30 day subcultures on MS basal medium with step down (3 and 1mg L^{-1}) concentration of 2,4-D for embryoid development. All operations were performed in a laminar air flow hood. The embryos developed were later transferred into shoot induction medium containing MS medium supplemented with 3ppm of benzyl aminopurine for 30 days. The shoots developed were subjected to root induction. For root induction, the plantlets with well developed shoots were transferred to root induction medium containing half strength MS medium with 0.3% w/v of activated charcoal. The regenerants with well developed root and shoot were then subjected to hardening. The plantlets with well developed root and shoot were hardened to acclimatize them to the natural environment, under green house condition by transplanting into earthen pots containing sterile soil:sand mixture in 1: 1. After the process of hardening, the plants were transferred to pots containing soil: sand mix supplemented with fertilizers.

In order to maintain uniformity in sample preparation the boot leaf of all the regenerants which reached maturity were

selected for biochemical and molecular studies.

Non tissue cultured controls-5141 B line highly susceptible to downy mildew were obtained by germinating seed in earthen pots containing 1: 1: 1 of soil:sand:manure mixture under greenhouse conditions.

Biochemical analysis for detection of variation in somaclones of pearl millet

The somaclones obtained were subjected to quantitative and qualitative analysis of proteins.

Extraction of proteins:

The soluble proteins were extracted in 10 mM Phosphate buffered saline (0.85% NaCI; pH 7.2) from l g of the regenerants and the controls using acid washed glass beads and grinded into fine paste. The mixture was centrifuged at 10,000g for 15 min at 4°C and the supernatant was collected. Protein was concentrated through dialysis.

Estimation of Protein:

Protein was estimated by the dye binding method (Bradford, 1976). Bovine Serum Albumin (Sigma) was used as standard.

Sodium dodecyl sulphate- Polyacrylamide gel electrophoresis (SDS-PAGE):

SDS-PAGE was done according to Laemmli[13] on 12% separating and 5% stacking gels. Eighty microgram of each protein sample was loaded into the well in the slab gel. Standard proteins for molecular weight determination obtained from Pharmacia Biotech, Uppsala, Sweden were run alongside the samples.

Molecular analysis for detection of variation in somaclones of pearl millet

DNA isolation:

Total genomic DNA of the regenerants and the control plants was extracted from fresh leaf tissue (100 mg) following the method of Lubeck and Lubeck[15] with modifications.

Quantification of nuclear DNA:

The DNA isolated was quantified in UV spectrophotometer (Hitachi 2000). Purity of the isolated DNA 'was evaluated by recording the absorbence ratio (260/280).

Random Amplification of Polymorphic DNA analysis (RAPD):

For RAPD-PCR, 7 primers of l0bp length were used which were obtained from Operon Technologies, USA and Genei Pvt. Ltd, Bangalore, India. The primers and their sequence are as follows

Primer number	Sequence	Primer number	Sequence
1	CCCACAGTCA	6	TCCGGCTTTC
2	CTCGCTGTCG	7	CCAGCTGTGA
3	GGCGTATGCG	8	GGGAATTCGG
4	GACGAGTACG	9	CCGATATCCC
5	GTGCGTATGG		

Taq DNA polymerase and dNTPs (dATP, dCTP, dGTP and dTTP were obtained from Bangalore Genei Pvt, Ltd, Bangalore, India.

The genomic DNA isolated from the somaclones and respective controls was quantified and diluted to a concentration of 50ng/µl. This was used for RAPD analysis. The reaction mixture consisted DNA (50ng/ µl) -1.0 µl, l0X Assay buffer-2.5 µl, dNTPs (200mM) -1.0 µl, *Taq* polymerase (3U/ml) -0.5 µl, Primer (25ng/ml) -1.0 µl, Sterile distilled water-19 µl, the total volume -25 µl

The reaction mixture was mixed thoroughly and the tubes were loaded in a thermal cycler (MJ Research Machine, INC, PTC 100TM, Peltier effect cycler). The thermal cycler was programmed as Initial denaturation at 94°C for 3 min, denaturation at 94°C for 30 sec. annealing at 36°C for l min, extension at 72°C for 2 min, final extension at 72°C for 10 min. The steps were programmed to run for 45 cycles.

After amplification, the RAPD products were analyzed by electrophoresis in

1.5% agarose gels and detected by staining with ethidium bromide.

Methylation analysis of genomic DNA:

lµg of DNA from all the regenerants and the control plants were taken in 200 µl volume eppendorf tube. To this, methylation sensitive isoschizomers - Hpa II, Msp I, Sma 1, Xma I procured from Genei, Bangalore, were added separately to different tubes. Reaction volume of 10µl consisting of 1000U of enzyme, IX buffer, l µg of DNA and volume make up with double distilled water was set up. Incubation was carried out at 37° C for 12-15h with continuous shaking in water bath.

The digested DNA was fractionated on 1 % agarose in Tris borate EDTA buffer at 75 V for 3 h and the digested products stained using ethidium bromide[27].

After electrophoresis, the gel was viewed and documented under Bioprofil Image Analysis System (Vilber Lourmat, France).

RESULTS

Biochemical Analysis for Detection of Variation in Somaclones of Pearl Millet

Total protein form different regenerants was extracted and fractionated on SDS- PAGE for comparison of different protein constitution with respect to electrophoretic patterns. Those plants which were sterile fractionated as a separate set and those in which seed setting was observed were fractionated as a separate set only for convenience and curiosity to observe the difference. The results obtained are not presented. The fractionation pattern was of four different types. For convenience of analysis they were grouped into different sets and evaluated.

Somaclones 9, 10, 12, 14, 15 and 16 gave rise to distinct pattern of protein fractionation with a variation in intensity of five polypeptides of molecular weight 13.79, 29.5, 31.46, 64.93 and 117.57 kDa. Similarly, another set consisting of somaclones 17, 18, 19, 21, 22 and 25 gave rise to fractionation of several patterns with distinct polypeptides showing variation with respect to absence or presence or intensity. The distinct bands were 40, 44, 49, 61, 67, 72, 81, 85 and 94 kDa.

The third set comprising of control plants and somaclones 1, 2, 3, 5 and 6 showed bands which had distinct presence and distribution within the regenerants as well as when compared with the control. The bands of molecular weight 16, 18, 27, 47, 51, 55, 72, 90, 105 and 110 kDa were differentiating bands in this set on fractionation. This set showed more resemblance to the parental control than the other sets. In the fourth set comprising of clones 7, 8, 20, 25, 23, 26, 28 and 29, more number of fractionated polypeptides were observed when compared to the other three sets However, distinct bands which differentiated the regenerants from each other were few with bands corresponding to 29, 31, 83, 89, 124 and 127 kDa.

Molecular Analysis for Detection of Variation in Somaclones of Pearl Millet

Quantification of Nuclear DNA:

Quantitative variation in genomic DNA content was observed among the regenerants and also in comparison with control. In spite of using constant sample size, variation in yield of nuclear DNA was observed between the range of 15 to 125 µg per 0.1 g of leaf tissue which showed significant variation. A maximum of 125 µg was found in sample 1 followed by 122 µg per 0.1 g of leaf in sample 21. Least amount of DNA was found in sample 15, which possessed 15 µg followed by 31 µg in sample 26. On observing the isolated genomic DNA using gel electrophoresis followed by ethidium bromide staining, a clear distinct band of 23 kb as found in all samples.

Random Amplification of Polymorphic DNA analysis (RAPD):

DNA isolated from regenerants and controls were estimated and the samples used for analysis using arbitrary decanucleotides to direct amplification. Following electrophoresis, the DNA banding pattern were photographed and illustrated in (Fig 1 to 5). Nine primers were used in this analysis of which 7 showed amplification. However, only five primers showed polymorphism. RADP banding differences was detected among the regenerants as well as in comparison with that of control.

Primer sequence CTCGCTGTCG

A single band corresponding to 0.40 kb was observed in all samples except in 9.25 and control (Fig 1). Another band corresponding to 0.5 kb was observed only in DNA from somaclone 18. No amplification was observed in somaclones 9, 25 and control.

Primer sequence GACGAGTACG:

A single band of 0.13 kb was observed in all samples except in 3, 8, 18, 20, 23 and 25 (Fig 2). In sample 21, 22, 24, 26 and 29 a new band of 0.56 kb was observed. In samples 3, 7, 8, 19, 23 and 25 no amplification was observed.

Primer sequence GTGCGTATGG:

Good amplification with increased polymorphism was observed with this primer. A faint band of 1.33 kb was found exclusively in sample 8 and 9 (Fig 3). Another band of 0.98 kb was found only in 3, 6 and 8. Band of 0.76 kb was observed with varying intensity in somaclones 8, 10, 12, 14, 15, 16, 18, 20, 23, 24 and 26. Increase in intensity of this band was found in somaclones 10, 21, 24 and 26. A band of 0.50 kb was found in somaclones 8, 9 and 20. Band of 0.47 kb was found in somaclones 2, 3, 5, 6, 7, 8, 9, 12, 14, 15, 16, 18, 21, 22, 23, 24, 26, 28, 29 and control. A band of 0.21 kb was found in somaclones 10, 14, 15, 16, 17, 23, 24, 25 and 26. A single band of kb was found exclusively in somaclones 12 and 22 only. Another band of 0.5 kb was found in somaclones 10, 12, 16, 17, 18, 21, 22, 25, 26 and control. In somaclones 1 and 19, diffused bands of range of 1.33 to 0.21 kb without distinct identity was observed.

Primer sequence CCAGCTGTGA:

A single band corresponding to 0.13 kb was observed in somaclones 1, 2, 13 and 15 (Fig 4). Another band of 0.10 kb was found in somaclones 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12. A band of 0.09 was found exclusively in sample 21.

Primer sequence GGGAATTGG:

An amplified band of 0.50 kb was found in all samples except in 9, 20, 25 and control. Another band of 0.10 kb was found in sample 18 alone.

Methylation analysis of Genomic DNA:

Restriction digested DNA samples extracted from individual regenerats and fractionated on 1 % agarose and stained with ethidium bromide are represented in Fig 5 to Fig 8. The samples were digested with methylation sensitive isoschizomers Sma I, Xma I, Msp I and Hpa II.

Agarose gels containing digested DNA samples derived from regenerants were stained with ethidium bromide. The Sma I digestion products were not resolved in somaclone 1, 2, 5, 6, 7, 8, 14, 15, 17, 18, 19, 20, 22, 24, 25 and 26 and a considerable amount of genomic DNA in these somaclones remained as high molecular weight fragments (21.2 kb) (Fig 5). Partial digestion of DNA somaclones *viz*., 10, 12, 16, 21,23,28,29 and control was observed by Sma I. However, only sample 3 and 9 were digested to completion by Sma I. Xma 1 showed complete and partial digestion of these somaclone undigested by Sma 1 (Fig 6). Samples from somaclones 1, 2, 5, 6, 7, 8, 15, 17, 19, 20, 21, 24, 25, 28 and 29 were completely digested by Xma I. While DNA from somaclones 10, 12, 14, 18, 22, 23, 26 and control were partialy digested. Sma I also gave rise to fragments of DNA of 1.56, 1.64, 4.15, 5 and 5.10 kb in samples 28, 16, 4, 14 and 12 respectively.

DNA digested with Hpa II and Msp I produced a range of digestion. Msp I digested all the samples to completion with an exception of somaclones 2, 5, 7, 12, 14, 17, 18 and 20 which were partially digested (Fig 7). Hpa II also digested DNA of all the somaclones with an exception of somaclone 8 which was not digested at all (Fig 8). DNA from somaclones 1, 3, 9, 14, 15, 16, 17, 19, 20, 21, 24, 25, 18 and 19 were completely digested whereas that somaclones from 2, 5, 6, 7, 7, 10, 12, 18, 22, 23 26 and control were partially digested.

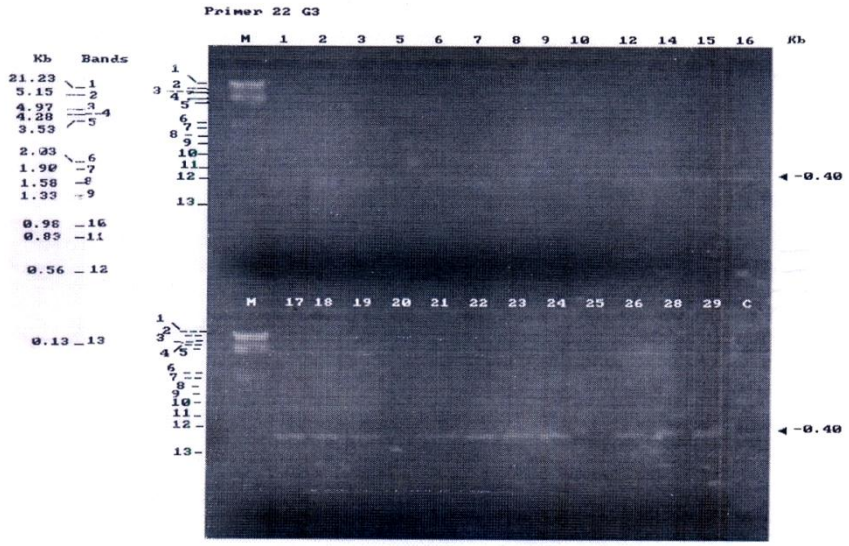

Figure 1: Agarose gel electrophoresis of RAPD reaction directed by primer CTCGCTGTCG using DNA extracted from 25 Pearl millet regenerants and non tissue cultured control plants 5141B. Novel bands are indicated along with their molecular weight. Marker lane-Lane M is also indicated.

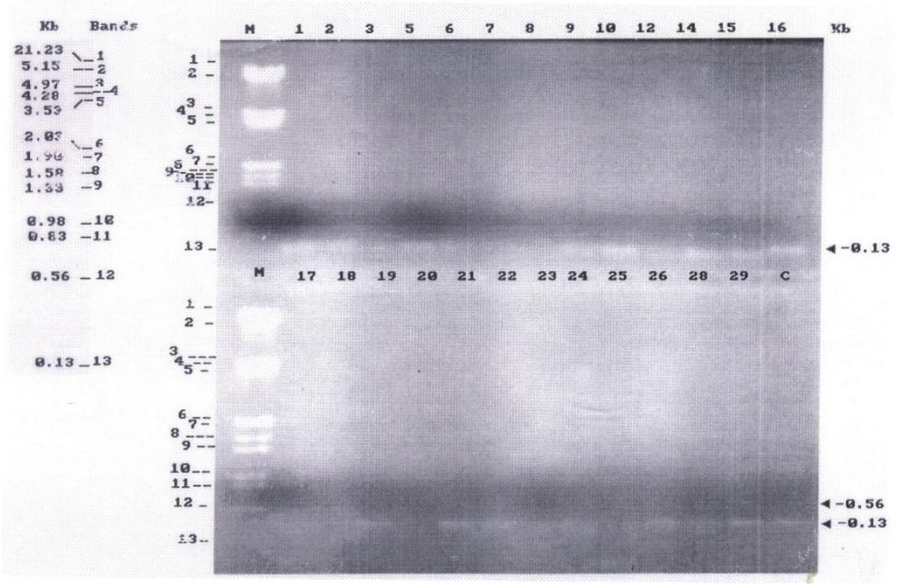

Figure 2: Agarose gel electrophoresis of RAPD reaction directed by primer GACGAGTACG using DNA extracted from 25 Pearl millet regenerants and non tissue cultured control plants 5141B. Novel bands are indicated along with their molecular weight. Marker lane-Lane M is also indicated.

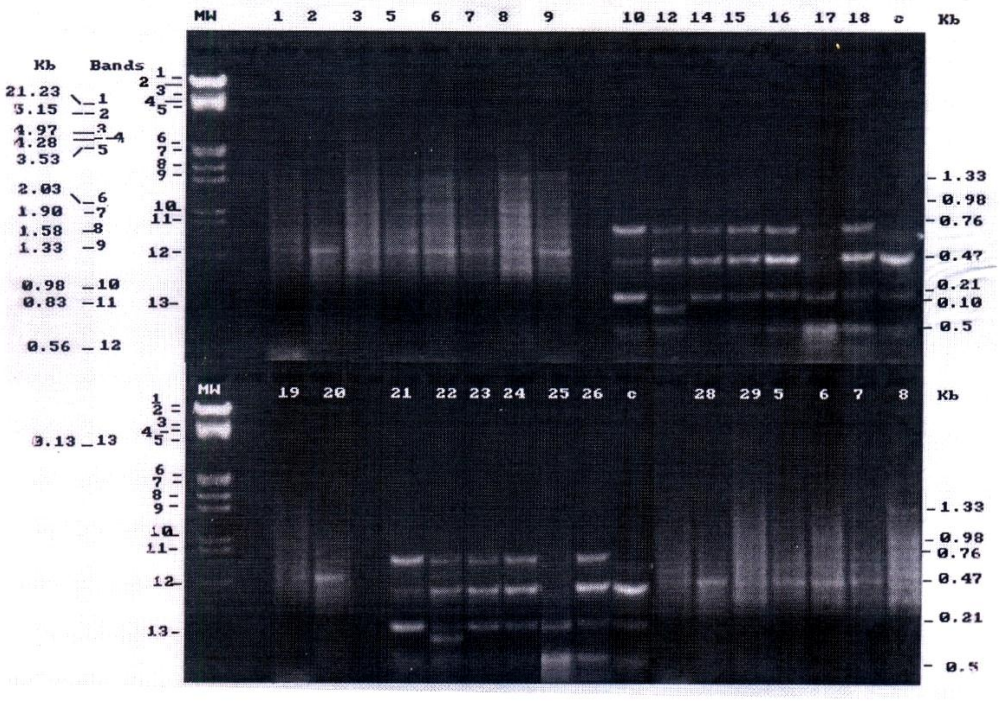

Figure 3: Agarose gel electrophoresis of RAPD reaction directed by primer CCAGCTGTGA using DNA extracted from 25 Pearl millet regenerants and non tissue cultured control plants 5141B. Novel bands are indicated along with their molecular weight. Marker lane-Lane M is also indicated.

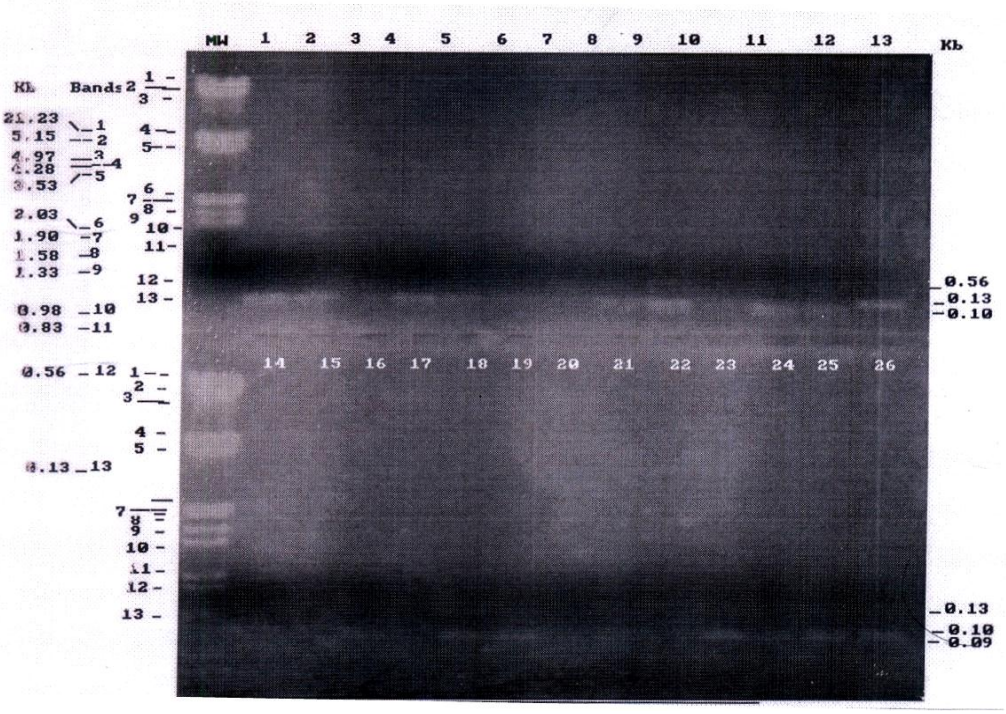

Figure 4: Agarose gel electrophoresis of RAPD reaction directed by primer GGGAATTGG using DNA extracted from 25 Pearl millet regenerants and non tissue cultured control plants 5141B. Novel bands are indicated along with their molecular weight. Marker lane-Lane M is also indicated.

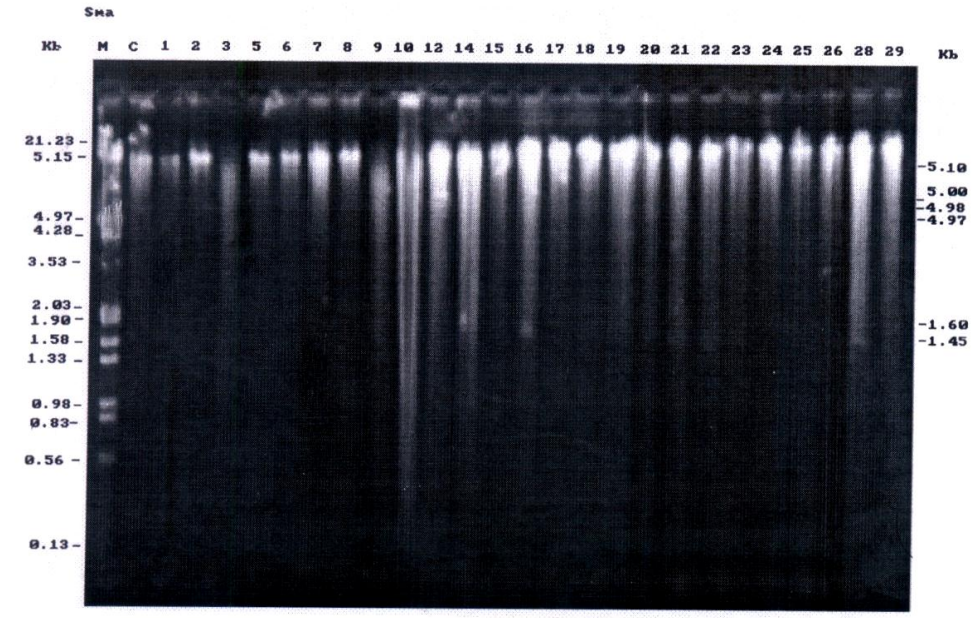

Figure 5: Ethidium bromide stained methylation sensitive restriction enzyme Sma 1 digested DNA samples extracted from 25 Pearl millet regenerants and non tissue cultured control plants 5141B. Novel bands are indicated along with their molecular weight. Marker lane-Lane M is also indicated.

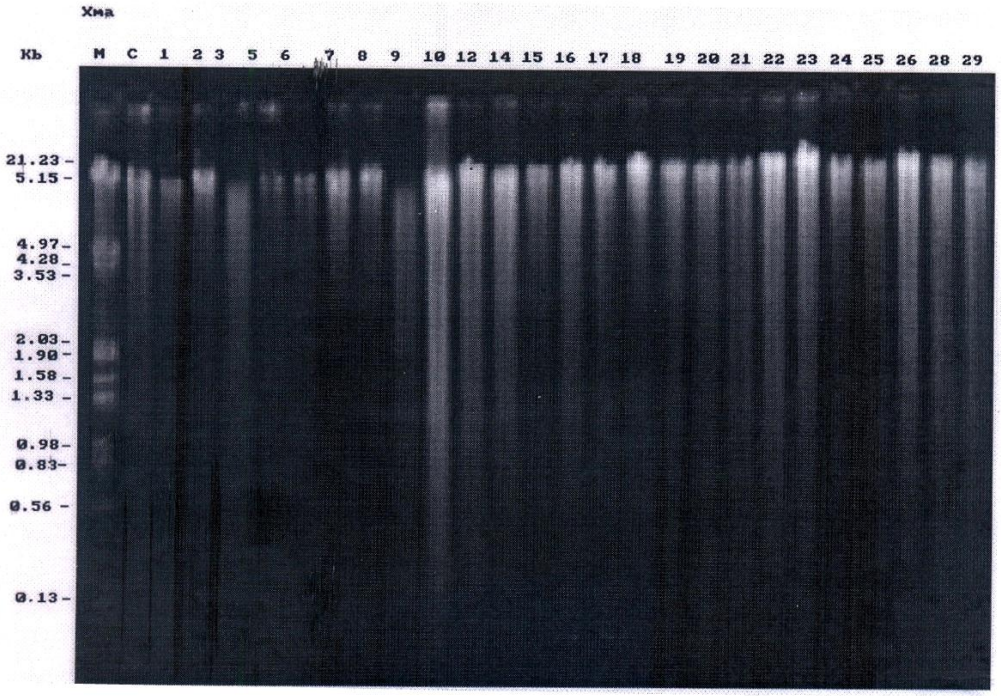

Figure 6: Ethidium bromide stained methylation sensitive restriction enzyme Xma 1 digested DNA samples extracted from 25 Pearl millet regenerants and non tissue cultured control plants 5141B. Novel bands are indicated along with their molecular weight. Marker lane-Lane M is also indicated.

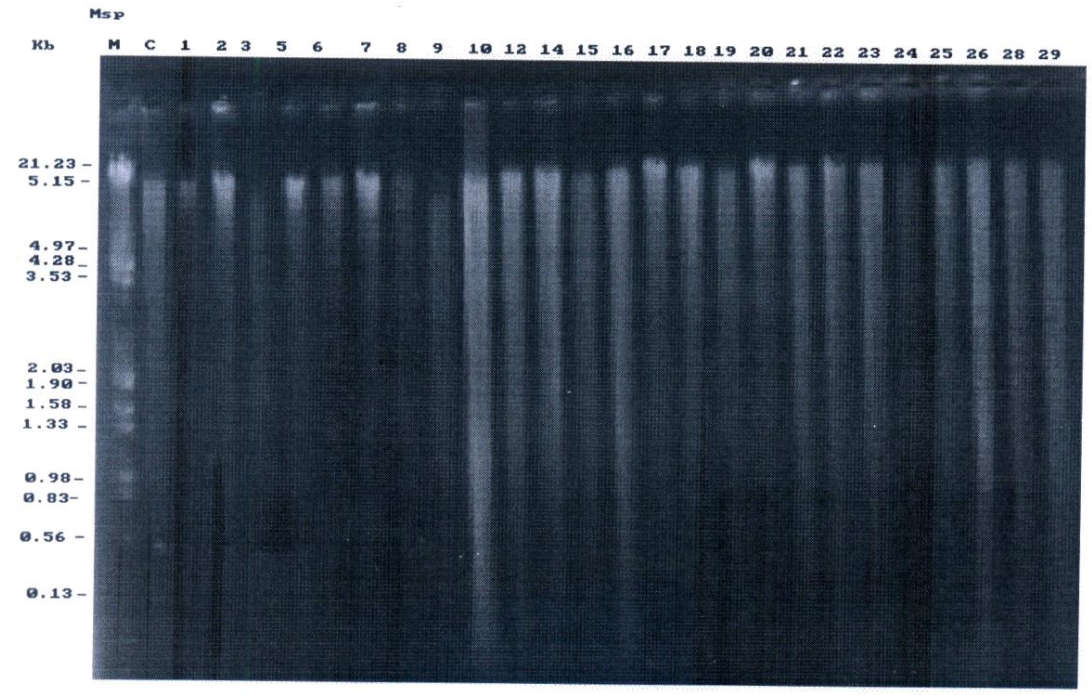

Figure 7: Ethidium bromide stained methylation sensitive restriction enzyme Msp 1 digested DNA samples extracted from 25 Pearl millet regenerants and non tissue cultured control plants 5141B. Novel bands are indicated along with their molecular weight. Marker lane-Lane M is also indicated.

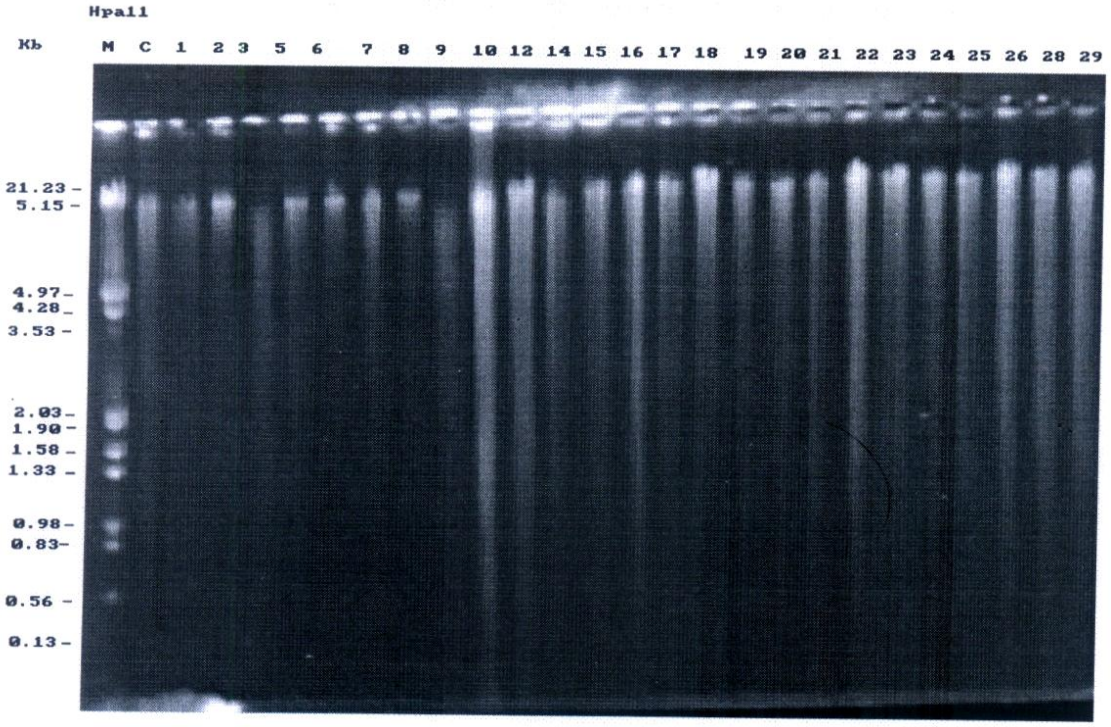

Figure 8: Ethidium bromide stained methylation sensitive restriction enzyme Hpa 11 digested DNA samples extracted from 25 Pearl millet regenerants and non tissue cultured control plants 5141B. Novel bands are indicated along with their molecular weight. Marker lane-Lane M is also indicated

DISCUSSION

A full fledged screening system forms the basic necessity for successful evaluation of somaclonal variation in tissue culture derived plants. The R0 generation in Pearl millet showed varied and significant agronomic traits which prompted us to evaluate these plants at molecular level. Significant variation were observed for plant height, panicle length, total number of tillers, number of fertile tillers, panicle length and shape and days to 50% flowering[29]. Such variations in agronomic characters have been reported in height and maturity[5, 8, 18]. Plant vigor was reduced in some of the regenerants. Such variation due to enhanced repressive physiological effects on plant vigor resulting from the *in vitro* process is not uncommon. A similar results occurred with maize and sorghum and few other crops[11]. Some of these traits might be non transferable to next generation whereas a few might. Thus the differential expression of morphogenic response to tissue culture among regenerants provides the potential for exploiting the genetically heritable variation to develop novel breeding material, particularly for single gene variants[8].

The alteration in genome is documented as an altered physiological status of the individual. Likewise the alteration in the genome of the clones would lead to altered level of protein content. An attempt was made to compare the total protein content among the regenerants and those present in the control as a manifestation of altered genome due to somaclonal variation. Considerably high frequencies of variation in the protein composition among the regenerants were observed in comparison with the parental sets. Some of the variations might be due to nuclear mutations, thus to be true somaclonal variation and few might also be epigenetic. The somaclonal variants analyzed are characterized either by the presence of new polypeptides or by the lack of parental polypeptides. The number of variant types detected was more suggesting the modifications of the post-translational processing rather than activation and or silencing of the genes might also be responsible for the observed variation. A further genetic analysis of relationship of the variant polypeptides and molecular analysis of the genes and post-transcriptional modifications are necessary to clarify the nature of these variations.

The genetic variation induced during tissue culture forms the basis for the variation in physiology and morphology of the regenerants. Such variation arises *de novo* during the period of dedifferentiated cell proliferation that takes place between the culture of an explant and production of regenerants. The genetic variation can be detected in somaclones by employing DNA-based marker techniques. The PCR based molecular technique RAPD[32] has been increasingly used for estimations of genetic diversity, for varietal finger printing, for linkage mapping and for identification of somatic hybrids. In several cases, RAPD technology as been used in the analysis of culture derived material and the banding patterns are been reported in several monocots. In this study, DNA was extracted from the parental as well as the regenerant and RAPD was carried out using 9 decanucleotide primers. Of these seven primers showed amplification and five of them showed polymorphism. Analysis revealed distinct identity and differentiation of each somaclone. For each primer evaluated, a multiple band profile or finger print was produced comprising from two to 10 bands plus a varying number of minor bands (Fig 1 to 4). With these primers the overall signal strength was good although some ambiguities arose in the scoring of minor bands. In some cases all the bands were weak and could be seen clearly after concentrating by loading the complete reaction products onto the gel. Over all, the complexity of the band profiles was similar to those obtained with other plant species[23]. Among the polymorphic bands many were found to be novel ones which were not observed in the parental plant.

RAPD techniques can potentially detect single base mutations or deletions at the level of the primer target and also insertions

and deletions within the amplified fragments[32]. In the present study, polymorphisms observed among the regenerants plants could be identified by the differences in the fragment sizes and presence or absence of the fragments (Fig 1 to 4). The present results indicate that some mutations such as deletion or insertion might occur in the amplified regions and / or base changes might induce the alteration of primer binding sites. Behavior of transposable elements could provide an explanation for the genetic changes among somaclones of plants[23]. This study demonstrates that analysis of genome of the somaclones can be successfully and effectively made using RAPD methodology when compared to other techniques since the information can be obtained quickly and the starting material required is very small, large number of samples can be screened at a time and a very large number of non-species specific primers are commercially available thus making scoring of genome easy.

Analysis of DNA content from the regenrants shows differences in total content in individual plant suggesting presence of polyploidy among the regenerants . However it has to be further confirmed using flow cytometry or cytological techniques to substantiate the statement.

The role of DNA methylation in controlling expression has been extensively reviewed[26]. Increase in methylation *in vitro* potentially regulates gene activity[2] and regulation. Methylation of a gene inactivates its transcription[22] and thereby controls gene expression during somatic embryiogenesis[6]. Present work was carried out to determine whether DNA methylation is altered as a result of tissue culture. DNA was extracted from R0 plants and parental genotype 5141 B and digested with the isoschizomer restriction endonuclease which are methylation sensitive and insensitive- Smal and Xma 1 and another set of Hpall and Msp 1. While both the sets recognise different sequences of digestion shows gross changes in methylation as shown in Fig 5 to 8**.** The tracks showing complete digestion of DNA with Sma 1 indicate presence of DNA sequence $C^{m5}CCGGG$. Whereas those uncut by this enzyme indicates absence of this sequence. Partially digested lanes indicate the mixture of DNA sequence possessing and not possessing this sequence. Likewise similar results in this pattern were obtained for DNA digested with Xma 1 which recognizes the sequence $CC^{m5}CGGG$. In some cases none of these two enzymes have shown digestion thus indicating absence of this DNA sequence in the clone. Hpa 11 and Msp 1 recognize the sequence CCGG. While Hpa l1 is unable to restrict when the internal cytosine is methylated *i.e.* CmCGG and Msp 1 is unable to cut mCCGG. Both enzymes are unable to digest the genome sequence when both the internal and external cytosines are methylated i.e. mCmCGG. Similar results have been obtained by Muller[19] on their studies in rice. Interestingly we have observed fragments released from genome of clones 1.56, 1.6, 4, 4.15, 5 and 5.10 kb in samples 28, 16, 4, 14 and 12 respectively on digestion with Sma 1. Further characterization of these sequences would enlighten about the genomic constitution of these fragments which might help in development of markers. In this study the methylation changes affecting the whole genome have been estimated by the degree of restriction digestion cleavage as seen in the ethidium bromide stained gels, whereas effects on individual gene methylation may be analyzed by southern transfer and hybridization using probes corresponding to specific sequences.

This study consolidates characterization of the clones derived from cell culture of the genotype 5141 B line of pearl millet with reference to phenotype, biochemical and molecular changes associated somaclonal variation. The work is beginning towards understanding the mechanisms involved at genetic level which lead to altered phenotype/ characters which are beneficial to the farmers. The unique variation observed in R0 will be traced for its inheritance in subsequent generation and an attempt will be made to identify marker of downy mildew resistance so as to enable screening of pearl

millet samples for their reaction to downy mildew pathogen.

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REFERENCES

1. Azeqour, M., Majourhat, K. and Baaziz, M., Morphological variations and isoenzyme polymorphism of date palm clones from *in vitro* culture acclimatized and established on soil in South Morocco. *Euphytica.* **123:** 57-66 (2002).
2. Bernacchia, G., Primo, A., Giorgetti, L., Pitto, L. and Cella, R., Carrot DNA methyl transferase is encoded by. two classes of genes with differing patterns of expression. *The Plant Journal*. **13:** 317-329 (1998).
3. Bird, A., Perceptions of epigenetics, *Nature.* **447:** 396-398 (2007).
4. Bradford, M. M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye-binding. *Analytical Biochemistry*. **72:** 248-254 (1976).
5. Cai, Q., Shen, Y., Gao, M. and Liang, Z., Preliminary studies on *in-vitro* induced mutation technique and its application in early maturity CMS line improvement in hybrid rice. In: *Genetic engineering and crop improvement* (eds: J. Xiong and S. Lin,). Wuhan, China International Foundation. pp. 1-12. April 11-15, (1994).
6. Cedar, H. and Razin, A., DNA methylation and development. *Biochemical Biophysical Acta*. **1049:** 1-8 (1990).
7. Chen, W.H., Chen, T.M,, Fu YM., Hsieh, R.M. and Chen, W.S., Studies on somaclonal variation in *Phalaenopsis*. *Plant Cell Reports.* **18:** 7-13 (1998).
8. Encheva, J., Ivanov, P., Tsvetkova, F., and Nikolova, V., Development of a new initial breeding material in sunflower *(Helianthus annus* L.) using direct organogenesis and somatic embryogenesis. *Euphytica.* **68:** 181-185 (1993).
9. Feuser, S., Meler, K., Daquinta, M., Guerra, M.P. and Nodari, R.O., Genotypic fidelity of micropropagated pineapple (*Ananas comosus*) plantlets assessed by Isozyme and RAPD markers. *Plant Cell, Tissue and Organ Culture.* **72:** 221-227 (2003).
10. Grant-Downton, R.T. and Dickson, H.G., Epigenetics and its implications for plant biology. 1. the epigenetic network in plants. *Annals of Botany*. **96**: 1143-1164 (2005).
11. Henry, Y., Vain, P. and De Buyser, J., Genetic analysis of *in vitro* plant tissue culture responses and regeneration capacities. *Euphytica.* **79:** 45-58 (1994).
12. Kennedy, D. and Norman, C., What don't we know? *Science.* 3**09**:75 (2005)
13. Laemmli, U. K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227:** 680-685 (1970).
14. Larkin, P.J. and W.R. Scowcroft., Somaclonal variation: a novel source of variability from cell cultures for plant improvement. *Theor. Appl. Genet.* **60:** 197–214 (1981).
15. Lubeck, M. and Lubeck, P. S., PCR-based methods - A promising tool for detection and identification of fungi in soil. In: *Monitoring antagonistic fungi deliberately released into the environment. Developments in plant pathology* (eds: D. F. Jensen, H.B. Jansson and A. Tronsmo) pp 113-121, Kluwer Academic Publishers, Dordrecht, Netherlands (1996).
16. Mangolin, C.A., Prioli, A.J, and Machado, M.F.P.S., Isozyme variability in plants egenerated from calli of *Cereus peruvianus* (Cactaceae). *Biochemical Genetics.* **35**: 189-204 (1997).
17. Martienssen, R.A. and Colot, V., DNA methylation and epigenetic inheritance in

plants and filamentous fungi. *Science.* **293**: 1070-1074 (2001).

18. Mohmmad, A. S. and Nabors, M. W., Somaclonal variant plants of wheat derived from mature embryo explants of three genotypes. *Plant Cell Reports*. **8:** 558-560 (1990).
19. Muller, E., Brown, P. T. H., Hartke, S. and Lorz, H., DNA variation in tissue culture derived rice plants. *Theoretical and Applied Genetics*. **80:** 673-679 (1990).
20. Murashige, T. and Skoog, F., A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Plant Physiology*. **15:** 473-497 (1962).
21. Nagarathna, K. C., Biotechnological approach to develop downy mildew disease resistance in pearl millet. *Ph.D. Thesis*, University of Mysore, Mysore, India, (1993).
22. Palmgren, G., Mattsson, O. and Okkels, F. T., Specific levels of DNA methylation in various tissues, cell lines and cell types of *Daucus carota. Plant Physiology*. **95:** 174-178 (1991).
23. Purwantoro, A., Supaibulwatana, K., Mii, M. and Koba, T., Cytological and RAPD (Random Amplified Polymorphic DNA) analysis of somaclonal variation in easter lilly *(Lilium longiflorum* Thumb.). *Plant Biotechnology*. **16:** 247-250 (1999).
24. Rapp, R.A. and Wendel, J.F., Epigenetics and plant evolution. *New Phytologist.* **168**: 81-91 (2005).
25. Reyna-López, G, E., Simpson, J. and Ruiz-Herrera J., Differences in DNA methylation patterns are detectable during the dimorphic transition of fungi by amplification of restriction polymorphisms, *Molecular and General Genetics*. **253**:703-710 (1997).
26. Richards, E. J., DNA methylation and plant development. *Reviews.* **13:** 319-323 (1997).
27. Sambrook, J., Fritsch, E. F. and Maniatis, T., Molecular cloning: *A laboratory manual*. (ed: C. Nolan) Cold Spring Harbor Laboratory Press. (1989).
28. Schaffer, W., Terminology associated with cell, tissue and organ culture, Molecular biology and molecular genetics, *In Vitro Cellular and Developmental Biology-Plant*, **26:** 97-101 (1990).
29. Thejaswini, P.H., Molecular studies on the host pathogen interactionwith reference to Pearl Millet and *Sclerospora graminicola*. *Ph.D. Thesis*, University of Mysore, Mysore, India, (2000).
30. Vogel, G., How does a single somatic cell become a whole plant? *Science*, **309:** 86 (2005)
31. Vos, P., Hogers, R. and Bleeker M., A new technique for DNA Fingerprinting, *Nucleic Acids Research.* **23:** 4407-4414 (1995).
32. Williams, J. G. K., Kubelik, A. R, Livak, K. J., Rafalski, J. A. and Tingey, S. V., DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research,* **18:** 6531-6536 (1995).
33. Wolffe, A.P. and Matzke, M.A., Epigenetics: regulation through repression. *Science.* **286:** 481-486 (1999).