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Full Length Research Paper

Simple sequence repeat (SSR) and inter simple sequence repeat (ISSR) analyses of genetic diversity in tissue culture regenerated plants of cotton

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Cotton is one of the main economic crop plants of Iran cultivated under continuous artificial selection and cultivation which may lead to genetic erosion and possible loss of useful genetic loci resulting in vulnerability to pests and diseases. For this reason increasing and improving the amount of genetic diversity in cotton germplasm through tissue culture is important. The present report considers genetic diversity induced in tissue culture regenerated plants of three cotton cultivars namely Mehr, Sindose and their hybrid Mehr X Sindose. Surface of seeds were disinfected with 70% ethanol for 2 min and then treated with 5% hypochlorite solution for 20 min. Finally, they were washed 3 to 4 times with sterile distilled water and inoculated aseptically on Murashige and Skoog (MS) basal medium free hormones. Single nodes resulted from seedlings cultured as explants. Inter simple sequence repeat (ISSR) and simple sequence repeat (SSR) primers used produced different number of bands in the genotypes studied showing different levels of molecular polymorphisms in each cultivar. Some common and few specific ISSR/SSR loci were indentified while some bands were present in all the genotypes except one indicating genetic changes in them. Analysis of molecular variance (AMOVA) test showed significant difference ($p < 0.05$) for ISSR markers but not for SSR markers. Molecular trees obtained showed genetic variations among the regenerated plants of each cultivar due to tissue culture.

Key words: Cotton, genetic diversity, ISSR, RAPD.

INTRODUCTION

Production of new genetic variants in crop plants is one of the possible sources of obtaining elite genotypes to be used in hybridization and breeding programs. Plant tissue culture leading to somaclonal and developmental variation has been considered as one of the possible sources of inducing genetic variability in crop plants.

Somaclonal variation is the occurrence of genetic variants among the regenerated plants of a single subculture derived from *in vitro* procedures and developmental genetic changes occur during several sub-

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culturing among the regenerated plants (Isabel et al., 1993), it is also called tissue or culture-induced variation (Bordallo et al., 2004).

Variability induced in regenerated plants during tissue culture often arises as a manifestation of epigenetic influence or changes in the genome of differentiating vegetative cells and is expected to generate stable plants carrying interesting heritable traits (Soniya et al., 2001). Four important variables influence the induced variability discussed, these are: Genotype, explant origin, cultivation period and the cultural condition in which the culture is made (Evans and Sharp, 2000). Variations induced may be manifested as cytological abnormalities, frequent qualitative and quantitative phenotypic mutation, sequence change, gene activation and silencing (Sheidai et al., 1998).

Identification of possible somaclonal variants at an early stage of development is very useful for quality control in plant tissue culture, transgenic plant production and in the introduction of variants (Rani et al., 1995; Soniya et al., 2001; Sheidai et al., 2010). Many strategies can be used to evaluate plant genetic structure from *in vitro* derived plant clones, including cytogenetic analysis and isoenzyme markers and different DNA molecular markers etc., but most of them have limitations. Karyology analysis cannot reveal alteration in specific genes or in small chromosome arrangements. Isoenzyme markers provide an appropriate method to detect genetic changes (Rani et al., 1995; Soniya et al., 2001). However, these markers are susceptible to ontogenetic variation and are limited in number, and only DNA segments coding for soluble proteins can be sampled. Various molecular markers including restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragments length polymorphism (AFLP), inter simple sequence repeats (ISSR) and simple sequence repeats (SSR) (Rani et al., 1995; Soniya et al., 2001; Noormohammadi et al., 2011) have been used in somaclonal and genetic diversity analyses.

Cotton is one of the main economic crop plants of Iran cultivated in many regions of the country. Due to continuous artificial selection, genetic diversity available may decrease leading to genetic erosion and possible loss of useful genetic loci which could result in vulnerability to pests and diseases (Wajahatollah and Stewart, 1997; Van Esbroeck and Bowman, 1998; Kumar et al., 2003; Vafaie-Tabar et al., 2003; Mehetre et al., 2004; Dongre et al., 2007; Rana et al., 2007; Preetha and Raveendren, 2008; Sheidai et al., 2008; Wei et al., 2008, Noormohammadi et al., 2011). Therefore, making opportunities to increase and improve the amount of genetic diversity in cotton germplasm through tissue culture is important. The present report considers genetic diversity induced in tissue culture regenerated plants of three tetraploid cotton cultivars namely Mehr, Sindose and their hybrid Mehr X Sindose.

MATERIALS AND METHODS

Three *Gossypium hirsutum* L. cultivars Mehr, Sindose and their hybrid Mehr X Sindose were used in this study. Surface of seeds were disinfected with 70% ethanol for 2 min and then treated with 5% hypochlorite solution for 20 min. Finally, they were washed 3 to 4 times with sterile distilled water and inoculated aseptically on MS (Murashige and Skoog, 1962) basal medium free of hormones. Single nodes from seedlings were aseptically collected and their regeneration was attempted on MS medium containing Indoel-3-acetic acid (IAA) (1 mg L^{-1}) and Zeatin (0.1 mg L^{-1}). The pH of all of media was adjusted to 5.7 and 0.8% (weight/ volume) agar was added prior to autoclaving at 103 Kpa for 20 min. Cultures were incubated under a 16 h photo-period with light intensity of 3000 lux at 28 ± 1°C. Fresh leaves of *in vitro* regenerated cotton plants from first, second and the third sub-cultures were collected for DNA extraction.

Microsatellite and ISSR assay

Total genomic DNA was extracted from fresh leaves using the CTAB method by DNeasy® Plant Mini Kit (Qiagen Inc., Valencia, CA). Cotton TMB1421, TMB1489, Gh427 and NAU2265 SSR markers were obtained from three TMB, Gh and NAU sources. The sequences of these SSRs are available at http://www.cottonmarker.org.

SSR-PCR reactions were carried out in Techne thermocycler (Germany). Each 20 µl PCR reaction contained 20 ng genomic DNA, 1X supplied PCR buffer (Bioron, Germany) 200 µM of each dNTP (Bioron, Germany), 0.25 unit of *Taq* DNA polymerase (Bioron,Germany) and 0.2 µM of each fluorescently labeled forward (ABI dyes: 6-FAM or HEX) (5´) and reverse non-labeled (3´) SSR primer pair.

Touch-down PCR was performed following the program: Initiation denaturation at 94°C for 10 min followed by 10 cycles of 94°C for 1 min, 60°C for 1 min (decreases of 0.5°C in each cycle) and 72°C for 2 min; 33 cycles of 94°C for 15 s, the annealing temperature 55zzC for 30 s and 72°C for 1 min. A final extension at 72°C for 7 min was performed. Capillary electrophoresis was performed on an automated sequencer (ABI Prism 3130 DNA sequencer, Applied Biosystems, Foster City, CA, USA) and the GeneScan internal size standard labelled with LIZ-500 (Applied Biosystems) was used. The ISSR primers used in the present study were selected in a set of ten primers; UBC807, UBC810, UBC811, UBC823, UBC834 and UBC849 commercialized by UBC (the University of British Columbia) as well as (CA) 7GT, $(GA)_9$T, $(GA)_9$ A and $(GA)_9$ C primers. PCR reactions were performed in a 25 µL volume containing 10 mM Tris–HCl buffer at pH 8; 50 mM KCl; 1.5 mM $MgCl_2$; 0.2 mM of each dNTP; 0.2 µM of a single primer; 20 ng genomic DNA and 1 unit of *Taq* DNA polymerase (Bioron, Germany). Amplifications reactions were performed in Techne thermocycler (Germany) with following program: 5 min initial denaturation step 94°C, 30 s at 94°C; 1 min at 50°C, 1 min at 72°C. The reaction was completed by final extension step of 7 min at 72°C. Amplification products were visualized by running on 2% agarose gel, following ethidium bromide staining. Fragment size was estimated by using a 100 base pairs (bp) molecular size ladder (Fermentas, Germany). ISSR bands more than 1700 bp were not scored for more accuracy of scoring.

Data analysis

Microsatellite allelic size was determined by using the program GENEMAPPER 3.1 from Applied Biosystems. The number of SSR alleles, the number of unique alleles, expected (H_e) heterozygosity (Nei, 1987) and polymorphic information content (PIC) (Botstein et al., 1980) by using GenAlex 6.4 (Peakall and Smouse, 2006). In addition, the effective number of alleles (N_e) and Shannon's index were determined for both SSR and ISSR loci by POPGENE program (Yeh and Boyle, 1999) in all loci and also each population separately.

Analysis of molecular variance (AMOVA) and the *Fst* pair-wise test (with 10100 permutations) were performed to reveal significant genetic difference between populations as well as among individuals of each population using GenAlex 6.4 (Peakall and Smouse, 2006) for AMOVA analysis.

Jaccard similarity as well as Nei's genetic distance (Nei, 1973) was determined among the cultivars studied and used for grouping of the genotypes by Unweighted Paired Group with Arithmetic Average (UPGMA) and NJ (Neighbor Joining) clustering methods and ordination based on principal components analysis (PCA) (Podani 2000; Weising et al., 2005). Cophenetic correlation and bootstrapping (100 replications) were performed to check the fit of dendrograms obtained. NTSYS Ver. 2.02 (1998) and DARwin ver. 5 (2008) were used for clustering and PCA analyses.

RESULTS

ISSR analysis

In Mehr cultivar and its tissue culture regenerated plants, the nine ISSR primers used produced 83 reproducible bands in Mehr cultivar and their tissue culture regenerated plants. Out of 83 bands, 65 bands were polymorphic and 18 bands were monomorphic among Mehr genotypes studied.

Some of the ISSR bands (loci) occurred only in one genotype, for example the ISSR band No. 2 (350 bp) of the primer UBC810 occurred only in the regenerated plants of the first subculture, while band No. 2 (400 bp) of the primer UBC807 occurred only in the regenerated plants of the second subculture.

In Sindose cultivar and its tissue culture regenerated plants, the ISSR primers produced 71 reproducible bands in Sindose cultivar and their tissue culture regenerated plants. Out of 71 bands, 50 bands were polymorphic and 21 bands were monomorphic among Sindose genotypes studied.

ISSR locus No. 2 (350 bp) of the primer UBC810 was present only in the regenerated plants of the third subculture, while band No. 4 (800 bp) of the primer (GA) 9C occurred only in the regenerated plants of the first subculture. Moreover, band No. 11 of the primer UBC-811 and band No. 8 (900 bp) of the primer UBC-823 were present in all the genotypes except one regenerated plant in the first and second subcultures respectively.

In the hybrids Mehr X Sindose and its culture regenerated plants, the ISSR primers produced 87 reproducible bands in the hybrid cultivar and their tissue culture regenerated plants. Out of these, 79 bands were polymorphic and 8 bands were monomorphic among hybrid genotypes studied. In total, 9 specific bands occurred in the hybrid and its regenerated plants, while 9 bands also were absent in one of the plants in this genotypes. For example, band No. 3 (500 bp) of the primer UBC-811 was only present in one of the regenerated plants of the third subculture (subcultures must have been described in Materials and Methods), while band No. 12 (1500 bp) of the same primer occurred only in the regenerated plants of the second subculture. Band No. 2 (500 bp) of the primer UBC-849 was present only in the regenerated plants of the second subculture.

The band No. 5 (1200 bp) of the primer (GA)9T was only absent in a single regenerated plant of the third subculture, while bands No. 6 and 7 (800 and 1400 bp respectively) of the primer UBC-849 were absent only in one regenerated plant of the first subculture.

The mean number of alleles obtained in Mehr cultivar and the hybrid Mehr X Sindose tissue culture regenerated plants was 1.06, while it was 0.99 in Sindose regenerated plants. Similarly, the mean number of effective alleles in Mehr regenerated plants was 1.244 while it was 1.217 in Sindose and 1.213 in the hybrid Mehr X Sindose tissue culture regenerated plants. The mean values of Nei's gene diversity (H) and Shanon's information indices (I) of the parents and their hybrid regenerated plants for ISSR markers determined showed 0.137 and 0.20 respectively in the Mehr; 0.128 and 0.19 respectively for Sindose regenerated plants and 0.123 and 0.19 for hybrid cultivar regenerated plants. Therefore the highest values of genetic diversity in ISSR markers were obtained in Mehr cultivar.

Analysis of molecular variation (AMOVA) showed significant difference ($p < 0.05$) for ISSR markers both among and between genotypes studied. The test showed 34% of total variance between genotypes and 66% within genotypes.

UPGMA and NJ trees of ISSR data produced similar results supported by principal component analysis (PCA) ordination plot (Figures 1 and 2). The Cophenetic correlation of NJ tree was higher ($r = 0.96$) and showed low bootstrap values (not shown), therefore it is discussed below (Figure 2).

The parental genotypes Mehr, Sindose and their hybrid differ in ISSR characteristics are (were) placed in separate clusters. In each cultivar, variation occurs in the regenerated plants of different subcultures, for example in Mehr cultivar, a single regenerated plant of the first subculture (M1) is placed far from the other plants of this subculture and is placed close to the regenerated plants of the second subculture (M2 plants) (subcultures have not been described). The same holds true for regenerated plants of the second subculture which stand far from the other plants of this subculture and shows affinity to M_3 plants. The regenerated plants of different

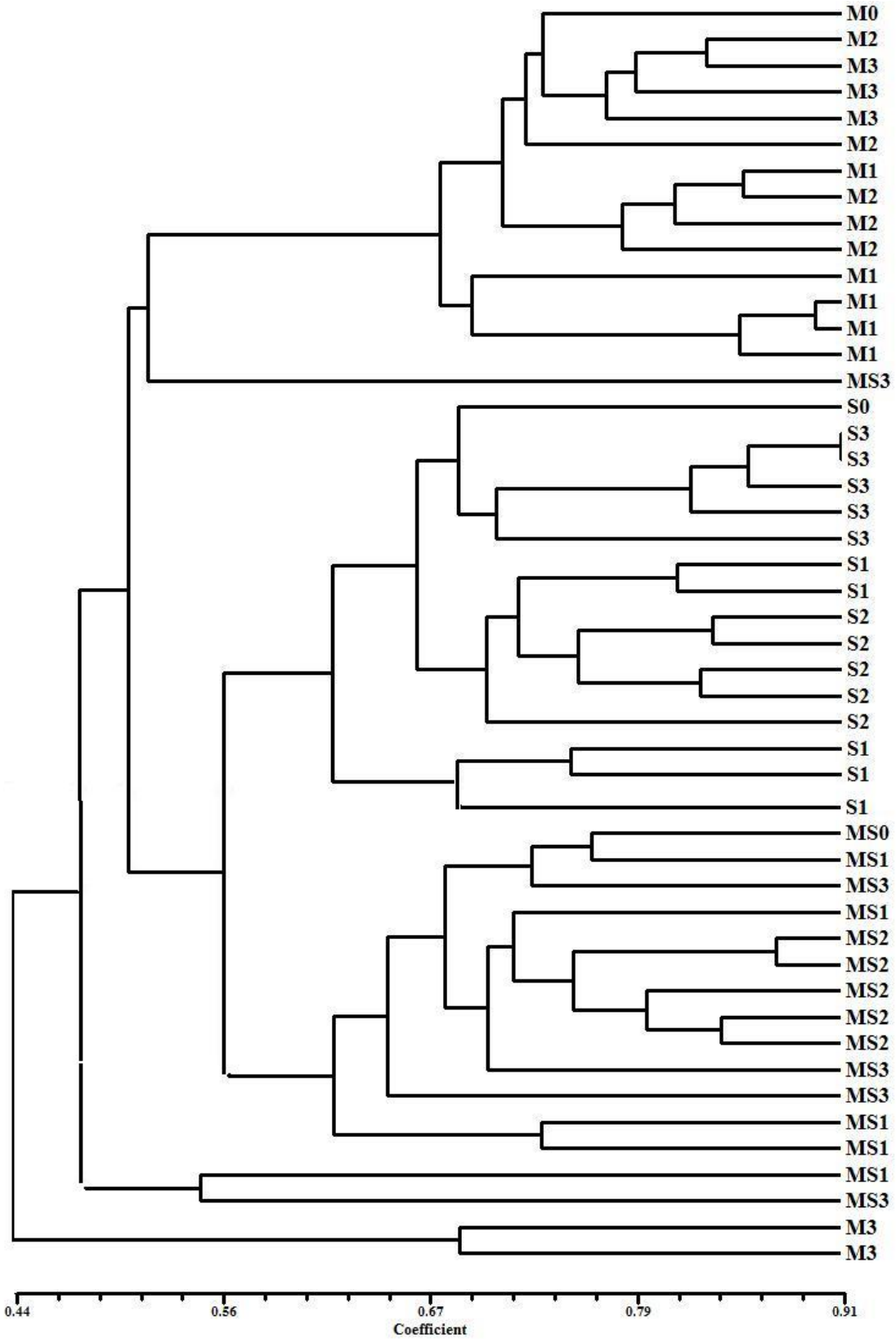

Figure 1. UPGMA tree of ISSR data. M = Mehr cultivars, S = Sindose cultivar, MS = Hybrid (0 = parental plant, 1-3 = Subcultures 1-3).

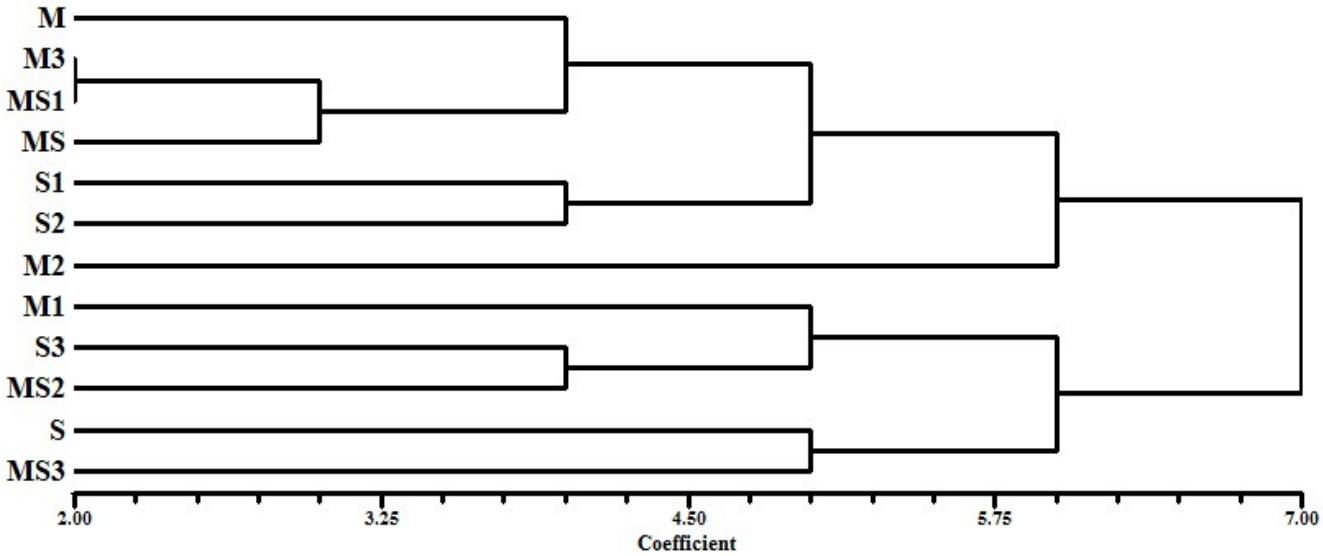

Figure 2. NJ tree of SSR data. M = Mehr cultivars, S = Sindose cultivar, MS = Hybrid (1 to 3 = Subcultures 1 to 3).

subcultures in Sindose and the hybrid cultivar studied also gave similar results.

Microsatellite diversity

Amplification products were obtained in all the genotypes studied at four SSR loci analyzed. We used SSR primers of the chromosome numbers 1, 2, 3 and 5 in cotton and obtained from 1 to 6 alleles (Cotton is a amphidiploid species, with a narrow genetic base considering cultivated materials. You cannot find 6 different alleles in one locus, analyzing two cultivars) in each locus. Amplification of the SSR markers produced 10 reproducible alleles in the Mehr cultivar and its tissue culture regenerated plants. The size of alleles obtained ranged from 217 bp (GH427-217 locus) to 235 bp (NAU2265-235 locus) in size.

Amplification of the SSR markers produced 10 reproducible alleles in the Mehr cultivar and its tissue culture regenerated plants. The size of alleles obtained ranged from 217 bp (GH427-217 locus) to 235 bp (NAU2265-235 locus) in size. SSR allele TMB1489-228 occurred only in the parental plants of Mehr, while NAU2265-235 allele regenerated plants of the first subculture. Three alleles of TMB1421-181, GH427-217 and NAU2265-219 were common to all Mehr genotypes studied.

In Sindose cultivar and its tissue culture regenerated plants, eight SSR alleles were obtained. Four SSR alleles of TMB1421-181, NAU2265-223, GH427-217 and NAU2265-219 were common to all Sindose genotypes studied (four alleles considering the four primer pairs or four allele for each primer pair), while others occurred at least in two genotypes. In the hybrid cultivar Mehr X Sindose, also eight SSR alleles were obtained. Three SSR alleles of NAU2265-223, GH427-217 and NAU2265-219 were common to all Sindose genotypes studied, while others occurred at least in two genotypes. No genotype specific band was observed in the hybrid genotypes studied but the allele TMB1489-122 was present in all and only absent in the hybrid regenerated plants of the second subculture.

The mean number of alleles obtained in Mehr cultivar was 1.14, while it was 1.10 in both Sindose and the hybrid cultivars regenerated plants. Similarly, the mean number of effective alleles in Mehr regenerated plants was 1.30 while it was 1.17 in Sindose and 1.24 in the hybrid Mehr X Sindose tissue culture regenerated plants. (without knowledge of the amount of plants analyzed it is not possible to decide).

The mean values of expected heterozygosity (H) and Shanon's Information Indices (I) for SSR markers determined was 0.179 and 0.269 respectively in the Mehr, 0.106 and 0.160 respectively for Sindose regenerated plants and 0.133 and 0.190 for hybrid cultivar regenerated plants. Therefore, the highest values of genetic diversity in SSR markers also were obtained in Mehr cultivar. Analysis of molecular variation (AMOVA) did not show significant difference ($p < 0.90$) for SSR markers among the genotypes studied.

NJ and UPGMA trees obtained from polled data of the regenerated plants (Figure 1) produced similar results, showing a mixture of regenerated plants obtained in different subcultures. For example, regenerated plants of the Mehr cultivar (M_1-M_3) stands far from each other, close to other genotypes regenerated plants. The same holds true for Sindose and the hybrid cultivar studied.

DISCUSSION

The presence of ISSR and SSR polymorphic bands in the parental and their F_1 hybrid as well as their tissue culture regenerated plants studied indicates the presence of genetic polymorphism in these genotypes. The occurrence of molecular differences in the regenerated plants of each subculture indicates the occurrence of somaclonal variation (This is not clear, because comparisons are not clearly described). Moreover, the occurrence of molecular variations in the regenerated plants of each cultivar due to continuous subculturing indicates the occurrence of developmental genetic changes. The groupings obtained in both ISSR and SSR trees further supports such genetic changes in the plants studied. The occurrence of specific bands/loci only in some of the plants/genotypes illustrates the occurrence of unique insertion/deletion in DNA material of these genotypes which may be used in planning hybridization.

The number of ISSR bands and degree of polymorphism obtained in the cotton cultivars studied is almost in agreement with the other studies performed in cotton. Wei et al. (2008) studied the genetic diversity in 48 cotton accessions of *G. barbadense* and *G. hirsutum* by ISSR markers and obtained 92 reproducible ISSR bands out of which 72 were polymorphic (a much greater number of cultivars, of two different species has been used). They also could differentiate the genotypes collected from different provinces in China based on ISSR data. In another study, Vafaie-Tabar et al. (2003) reported 79% average genetic similarity among Indian tetraploid cotton cultivars while Rana and Bhat (2005) reported 74% average genetic similarity. According to Rana and Bhat (2005), the other studies on tetraploid cotton cultivars outside India also report similar ranges of average genetic similarity.

Studies performed in the tissue culture regenerated plants in other cotton genotypes including Sahel, Siokra and their hybrid (Tafvizi et al., 2010) and Bakhtegan, Zeta2 and their hybrid (Sheidai et al., 2008) also agrees with the present finding, since RAPD analysis of genetic diversity and somaclonal variation performed in these cultivars revealed the occurrence of molecular changes during tissue culture in these plants.

The genetic diversity indices determined for both ISSR and SSR markers revealed the highest values of genetic diversity in Mehr cultivar and its tissue culture regenerated plants, while its hybrid with Sindose shows a lower value of genetic polymorphism. This result is interesting as a hybrid cultivar is expected to show higher level of genetic diversity compared to its parental genotypes due to combination of two almost different genomes obtained from its parents. However, one of our parents (Mehr cultivar) still shows more genetic diversity in its tissue culture regenerated plants which may indicate the effect of genetic background/ genotype specificity in achieving genetic diversity during tissue culture. AMOVA test shows genetic distinctness of the genotypes studied as they differ significantly in their molecular content. In a similar investigation, Rana and Bhat (2005) could differentiate the Indian diploid and tetraploid cotton genotypes by the help of RAPD markers identifying a higher degree of genetic diversity in diploid genotypes compared to tetraploid cultivars.

Dendrograms obtained in both ISSR and SSR agree in one point that is separation of some of the regenerated plants from the other plants in the same cultivar and in the same subculture. This indicates the possible occurrence of genetic recombination or insertion/deletion in molecular content of each plant irrespective of its parental genotype.

Since, even single base change at the primer annealing site is manifested as appearance or disappearance of ISSR and SSR bands, these bands may indicate the occurrence of genetic changes in the genome of the progenies either through the loss or rearrangement of some of their nucleotides. Chromosomal crossing over during meiosis may result in loss of primer attachment pair sites in the offspring leading to novel ISSR pattern in the offspring (Smith et al., 1996). Sushir et al. (2008) carried out cytogenetic and RAPD analysis of F_1 and F_2 progenies of the interspecific cross between *Gossypium arboretum* X *G. anomalum* and reported that among nine F_2 segregates, $F_{2\ to\ 1}$ progeny plants showed one additional band than F_1 and $F_{2\ to\ 5}$ progeny plant showed the recombination event. On the contrary in plants $F_{2\ to\ 6}$ and $F_{2\ to\ 8}$ loss of priming sites happened showing that recombination between A and B genomes of *G. arboreum* and *G. anomalum* respectively is possible (Sushir et al., 2008).

Sheidai et al. (2008) also used RAPD markers to analyse different cotton genotypes and reported the occurrence of novel RAPD bands in the hybrids not observed in their parents and also reported the absence of RAPD bands in the hybrids which were present in their parents. This phenomenon is not confined to cotton and occurs in other plant species too, for example Wang et al. (2004) reported the presence of some RAPD bands in the parental genotypes of *Fagopyrum* which were not observed in their hybrid and also noticed the appearance of some novel RAPD bands in the hybrids which were not present in the parental genotypes.

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