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Full Length Research Paper

Molecular, C-value and morphological analyses of somaclonal variation in three olive cultivars

Farah Farahani[1], Reza Yari[2] and Masoud Sheidai[3]*

[1]Department of Microbiology, Islamic Azad University, Qom branch, Qom, Iran.
[2]Biology Department, Islamic Azad University, Boroujerd Branch, Boroujerd, Iran.
[3]Faculty of Biological Sciences, Shahid Beheshti University, Tehran, Iran.

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The occurrence of somaclonal variation was studied in three olive cultivars (*Olea europaea* L.) by using Random amplified polymorphic DNA (RAPD), C-value and morphological analyses. The tissue culture of the cultivars was performed using internode cuttings. The experiment was carried out up to the fifth and seventh subculture in different cultivars. Significant difference was observed in morphological characters among the regenerated plants of different subcultures in all cultivars. Grouping of the genotypes based on morphological data separated some of the regenerated plants from the others due to their morphological differences, however this was not correlated with the time period of subculturing. RAPD analysis produced polymorphic and specific bands in the genotypes studied. Grouping of the genotypes based on RAPD data revealed that in all cultivars the parental genotype and regenerated plants of the first and second subcultures are placed close to each other while the regenerated plants of the latter subcultures stand far from the other genotypes due to their genetic difference. No significant difference occurred in C-value in mother plants and the regenerated plants, indicating that genetic differences are mainly of nucleotide polymorphism/mutations and not due to quantitative change in DNA amount during tissue culture process.

Key words: Olive, Random Amplified Polymorphic DNA (RAPD), tissue culture.

INTRODUCTION

The olive tree (*Olea europaea* subsp. *Europaea*) is one of the ancient and important crop plants of many countries including Iran (Green, 2000; Green and Wickens, 1989; Zohary, 1994; Besnard et al., 2002a). The knowledge of available genetic diversity in the olive germ plasm as well as inducing new genetic variability by different means is very important for future breeding of this valuable plant.

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 (Isabel et al., 1993). This 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 are expected to generate stable plants carrying interesting heritable traits (Soniya et al., 2001).

Evans and Sharp (2000) considered four basic and influencing variables in somaclonal variation, including the genotype, explant origin, cultivation period and the tissue culture condition.

Different strategies exist for evaluation of the somaclones, including cytogenetic and C-value analyses, isoenzyme markers and various DNA molecular markers etc.

Various molecular markers have also been used to study genetic diversity in olive trees such as izoenzymes, RAPD (Random Amplified Fragment Length Polymorphisms) markers, rDNA genes, chloroplast DNA, microsatellite markers, internal transcribed spacer 1 (ITS-1) sequences, intersimple sequence repeats (ISSR), etc. (Angiolillo et al., 1999; Hess et al., 2000; Besnard et al., 2001; Besnard et al., 2002b; Belaj et al., 2002; De la Rosa et al., 2002; Lumaret et al., 2004). RAPD markers

*Corresponding author. E-mail: msheidai@cc.sbu.ac.ir, msheidai@yahoo.com.

have been used widely in studying the genetic diversity of somaclonal variations in various plant species (Rani et al., 1995; Soniya et al., 2001).

Three Iranian olive cultivars namely Zard, Roghani and Eiks cultivars which are under cultivation in different regions of Iran were used for studying somaclonal variation and if tissue culture may bring about new genetic changes in the genome of these cultivars to be used in olive breeding program.

MATERIALS AND METHODS

Tissue culture and morphological characters

Tissue culture of 3 olive cultivars 'Zard', 'Roghani' and 'Eiks' (*Olea europaea* L.) were performed. One centimeter of internode samples were cut and cultured after sterilization and ddH_2O and NaOCl were added to. The DKW medium (Driver and Kuniyuki, 1984) containing 30 gL^{-1} sucrose, 7.0 gL^{-1} agar, 0.1 gL^{-1} Inositol, 0.2 ml^{-1} 2-ip was used for shooting and rooting explants production. The medium was sterilized by autoclaving for 20 minat 121°C. All media containing adjusted pH to 5.8 before autoclaving. Three to five samples were placed into glass bottles and maintained at 25±2°C under a 16/8 h light photoperiodic under a light intensity 3000 lux in a germinator. After 30 days some samples were transferred to fresh medium and processed till the seventh subculture for detection of morphological variations. Morphologic characteristics such as number of leaves, length and number of shoots, rooting, internode length in cm, length and width leaf in mm were evaluated in each subculture for both cultivars. The experiment was repeated for 3 times for each treatment used and morphological data were analyzed by analysis of variance test (ANOVA) followed by least significant difference test (LSD).

RAPD analysis

For RAPD analysis, fresh leaves were selected randomly from trees and DNA extraction was done by use of modified CTAB method (Murry and Tompson, 1980). The PCR reaction mixture consisted of 1 ng template DNA, 1 × PCR buffer (10 mM Tris-HCL pH 8.8, 250 mM KCL), 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-001 (Corbet, Australia). Template DNA was initially denatured at 92°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 2% agarose gels using 0.5 ×TBE buffer (44.5 Mm Tris/Borate, 0.5 Mm EDTA, pH 8.0) or 6% polyacrylamide gels. The gels were stained with ethidium bromide and visualized under UV light (Sambrook et al., 2001). A 100 bp DNA ladder (Gene Ruler, Fermentas) was used as the molecular standard in order to confirm the appropriate RAPD markers. These markers were named by primer origin, followed with the primer number and the size of amplified products in base pairs. RAPD bands were treated as binary characters and coded accordingly (presence =1, absence = 0). Jaccard similarity as well as Nei's genetic distance (Nei, 1972) were determined among the cultivars studied and used for grouping of the genotypes by clustering methods and ordination based on principal coordinate analysis (PCO) (Podani, 2000; Weising et al., 2005).

The level of genetic variation was determined by Nei's gene diversity (I) (Nei, 1979) as well as Shannon information index (H) (King and Schaal, 1989). UPGMA (Unweighted Paired Group using Arithmetic Average) and NJ (Neighbor Joining) clustering as well as PCO (Principle Coordinate Analysis) was performed for grouping of the genotypes. The fit of dendrograms obtained were checked by cophenetic correlation and bootstrap values (Podani, 2000). NTSYS ver. 2 (1998) was used for multivariate analyses and the genetic diversity was determined by POPGENE ver. 1.32 (1997).

Flow cytometry

Sample preparations were carried out in a two-step procedure. In the first step, 50 mg of fresh and non-fixed samples (leaves) were used. In the first step we placed plant tissue onto a plastic petri dish and added 0.5 ml of Cystain DNA and chopped the tissue with a sharp razor blade in order to release the nuclei from cells then added 1.5 ml of Cystain DNA and incubated at room temperature for 5 min. The sample was filtered through a Partec 50 µm Cell Trics filter and analysed in Partec flow cytometer (PI), Cod.05-5004 using UV excitation and measured blue emission. For standardization, we applied pea leaf as reference standard due to genome stability (Baranyi and Greilhuber, 1995, 1996).

For each individual, the 2C DNA content was estimated by comparing the mean peak intensity of its nuclei with the mean peak intensity of the *Pisum sativa* nuclei (4.40 pg, Koce et al., 2003). The resulting values were expressed as a peak ratio, which is a ratio of the mean position of the G_0/G_1 peak in the DNA histogram of the tested plant to the mean position of the G_0/G_1 peak in the histogram of the reference plant. To estimate genome size of the samples, each analysis was repeated at least three times (Bures et al., 2004).

The 2C DNA nuclear content of the samples was calculated as follows: Sample 2C relative/sample peak mean 2C DNA content × 2C DNA content of the standard. One-way ANOVA and Least Significant Difference tests were employed to assess the significance of genome size difference between wild and cultivated olives, by using SPSS ver. 9 (1998).

RESULTS

Morphological characters

ANOVA test followed by LSD showed significant difference ($p<0.01$) in morphological characters among the regenerated plants of different subcultures in three cultivars studied. NJ clustering and PCO plot (Figures 1 to 6) of the genotypes based on morphological data separated regenerated plants of the second subculture from the other genotypes of 'Eiks cultivar', the fourth and sixth subcultures in the 'Roghani' cultivar and the third subculture in 'Zard' cultivars from the other genotypes due to morphological differences.

RAPD analysis

RAPD analysis among 'Roghani' genotypes showed that all 12 primers used produced 69 bands in total, out of which 26 bands were monomorph, 43 bands were polymorph. Four specific RAPD bands were obtained, Band No. 3 (850 bp) of the RAPD primer OPC-03 which

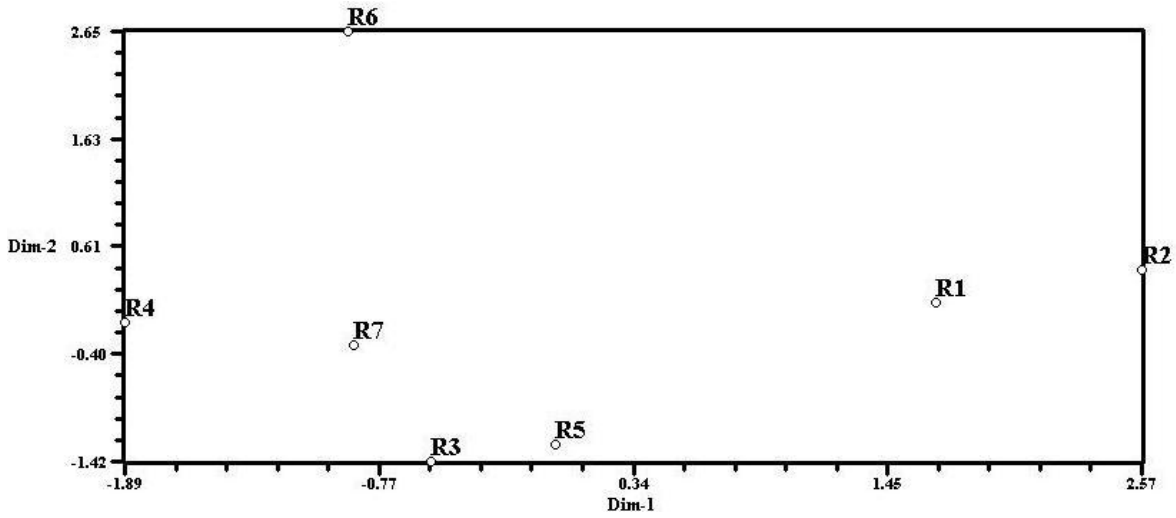

Figure 1. PCO plot of Roghani genotypes based on morphological data. R1-R6 = Regenerated plants of subcultures 1-6, respectively.

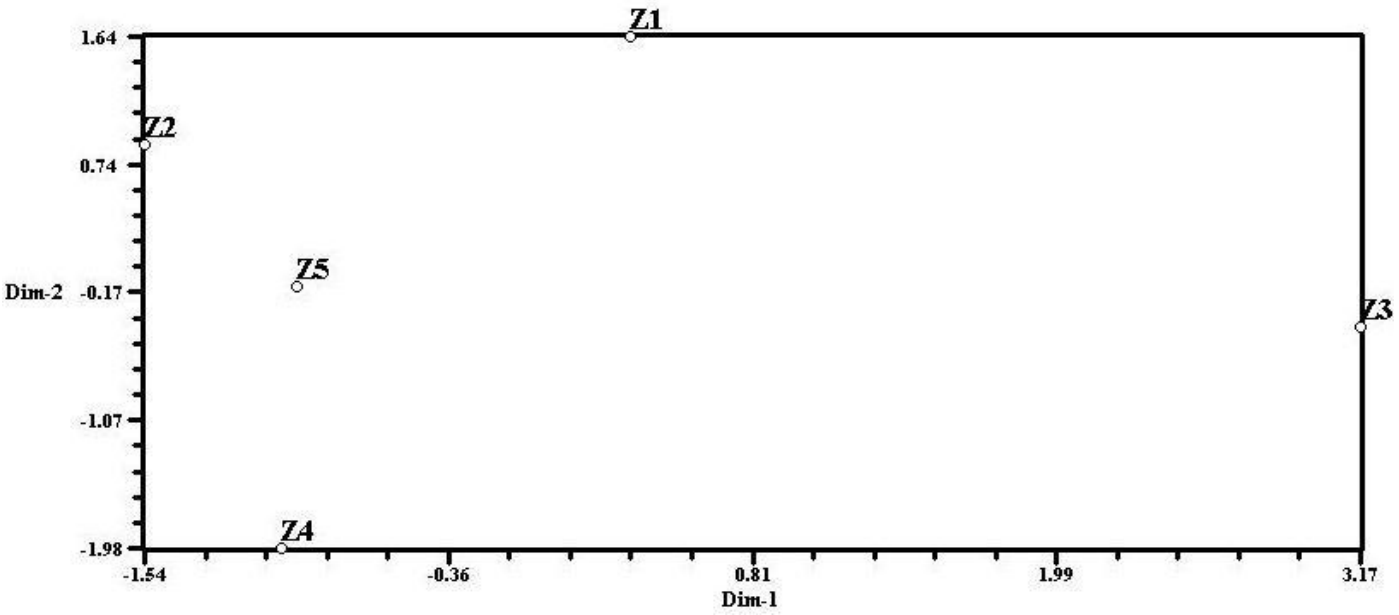

Figure 2. PCO plot of Zard genotypes based on morphological data. Z1-Z5 = Regenerated plants of subcultures 1-5, respectively.

was present only in the regenerated plants of the third subculture, band No. 3 (500 bp) of the RAPD primer OPC-04 which occurred only in the regenerated plants of the seventh subculture and band No. 3 (900 bp) of the RAPD primer OPA-15 occurring only in the regenerated plants of the second subculture.

In case of 'Eiks cultivar', a different RAPD profile was obtained. In total 45 RAPD bands were obtained, out of which 18 bands were polymorph. Seven specific RAPD bands were obtained, for example band No. 5 (300 bp) of the primer OPM-19 occurred only in the regenerated plants of the third subculture while, bands No. 1 and 2 (1 and 2 kb respectively) of the primer OPC-08 as well as band No. 1 (4 kb) of the primer OPC-05 occurred only in the regenerated plants of the fifth subculture.

Band No. 6 (700 bp) of the primer OPC-06 was absent only in the regenerated plants of the fifth subculture, while band No. 1 (2.50 kb) of the primer OPA18 was absent in the regenerated plants of the third subculture.

In the 'Zard' cultivar, 66 RAPD bands were obtained, out of which 31 bands were monomorph and 35 bands were polymorph. Five specific RAPD bands were

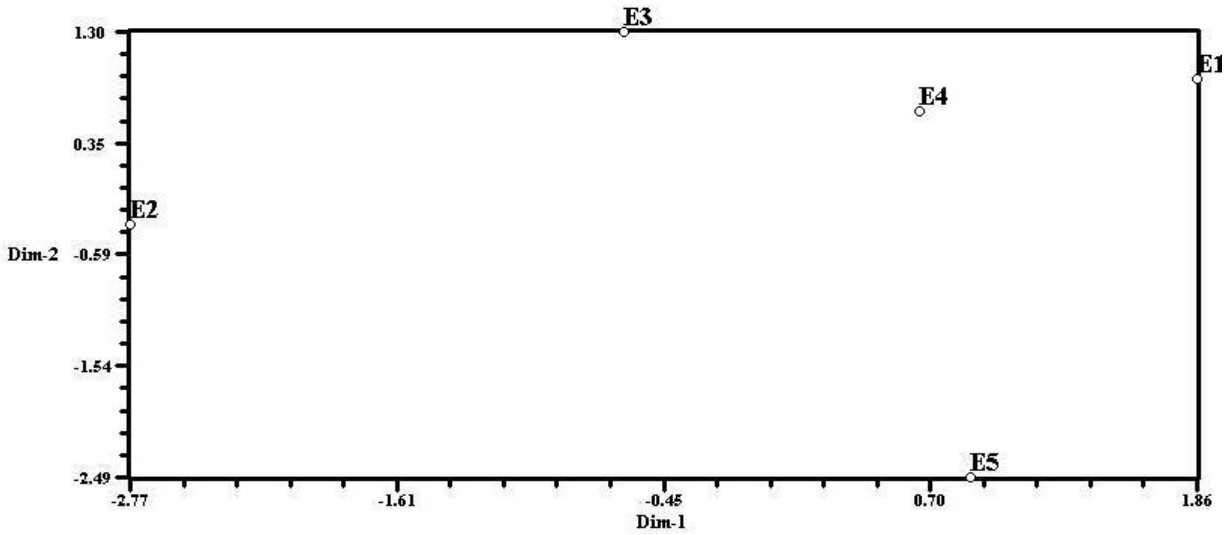

Figure 3. PCO plot of Eiks genotype based on morphological data. E1-E5 = Regenerated plants of subcultures 1-5, respectively.

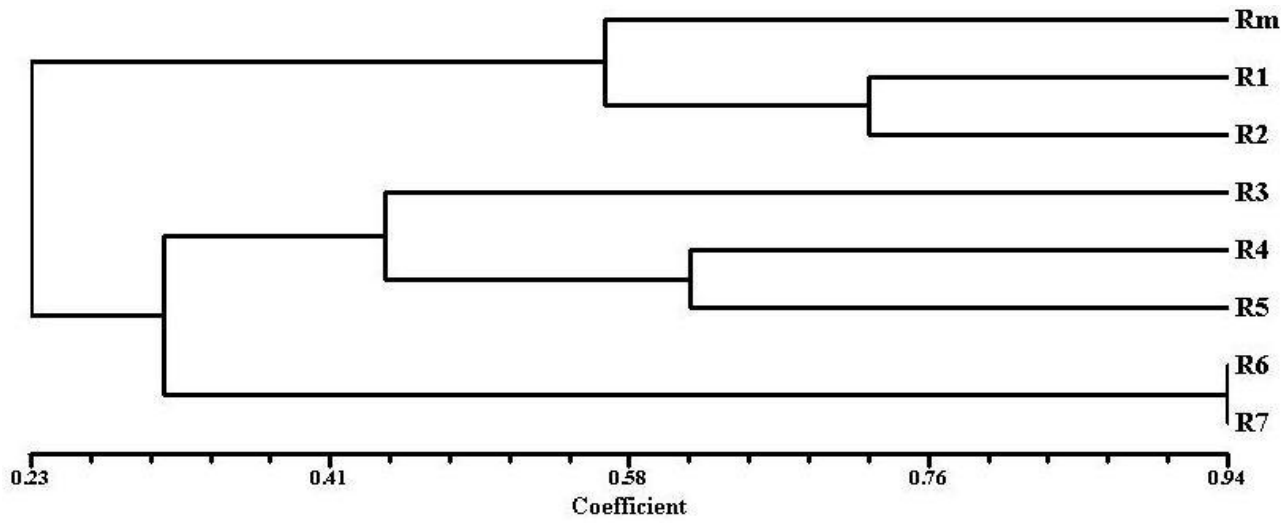

Figure 4. UPGMA tree of Roghani genotypes based on RAPD data. Rm = Parental plants, R1-R7 = regenerated plants of subcultures 1-7, respectively.

obtained, for example, bands No. 1, 7 and 8 (750 bp to 15 kb) of the primer OPA-06 occurred only in the regenerated plants of the fifth subculture while, bands No. 2 and 13 (13 kb and 380 bp respectively) of the same primer occurred only in the regenerated plants of the third subculture. Moreover, bands No. 3 and 6 (450 and 900 bp respectively) of the RAPD primer OPI-07 as well as bands No. 5 and 6 (400 and 450 bp respectively) were present in all the genotypes but were absent only in the regenerated plants of the fifth subculture.

UPGMA clustering (Figures 4 to 6) and PCO plot of the genotypes based on RAPD data using Jaccard similarity index as well as Nei's genetic distance produced similar results in all 3 genotypes grouping the parental genotype and regenerated plants of the first and second subcultures close to each other, while the regenerated plants of the third, fourth and the latter subcultures joined them with some distance. The regenerated plants of the sixth and seventh subcultures of the 'Roghani' cultivar and fourth and fifth subcultures of 'Zard' and 'Eiks' cultivars, which are the latest subcultures, stand far from the other genotypes due to their genetic difference.

The Nei's genetic identity and genetic distance determined between different subcultures in the three

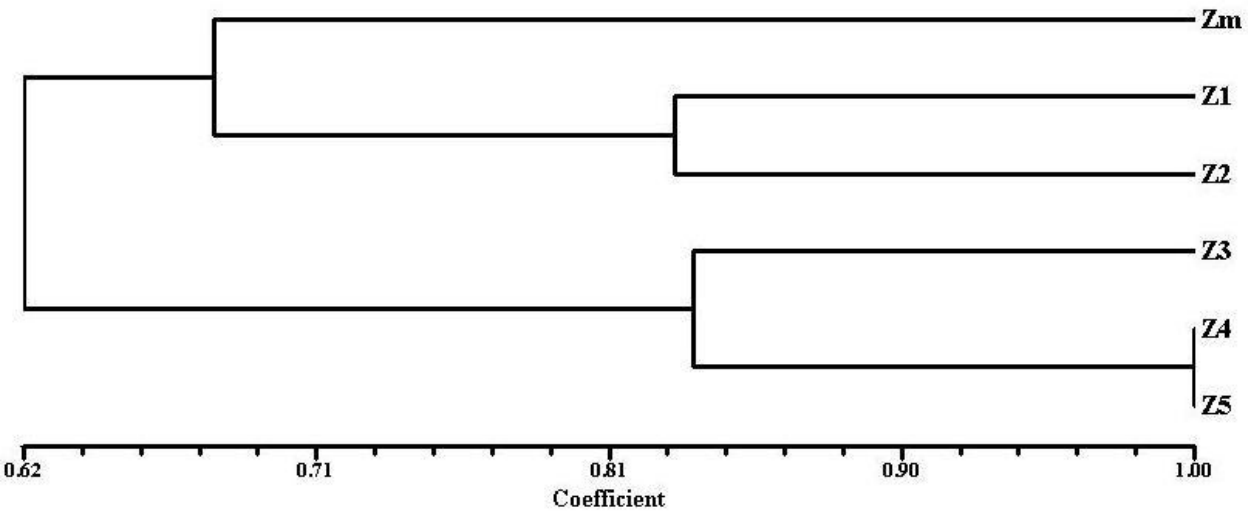

Figure 5. UPGMA tree of Zard genotypes based on RAPD data. Zm = Parental plants, Z1-Z5 = regenerated plants of subcultures 1-5, respectively.

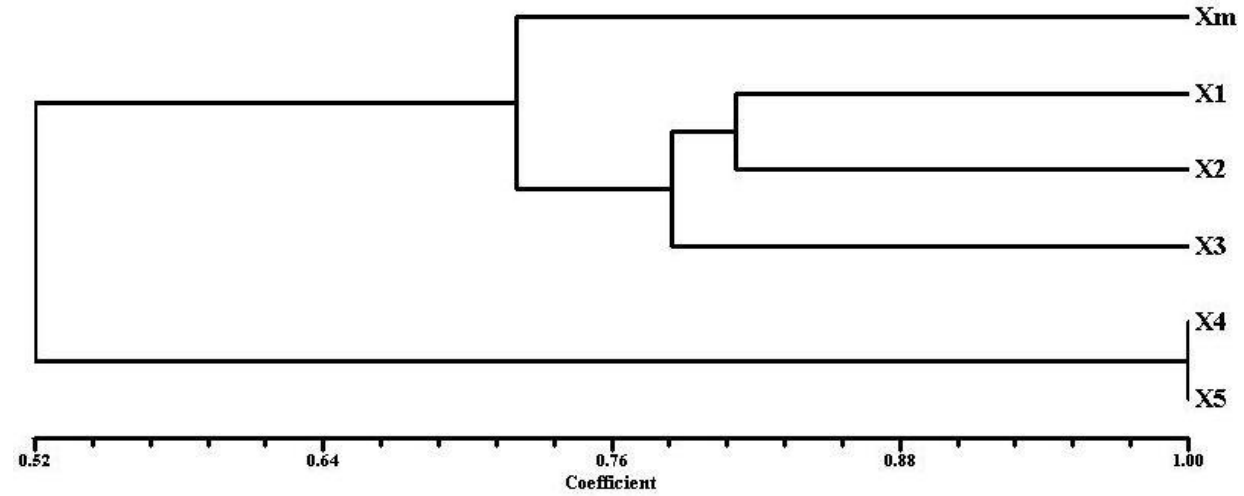

Figure 6. UPGMA tree of Eiks genotypes based on RAPD data. Xm = Parental plants, X1-X5 = regenerated plants of subcultures 1-5, respectively.

cultivars studied revealed that in 'Zard' cultivar, the lowest genetic distance occurs between the regenerated plants of the fourth and fifth subcultures (0.12), while the highest value of genetic distance occurs between parental plants and the regenerated plants of the fourth subculture (0.52). Similarly, the lowest value of genetic distance in the 'Eiks' cultivar was observed between the regenerated plants of the fourth and fifth subcultures (0.00), while the highest value of genetic distance occurred between parental plants and the regenerated plants of the fourth and fifth subcultures (0.67). In 'Roghani' cultivar the lowest value of genetic distance was observed between the regenerated plants of the fifth and sixth subcultures (0.00), while the highest value of genetic distance occurred between parental plants and the regenerated plants of the fifth and sixth subcultures (0.98).

Nei's genetic diversity (H) and Shanon index (I) determined showed H = 0.26 and I = 0.37 for 'Eiks', H = 0.41 and = 0.63 for 'Roghani' and H = 0.22 and i = 0.32 for 'Zard' cultivars.

C-value analyses

The parental plants of Roghani cultivar showed the mean

C-value of 5.42 pg, while the regenerated plants showed C-values ranging from 5.37 to 5.54 pg with the mean value of 4.42 pg. The parental plants of 'Zard' cultivar showed the mean C-value of 5.35 pg, while the regenerated plants showed C-values ranging from 5.38 to 5.51 pg with the mean value of 5.41 pg. Similarly the parental plants of 'Eiks' cultivar showed the mean C-value of 5.49 pg, while the regenerated plants showed C-values ranging from 5.36 to 5.40 pg with the mean value of 5.41 pg.

The mean C-value did not differ significantly among regenerated plants of different subcultures and also between the mother plants and tissue culture plants in all 3 cultivars studied.

DISCUSSION AND CONCLUSION

In the three cultivars studied, the regenerated plants of different subcultures differed significantly but in each cultivar, the subculture bringing about the highest morphological variations differed. This indicates that morphological changes are not completely correlated with time period of subculture and the latter subcultures not necessarily bring about more changes. Anyhow, significant morphological differences obtained among the regenerated plants of different sub-cultures 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 olive which may be used for breeding purposes.

Leva (2009) analysed thirty-two morphological traits among the regenerated olive plants obtained by somatic embryogenesis the plants produced by conventional cutting propagation method. Stable variant morphological phenotypes such as plant height, canopy dimensions, leaf, inflorescence and fruit dimensions were observed in the somaclonal plants.

The presence of specific RAPD bands/loci in some of the genotypes indicates the occurrence of molecular changes due to tissue culture and such loci can be considered as genetic finger prints for genotypes discrimination. Similarly the absence of RAPD bands/loci only in one genotype and their presence in the other genotypes indicates the loss of certain DNA nucleotides from the genome or due to point mutations or by the insertion or deletion of sequence or transposable elements (Peschke et al., 1991), due to 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 different degree of genetic changes in different regenerated plants. The appearance of non-parental bands in some of the subcultures could be because they were all derived from the same callus (Soniya et al., 2001).

It seems that the genetic variations induced in the regenerated plants increase with the time-period of the sub-culture, as regenerated plants of latter sub-cultures stand far from the parental genotype and plants of the earlier sub-cultures as it is supported by clustering and Nei's genetic distance obtained. The findings here are in line with the earlier reports on application of RAPD in describing genetic polymorphisms among regenerated plants in olive cultivar (Peyvandi et al., 2010) as well as several other plant species including *Apium* species, and *Prunus* species (Soniya et al., 2001).

Peyvandi et al. (2010) reported somaclonal variation in the plants of *Olea europaea* L. *cv. Kroneiki* derived from somatic embryogenesis by RAPD analysis and showed different degree of genetic difference among subcultures. The genetic similarity coefficient obtained indicated that regenerated plants were less than 75% similar to mother plants. The present study also shows that different olive genotypes differ in the level of somaclonal variation as the H and I values of the 3 genotypes studied varied, with 'Roghani' cultivar showing the highest values of genetic diversity induced during tissue culture.

Explant source is also considered as one of the critical variable for somaclonal variation. Different explants may bring about dissimilar regeneration rates and the 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 olive and compare the level of genetic variation obtained.

C-value analysis showed that no significant change has occurred during subculturing in the 3 cultivars and their tissue culture regenerated plants, indicating that the genetic changes accompanied by somaclonal variation is mainly due to the changes in the nucleotide content of the genome due to mutations (insertions/deletions) and not due to quantitative changes.

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