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ARTICLE

Genetic and morphological variations induced by tissue culture in tetraploid cotton (*Gossypium hirsutum* L.)

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ABSTRACT Three parental cotton cultivars including Bakhtegan, Zeta-2 and their hybrid Bakhtegan × Zeta-2 were used in tissue culture and the genetic and morphological diversity of the parental genotypes and the regenerated plants of different sub-cultures were studied. The seeds excised from three different cotton cultivars were cultured in MS free hormones, using the single nodes from seedlings as explants. Thirty random primers were used for molecular studies. The regenerated plants differed significantly in morphological characters like the length of shoots and number of leaves and differed in the number of RAPD loci identified as well as degree of polymorphic bands. Different sub-cultures produced different level of genetic diversity in the cultivars studied. With an increase in the time period of sub-cultures an in crease in the amount of genetic variation occurred in the regenerated plants of the tree cotton cultivars studied.
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KEY WORDS

cotton,
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Cotton is an important economic and fiber crop, grown in 70 countries in the world. Over 180 million people are associated with the fiber industry that produces 20 to 30 billion dollars worth of raw cotton. Both diploid (*Gossypium herbaceum*) and tetraploid (*G. hirsutum*) cultivars are cultivated in different regions of Iran and are considered as important crop plants of the country. Although great progress has been made in the field of improvement of cotton with conventional breeding methodology, it is time-consuming and commercialization of new cotton varieties often takes 6 to 10 years. Compatibility limitations narrow the gene pool available for this process (Zhang et al. 2000).

Southern corn leaf blight crisis of the 1970s has taught plant breeders and geneticists the danger of genetic uniformity in a crop species. What was a minor disease for many years turned into an epidemic in the corn industry simply because the seed companies were using a male sterile cytoplasm, which was highly susceptible to a race of leaf blight, in their hybrid seed production. Currently, the cotton industry, with its overwhelming acreage of transgenic cultivars, shares a resemblance to the corn industry of the 1970s. Most transgenic cotton cultivars are developed through a two-step process; the transgene is first "engineered" into embryonic cells that are capable of regenerating into plants and then introduced into the desired cultivars using backcross breeding. In cotton, the ability to produce embryogenic cells is genotype dependent with only a few genotypes known to be capable of regenerating plants from cell culture. Because of this limitation, a majority of the transgenic cultivars are closely related with one of their parents, not only further narrowed the genetic base of cotton, but remains a major obstacle for cotton transformation programs (Zhang et al. 2000).

Plant tissue culture leading to somaclonal variation has been considered as one of the possible sources of inducing genetic variability in crop plants to be used in breeding programs. Somaclonal variation is used to describe the occurrence of genetic variants derived from *in vitro* procedures (Larkin and Scowcroft 1981; Isabel et al. 1993), it is also called tissue or culture-induced variation (Kaeppler et al. 2000; Bordallo et al. 2004).

Somaclonal variation is somaclonal variability often arises in tissue culture as a manifestation of epigenetic influence or changes in the genome of differentiating vegetative cells induced by tissue culture and is expected to generate stable plants carrying interesting heritable traits (Soniya et al. 2001). Evans and Sharp (2000) reported four critical variables for somaclonal variation: genotype, explant origin, cultivation period and the cultural condition in which the culture is made.

Identification of possible somaclonal variants at an early stage of development is considered to be very useful for quality control in plant tissue culture, transgenic plant production and in the introduction of variants. Many strategies can be used to evaluate plant genetic structure from *in vitro* derived plant clones, including cytogenetic analysis, isoenzyme markers and different DNA molecular markers etc., but

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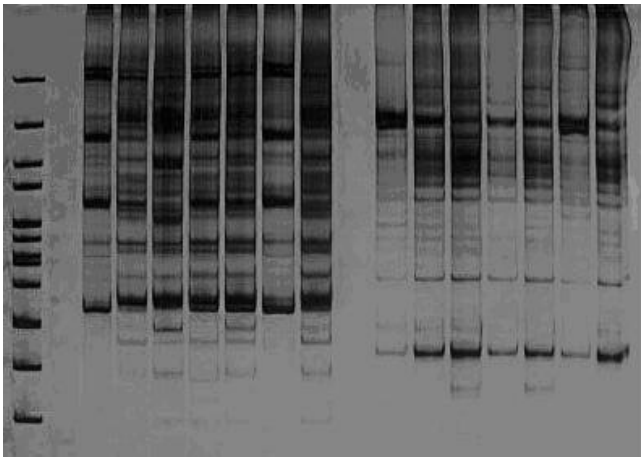

Figure 1. RAPD profile of primers OP16 and B05 in cotton cultivars studied. The columns from right to left are: Bakhtegan, B4, Zeta2, Z3, Hybrid, H3, H4 and No DNA for both primers respectively. The last column in the left is Ladder.

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. However, these markers are susceptible to ontogenetic variation and are limited in number, and only DNA segments coding for soluble proteins can be sampled. RFLP (Restriction Fragment Length Polymorphism) markers are reliable for sampling various genome regions and are potentially unlimited in number. However, this technique is slow, expensive, and requires large quantities of tissue. RAPD (Random Amplified Polymorphic DNA) analysis using PCR in association with short primers of arbitrary sequence has been demonstrated to be sensitive in detecting variation among individuals. The advantages of this technique are: a) a large number of samples can be quickly and economically analyzed using only micro-quantities of material; b) the DNA amplicons are independent from the ontogenetic expression; and c) many genomic regions can be sampled with a potentially unlimited number of markers (4). RAPD markers have been used widely in studying the genetic diversity of somaclonal variations in various plant species (Rani et al. 1995; Soniya et al. 2001).

Our earlier studies on the genetic diversity available among diploid and tetraploid cotton cultivars include morphometry, karyotype and meiotic analysis as well as molecular studies by using RAPD molecular markers (Sheidai et al. 1996; Sheidai and Alishah 1998; Sheidai et al. 1998, 2002, Sheidai and Koobaz 2003; Sheidai et al. 2004, 2006). The present study reports RAPD analysis of somaclonal variation in regenerated plants from cotton single node explants in three tetraploid cotton cultivars of Iran, namely Bakhtegan, Zeta-2 and their hybrid Bakhtegan × Zeta-2 for the first time.

Materials and Methods

Three *Gossypium hirsutum* L. cultivars of Bakhtegan, Zeta-2 and their hybrid i.e. Bakhtegan ×Zeta-2 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 3-4 times with sterile distilled water and inoculated aseptically on MS (Murashige and Skoog 1962) basal medium free hormones. Single nodes were resulted from seedlings cultured as explants. Regeneration of single nodes was attempted on MS medium containing IAA and BA. 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.

DNA was extracted from fresh leaves of *in vitro* regenerated cotton plants from 1, 2, 3, 4 and 5th sub-cultures of Bakhtegan (named accordingly B1-B5), Zeta-2 (Z1-Z5) and hybrid cultivar (H1-H5) and the mother plants by acetyl-trimethyl ammonium bromide (CTAB)-based procedure. For RAPD analysis, the PCR reaction mixture consisted of 1 ng template DNA, 1 x PCR buffer (10 mM Tris-HCJ pH 8.8, 250 mM KC12), 200 µM dNTPs, 0.80 µm 10-base random primers and 1 unit of Taq polymerase, in a total volume of 25 µl. DNA amplification was performed on a palm cycler GP-00 1 (Corbet, Australia). Template DNA was initially denatured at 94°C for 3 min, followed by 35 cycles of PCR amplification under the following parameters: denaturation for 1 min at 92°C, primer annealing for 1 min at 36°C and primer extension for 2 min at 72°C. A final incubation for 10 min at 72°C was performed to ensure that the primer extension reaction proceeded to completion.

The PCR amplified products were separated by electrophoresis on a 1.5% agarose gels using 0.5 X TBE buffer (44.5 mM Tris/Borate, 0.5 mM EDTA, pH 8) or 12% polyacrylamide gels. The gels were stained with ethidium bromide and visualized under UV light or silver stained for added sensitivity. RAPD markers were named by primer origin, followed with the primer number and the size of amplified products in base pairs. Thirty random primers of Operon technology (Alameda, Canada) were used.

The experiment was repeated for 3 times and reproducible RAPD bands were used for further analysis. The bands obtained were treated as binary characters and coded accordingly (presence =1, absence = 0). Jaccard similarity was determined among the genotypes studied to be used in clustering. The genotypes showing similarity in their RAPD characteristics were grouped by using UPGMA (Unweighted Paired Group with Arithmetic Average) and ordination based on principal coordinate analysis (PCO, Sheidai et al. 2007).

Analysis of variance (ANOVA) followed by the least significant test (LSD) was performed to indicate significant difference in quantitative morphological characters. In order to study the intra-subculture morphological variation among

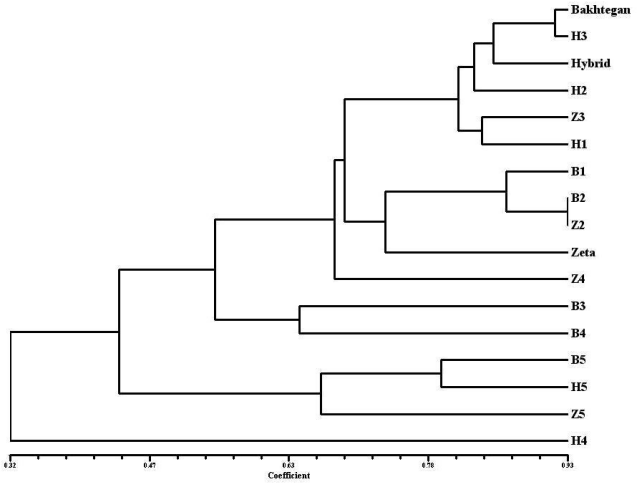

Figure 2. UPGMA clustering of the cotton cultivars based on RAPD data. (Genotype code as in the text).

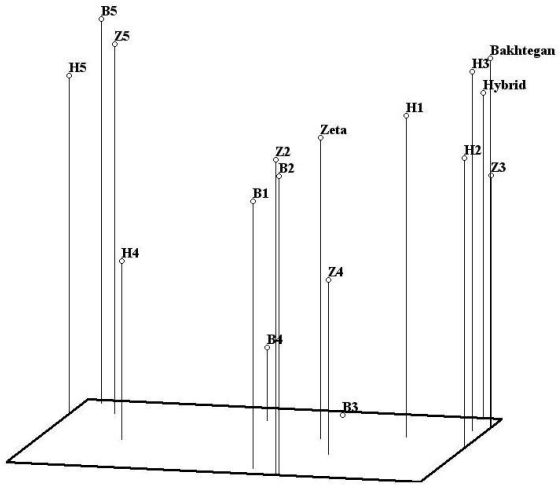

Figure 3. PCO ordination of the cotton cultivars based on RAPD data. (Genotype code as in the text).

different regenerated plants, UPGMA clustering and PCO ordination was performed. Morphological characters used are: Number of leaves, stem length, leaf shape, leaf color and number of nodes. Taxonomic Distance was determined among the genotypes studied based on standardized morphological data (Mean = 0, variance = 1), followed by clustering. SPSS Ver. 9 (1998) and NTSYS Ver. 2.02 (1998) were used for statistical analyses.

Results

Thirteen RAPD primers out of 20 primers used, produced 319 bands (Fig. 1) in all the genotypes studied. Out of 319 bands obtained, 251 were polymorphic (78.7.00%) while, 68 bands were monomorph (21.30%, Fig. 1). Among the primers used, OPI-07 produced the highest number of bands (41) while primers OPA-18 produced the lowest number (12). The highest percentage of polymorphic bands was observed in OPB-05 (96.66%) and while the lowest percentage was observed in OPA-13 (60.86%).

In total 9 specific bands were obtained out of witch, two specific bands were produced by primers OPR-15 and OPI-07 while primers OPH-02, OPH-16, OPH-19 and OPH-12 each produced one specific bands. Bakhtegan parental genotype possessed 3 specific bands which were not present in Bakhtegan sub-cultures' regenerated plants Hybrid cultivar also possessed 12 specific bands which were not present in its sub-cultures' regenerated plants.

Some bands were present in all the genotypes except one, for example the bands 5 and 10 of primer A13 were absent only in the regenerated plants of the fourth sub-culture (H4) in the hybrid cultivar of Bakhtegan X Zeta-2.Bbands 4, 6, 7 and 11 of the primer A18, were present in all the genotypes except in the regenerated plants of the sub-culture (B3) in the Bakhtegan cultivar and band 5 of the primer A18 was only absent in the regenerated plants of the second sub-culture in the hybrid cultivar of Bakhtegan X Zeta-2. Similar cases were observed for all 13 primers producing bands. Detailed molecular and morphological comparison of the parental genotypes and their subcultures' regenerated plants are presented bellow.

Bakhtegan and its sub-cultures' regenerated plants (B1-B5)

Comparison of the Bakhtegan parental genotype and its sub-cultures' regenerated plants (B1-B5) revealed their genetic differences. For example primer A13 produced twenty-two loci in the Bakhtegan cultivar and the regenerated plants of the third (B3) and forth (B4) sub-cultures, while regenerated plants of the first, second and third sub-cultures (B1, B2 and B5) did not produce any bands which were considered missing and were not included in further analysis. Out of 22 loci obtained, 14 loci were common in Bakhtegan, B3 and B4 regenerated plants, while 8 loci were polymorphic and differed in the genotypes studied. Similarly the primer A18 produced 12 loci in the Bakhtegan cultivar and the regenerated plants of the B3 and B4 only. Four loci were common in Bakhtegan, B3 and B4 regenerated plants while the other 8 loci were polymorph and varied in these genotypes.

The primers B05 and C09 produced bands in the parental genotype as well as B4 and B5 regenerated plants. The primer B05, produced 30 bands/loci, 3 of which were common in Bakhtegan, B4 and B5 regenerated plants and the other 27 loci were polymorph. The primer C09, produced 22 bands, 10 of which were common and other 13 loci were polymorph. Study of the other primers also showed variations in the number of common and polymorphic bands in Bakhtegan its

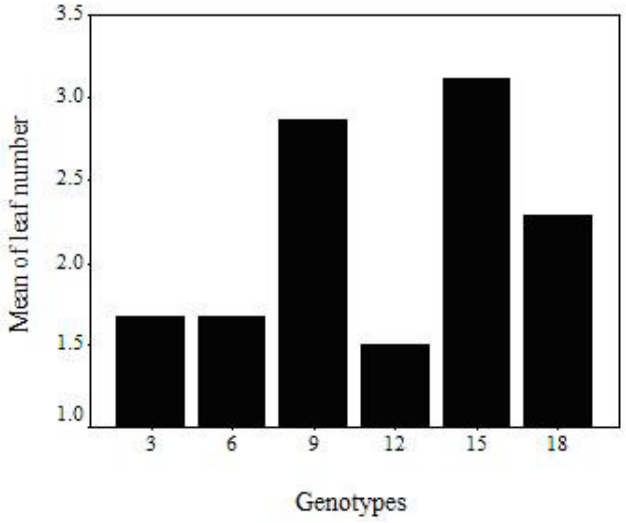

Figure 4. Mean comparison of Bakhtegan cultivar and its subcultures' regenerated plants for morphological character of leaf number. (Genotypes codes are: 3 = Bakhtegan, 6 = regenerated plants of the sub-culture B1, 9 = regenerated plants of the sub-culture B2, 12 = regenerated plants of the sub-culture B3, 15 = regenerated plants of the sub-culture B4 and 18 = regenerated plants of the sub-culture B5).

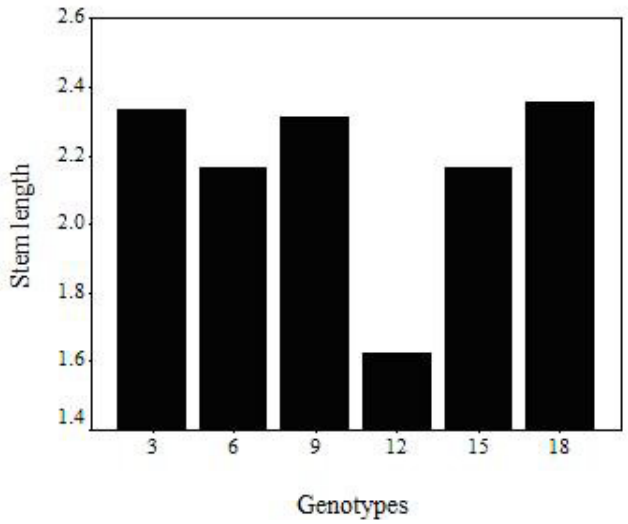

Figure 5. Mean comparison of Bakhtegan cultivar and its subcultures' regenerated plants for morphological character of stem length. (Genotypes codes are: 3 = Bakhtegan, 6 = regenerated plants of the sub-culture B1, 9 = regenerated plants of the sub-culture B2, 12 = regenerated plants of the sub-culture B3, 15 = regenerated plants of the sub-culture B4 and 18 = regenerated plants of the sub-culture B5).

different sub-cultures regenerated plants. The clustering and PCO ordination of the genotypes studied produced similar results (Figs. 2 & 3), grouping Bakhtegan and its different sub-cultures' regenerated plants in different clusters or groups far from each other.

Morphological analysis revealed the presence of a significant difference in the number of leaves and stem length among the Bakhtegan genotypes (Figs. 4 & 5). Further comparison revealed that different plantlets of each genotype also differ significantly in their morphological characters, indicating the presence of intra-subculture morphological variations. Grouping of the regenerated plants of each sub-culture showed that these plantlets are placed far from each other and mix with the other sub-cultures regenerated plants (Fig. 6).

Zeta2 cultivar and its sub-cultures' regenerated plants (Z1-Z5)

Detailed comparison of the Zeta2 genotype and its different sub-cultures' regenerated plants (Z 1- Z 5) also revealed the their genetic differences. For example the primer A13 produced 22 bands/loci in the parental genotype of Zeta2 and the regenerated plants of the third (Z3) and forth (Z4) sub-cultures only. The regenerated plants of sub-cultures Z1, Z2 and Z5 did not produce any bands which were considered missing and not entered in further analysis. Out of 22 loci obtained, 18 loci were common in Zeta-2, Z3 and Z4, while 4 loci were polymorph and differed in these genotypes.

A similar situation was observed for the primers A18 and B05. Out of twelve loci identified by primer A18, 4 loci were common in Zeta-2, Z3 and Z4 and 8 loci were polymorph, while in the primer B05, 27 loci were identified out of which, 23 loci were common in Zeta2, Z3 and Z4 and 4 were polymorph.

For the other primers used different results were obtained. For example the primers C09 and H02 produced bands in the regenerated plants of sub-culture Z3, while no band was produced in the regenerated plants of the sub-cultures Z1, Z2 and Z4.

The primer C010 produced bands in the regenerated plants of the sub-cultures Z3 and Z4 while no band was produced in the regenerated plants of the sub-cultures Z1, Z2 and Z5. These genotypes also differed in the number of common and polymorphic loci.

Morphological analysis revealed the presence of a significant difference in the number of leaves and stem length among the Zeta2 genotypes. Detailed comparison of morphological characters among different regenerated plants of each genotype also revealed the presence of intra-subculture morphological variations similar to the Bakhtegan genotypes (Fig. 7).

Hybrid cultivar and its sub-cultures' regenerated plants (H1-H5)

Comparison of the hybrid cultivar *i.e.* Bakhtegan X Zeta2 with its sub-cultures' regenerated plants (H1-H5) agrees with the results obtained for the parental genotypes of Bakhtegan and Zeta-2 as mentioned before. For instance, the primer A13 and B05, produced bands in the hybrid and the regenerated

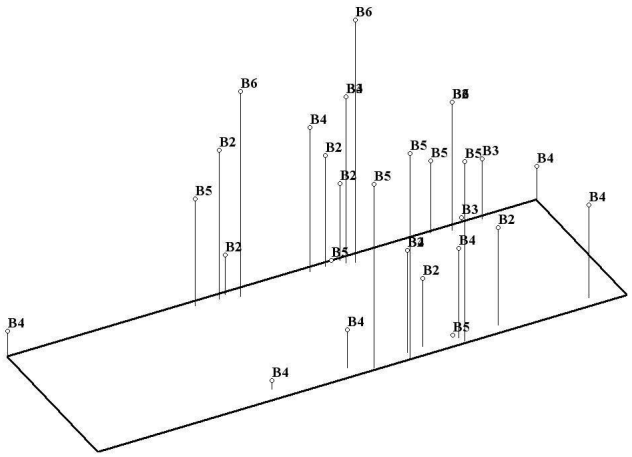

Figure6. PCO ordination of Bakhtegan regenerated plants based on morphological characters.

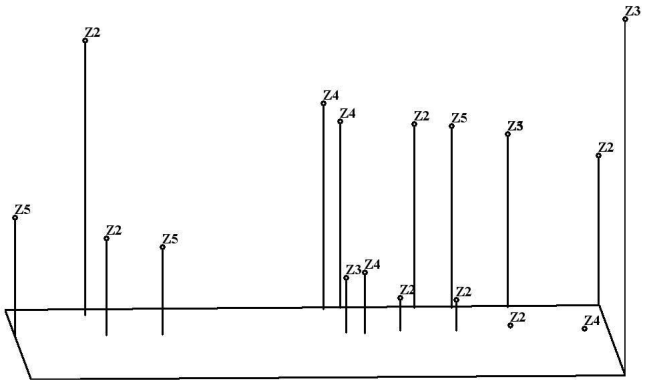

Figure7. PCO ordination of Zeta2 regenerated plants based on morphological characters.

plants of the sub-cultures H3 and H4, while no band was produced in the regenerated plants of H1, H2 and H5 subcultures. The primer C09, produced bands in the hybrid and the regenerated plants of the other sub-cultures except H4 and the primer C10, produced bands in the hybrid and the regenerated plants of the sub-cultures except the H5.

The number of common and polymorphic bands differed among the hybrid genotype and its sub-cultures' regenerated plants. For example, the primer A13 produced 23 bands/loci out of which, 15 were common and the other 8 loci were polymorph. Some of the polymorphic bands were absent in the H4 regenerated plants and some other bands were absent in the H2 regenerated plants.

The primer A18 produced 12 bands/loci out of which, 8 were common and 4 other loci were polymorph. One band was present only in the hybrid cultivar but absent in its subcultures. There were also some other bands which were present in the parental hybrid genotype but absent in the sub-cultures H2, H3 and H4 regenerated plants.

Morphological analysis revealed the presence of a significant difference in the number of leaves and stem length among the Zeta2 genotypes. Detailed comparison of morphological characters among different regenerated plants of each genotype also revealed the presence of intra-subculture morphological variations similar to Bakhtegan and Zeta-2 genotypes.

Discussion

The presence of specific bands indicates the presence of specific loci in the genotypes studied, while their absence in the following sub-cultures' regenerated plants indicates the occurrence of genetic changes during sub-culturing as a result of somaclonal variation.

Since, even single base change at the primer annealing site is manifested as appearance or disappearance of RAPD bands, it could be suggested that tissue culture conditions have induced varied amount of genetic changes in different regenerated plants. Some of these changes appeared identical in different plants as represented by appearance of non-parental bands. The reason for such commonness of genetic variation in these plants could be because they were all derived from the same callus (Soniya et al. 2001). The variations observed in the RAPD pattern may be due to different causes including loss/ gain of a primer annealing, due to point mutations or by the insertion or deletion of sequence or transposable elements (Peschke et al. 1991).

Grouping of the three parental cultivars and their subcultures regenerated plants indicate the genetic distinctness of the genotypes studied as they are placed in different clusters/groups far from each other. It also seems that the genetic variations induced in the regenerated plants increase with the time-period of the sub-culture. For example, the regenerated plants of the sub-cultures B1 and B2 show comparatively lower degree of genetic difference from the Bakhtegan parental genotype as they are placed in the clusters much closer to the Bakhtegan compared to the regenerated plants of the latter sub-cultures like B4 and B5. The regenerated plants of the sub-culture B5 show the highest degree of genetic difference with the others as they are placed in a cluster very far from the other Bakhtegan genotypes. A similar situation is present for the Zeta-2 and hybrid cultivars and their sub-cultures regenerated plants.

Detailed comparison of the genotypes studied also show different effects of sub-cultures on the genetic variation induced. For example, in the hybrid cultivar Bakhtegan x Zeta-2, the primer A18 produced 4 polymorphic loci one of which was present only in the hybrid but absent in its subcultures, possibly due to genetic changes occurred during sub-culturing process. There were also some other bands

present only in the hybrid parental genotype but missing in the H2, H3 and H4 regenerated plants, indicating the presence of genetic variation among different sub-cultures of the hybrid Bakhtegan X Zeta2.

The findings here are in line with the earlier reports on application of RAPD in describing genetic polymorphisms among regenerated plants in several other plants, viz. *Apium* species, and *Prunus* species (Soniya et al. 2001). The present study also shows that different cotton genotypes differ in the level of somaclonal variation and in each genotype a specific subculture may bring about the highest genetic variation. Explant source is also considered as one of the critical variable for somaclonal variation. Since explants may present dissimilar regeneration rates, selection procedures can differ among different explants types. For example, plants regenerated from chrysanthemum petal epidermis-induced calli showed greater somaclonal variation than those from apex-induced calli (De Jong and Custers 1986). Therefore it may be suggested that different sources of explants may be tried in cotton and compare the level of genetic variation obtained.

Significant morphological differences obtained among the regenerated plants of different sub-cultures and also within each sub-culture may indicate that the molecular/ genetic variation obtained is partly responsible for morphological variations, and also show the possible use of tissue culture in inducing new morphological (possibly new agronomic) characters in the cotton which may be used for breeding purposes.

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