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Molecular Analysis of Genetic Stability in Long-Term Micropropagated Shoots of *Olea europaea* L. (cv. Dezful)

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Abstract: Somaclonal variation of Micropropagated plants of olive trees (cv. Dezful) were evaluated between subcultures as well as two different carbohydrate sources by using RAPD technique and physiological characters. In this study, the highest polymorphic RAPD bands were observed between regenerated plants of first subculture and seventh subculture's while, the lowest changes were shown between two carbohydrate treatments in each subculture and it was also true between mother trees and first subculture plants. Different subcultures produced different level of genetic diversity in the cultivar studied. The genotypes also differed significantly in physiological characters like number of nods and branches by increasing the number of subcultures. The data reported in this study revealed that micropropagation may affect both genetic profile and physiological traits of the olive plants studied.

Key words: Carbohydrate source, olive, RAPD markers, somaclonal variation

INTRODUCTION

Olive (*Olea europaea* L.) cultivars like other important crops need the genetic improvement and obviously it is indispensable to use *in vitro* technologies. *In vitro* techniques have proven to be useful to clone selected woody plant genotypes producing plants theoretically identical to the original material (Kaeppler *et al*., 2000; Bordallo *et al*., 2004). This technique, after an initial difficulty for the stabilization of the explants and the successive growth, today has satisfactory results. Micropropagation by auxiliary bud stimulation for *in vitro* propagation of olive cultivars were reported and they are actually a commercial reality in the nursery production of some Mediterranean's countries by using mature materials (Leva *et al*., 2002; Rugini and Pesce, 2006).

The value of tissue-culture-induced variation or somaclonal variation to crop improvement depends on establishing a genetic basis for this variation. Factors such as explant source, time of culture, number of subcultures, phytohormones, genotype, media composition, the level of ploidy and genetic mosaicism are capable of including *in vitro* variability (Silvarolla, 1992). The occurrence of somaclonal variation is a potential drawback when the propagation of an elite tree is intended, where clonal fidelity is required to maintain the advantages of desired elite genotypes (e.g., superior growth, wood properties, disease resistance and other quality traits). On the other hand, stable somaclonal variation of a specific type may be advantageous for the improvement of certain traits (Antonetti and Pinon, 1993; Kanwar and Bindiya, 2003). Among the mechanisms causing this variation, the activation of transposable elements (Hirochika *et al*., 1996), DNA hypomethylation (Jaligot *et al*., 2000; Keyte *et al*., 2006; Lukens and Zhan, 2007), genome adaptation to different regulatory microenvironments (Bogani *et al*., 1996) and the presence of hot spots of DNA instability (Linacero *et al*., 2000) have been reported.

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 and in the introduction of variants. Recently somaclonal variation at phenotypic, cytological, biochemical and molecular levels were detected among

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micropropagated plants in many taxa (Rival *et al*., 1998; Rani *et al*., 2000; Ray *et al*., 2006; Joshi and Dhawan, 2007). Molecular markers are widely used to detect and characterize somaclonal variation at the DNA level (Cloutier and Landry, 1994; Waruiru *et al*., 1997; Bednarek *et al*., 2007) of the available techniques such as isozyme, RFLP and RAPD, while RAPD markers is among the most useful ones because; large number of samples can be quickly and economically analyzed by using only micro-quantities material, the DNA amplicons are independent from the ontogenetic expression and many genomic regions can be sampled with a potentially unlimited number of markers (Isabel *et al*., 1993) changes in the RAPD pattern may result from the loss/gain of a primer annealing provoked by point mutations or by the insertion or deletion of sequences or transposition elements (Kaeppler *et al*., 2000).

Up to now the genetic stability of the *in vitro* olive cultivars was tested by agronomic and morphological observation (Leva *et al*., 2002; Rugini *et al*., 2006) and molecular markers on field performance of micropropagated olive plants (Leva *et al*., 2002).

The influence of different carbohydrate sources of medium and the numbers of subcultures on somaclonal variation in olive micropropagated shoots using physiological and molecular characteristics is reported for the first time.

MATERIAL AND METHODS

The experiences were conducted in National Institute of Genetic Engineering and Biotechnology of Iran during 2006- 2008.

Plant material and micropropagation: Actively growing shoots of the Iranian olive cultivar Dezful were collected from a four-year old green house grown tree which located in National Institute of Genetic Engineering and Biotechnology (NIGEB, Tehran, Iran). Leaves were removed and sterilized with bleach (20%) for 5 min then rinsed in sterile distilled water (three times). Apical buds of sterile shoots were removed and shoots were cut into single nod segments. The explants were cultured in DKW medium (Driver and Kuniyuki, 1984) supplemented with 2-iso pentenyl adenin (2 i.p.) (4 mg L^{-1}) Two different treatments of Mannitol (30 g L^{-1}) and sucrose (30 g L^{-1}) were used as a carbohydrate source in tissue culture medium. The pH was adjusted to 5.8 before agar addition (6%) and autoclaving. *In vitro* shoots (4-5 nods) raised from explants were used for further experiments after 50 days treatment.

In order to study the influence of the number of subcultures and different carbohydrate sources on the generation of somaclonal variants, two nodal explants of sterile shoots were sub-cultured for seven times with 45 days interval period in the same tissue culture medium (DKW) including mannitol or sucrose treatment. All samples were kept in a growth chamber with 16 h light/8 h dark photoperiod and 24±2°C. After each subculture, the number of nods and branches raised from each explants were measured for further studies.

Molecular analysis: Five explants were randomly collected from the first, forth and seventh subcultures and 5 plants were collected randomly from the parental genotype cv. Dezful. DNA was extracted using the CTAB method, described by Murray and Thompson (1980) with modification by De-la-Rosa *et al*. (2002). Approximately 1 g of fresh tissue from each explant were powdered in liquid nitrogen. DNA was qualified by electrophoresis (3 V cm^{-1}) in 0.8% agarose gels (w/v). The DNA was visualized by ethidium bromide staining and the original DNA solutions were then diluted to 10 ng L^{-1} for PCR reactions.

For RAPD analysis, the PCR reaction mixture consisted of 20 or 40 ng template DNA, 1 x PCR buffer (10 mM Tris-HCl pH 8.8, 250 mM KCl2), 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 in a Thechne (Thechne, Germany) thermocycler. The reactions were subjected to 35 amplification cycles, after an initial denaturation at 94°C for 3 min. Each cycle consisted of 1 min at 92°C, 1 min at 36°C and 2 min at 72°C, with a final 10 min at 72°C. The PCR amplified products including of five explants from each sub-culture were separated by electrophoresis on 2% agarose gels due to examine RAPD profiles of repeated samples from each sub-culture. The representative samples from each sub-culture for obtaining more band resolution were separated by electrophoresis on 6% polyacrylamide gels (Sambrook *et al*., 1989). A DNA ladder (100 bp, Gene Ruler™, Fermentase) was used as molecular weight marker and the gels were stained with silver staining (Sambrook *et al*., 1989). After optimization of amplification conditions, 20 random primers of Operon technology (Alameda, Canada) were selected in kit C (OPC-01 to OPC-20).

Data analysis: Only consistently reproducible, well-resolved fragments, in the size range of 100 to 2000 bp were scored after three times repetitions as the present or absent for RAPD markers in each subculture and

treatment studied as well as in the mother cultivar trees. Bands of equal molecular weight and mobility generated by the same primer were considered to be identical. Data was scored as 1 for presence and 0 for the absence of DNA band in each plant. Genetic similarities between samples were measured by the Jaccard's and Simple Matching similarity coefficient and the similarity matrix obtained was used to construct dendrogram using the UPGMA (Unweighted Paired Group with Arithmetic Average). The two-dimensional Principal Coordinate Analysis (PCO) was constructed to complement the cluster analysis information using the NTSYS PC 2.1 software package (Rohlf, 1998).

Analysis of Variance (ANOVA) followed by the Least Significant Test (LSD) was performed to indicate significant difference in physiological characters (the number of nods and branches) between subcultures and treatments. χ^2-test was used to determine significant difference in the number of RAPD bands between subcultures and treatments used. SPSS Ver. 9 (1998) was used for univariate statistical analyses.

RESULTS

Ten primers out of 20 RAPD primers used produced 715 reproducible bands in all subcultures and treatments used (Table 1, Fig. 1). Out of 715 bands obtained, 41 were polymorph (34.2%) while, the rest were monomorph (65.8%). Among the primers used, OPC-20 produced the highest number of bands (97) while primer OPC-06 produced the lowest number (54. The highest number of polymorphic bands (7 bands) was observed in the primers OPC-19 and OPC-08 while the lowest number (2 bands) was observed in OPC-16, OPC-15 and OPC-06 (Table 1).

In total 5 specific bands were obtained, 4 of which were produced by the primers OPC-15 (2 bands) and OPC-14 (2 bands) these specific bands occurred in the first subculture with sucrose and mannitol 30 g L^{-1} treatments as well as mother olive trees.

Some bands were present in all the genotypes except one, for example the bands produced by primers OPC-19 (4 bands) and OPC-08 (7 bands) were present in all except in the regenerated plantlets of the seventh subculture in both treatments.

Among the genotypes studied the highest number of RAPD bands occurred in the mother olive trees (111 bands) as well as the regenerated plants of the first subculture (112 bands) while the lowest number of RAPD bands (87 bands) occurred in the regenerated plants of the seventh subculture (Table 1).

The χ^2-test performed between the two treatments of mannitol 30 g L^{-1} and sucrose 30 g L^{-1} in each subculture

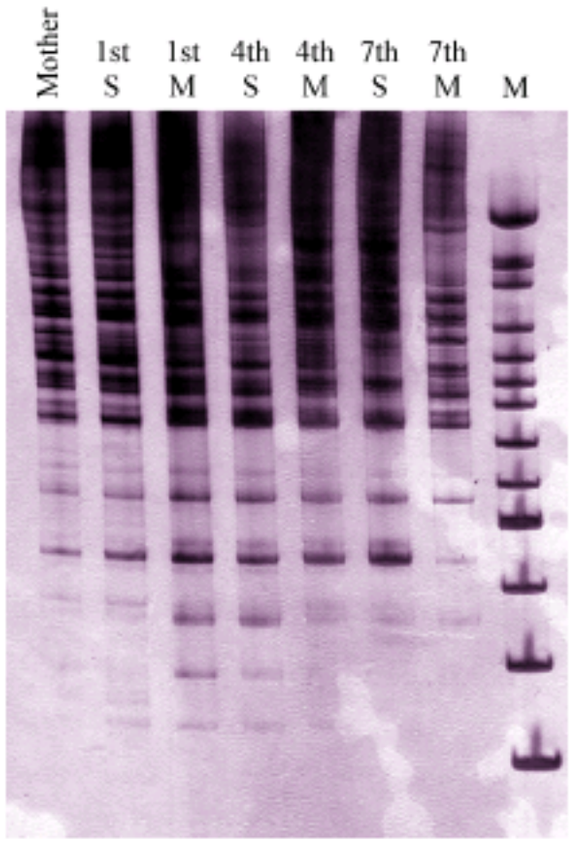

Fig. 1: RAPD profile of primers OPC-20 of generated plants in first, forth and seventh subcultures with two treatments and mother olive trees studied. S: Sucrose, M: Mannitol

Table 1: Total and polymorphic number of RAPD bands in 10 RAPD primers studied in three subcultures (first, forth and seventh), two treatments (Mannitol 30 g L^{-1} and Sucrose 30 g L^{-1}) and mother olive trees (cv. Dezful)

	Total bands								
	1st subculture		4th subculture		7th subculture				
Primers	Sucrose 30 g L^{-1}	Mannitol 30 g L^{-1}	Sucrose 30 g L^{-1}	Mannitol 30 g L^{-1}	Sucrose 30 g L^{-1}	Mannitol 30 g L^{-1}	Mother trees	Total	Polymorphic bands
OPC-05	10	10	7	7	8	8	11	61	5
OPC-06	7	7	9	8	7	7	9	54	2
OPC-08	14	14	14	14	8	7	12	83	7
OPC-12	12	12	11	11	11	9	12	78	4
OPC-14	10	10	8	8	8	8	8	60	5
OPC-15	12	12	4	6	10	10	10	64	2
OPC-16	9	9	9	9	8	8	10	62	2
OPC-18	14	14	13	13	12	12	15	93	4
OPC-19	11	11	11	11	4	4	11	63	7
OPC-20	13	13	15	15	14	14	13	97	3
Total	112	112	101	102	90	87	111	715	41

did not show any significant differences in the number of RAPD bands while the number of band varied significantly ($p< 0.05$) among the regenerated plantlets of the first, forth and seventh subcultures.

Analysis of Variance (ANOVA) and LSD tests showed a significant difference ($p<0.05$) in the physiological traits like the number of nods and branches of regenerated plants among the subcultures studied. For example in mannitol treatment, the mean number of nods significantly increased with an increase in the number of subculture. However, no significant difference was observed in the mean number of branches among 3 subcultures in mannitol treatment (Table 2).

A significant decrease ($p<0.05$) was observed in the number of branches in sucrose treatment among regenerated plants of the longer period subcultures (Table 2), while no significant differences was observed in mean number of nods among the subcultures studied.

Different similarity coefficients determined among the samples studied, showed the highest value of similarity between regenerated plants of mannitol and sucrose treatments in each subcultures (for example, r = 0.99 in simple matching coefficient) while the lowest value of similarity occurred between regenerated plants of first subculture and seventh subculture in both treatments studied (for example, r = 0.70 in Simple Matching coefficient) (Table 3).

The cophenetic correlation showed a high value (r = 0.98) for UPGMA method indicating a good fit of clustering to the original similarity of the samples. In general two major cluster or groups are formed (Fig. 2, 3) The first major cluster is comprised of two sub-clusters. The mother plants and the regenerated plants of the first subculture in both mannitol and sucrose treatments form the first sub-cluster, while the regenerated plants of the forth subculture are placed in the second sub-cluster with more than 0.80 similarity.

The second major cluster is comprised of the regenerated plants of the seventh subculture joining to the members of the first cluster with some distance (0.75).

Table 2: The mean number of nods and branches in first, forth and seventh subcultures

No. subculture	Carbohydrate (g L^{-1}) Mannitol	Sucrose	Branch	Nod
1st	30	0	1.44a	4.07b
	0	30	1.27a	3.66bc
4th	30	0	1.28a	5.31a
	0	30	1.00b	3.14c
7th	30	0	1.36a	5.44a
	0	30	1.06b	3.56bc

Different letter(s) in row indicate significant differences ($p\leq0.05$)

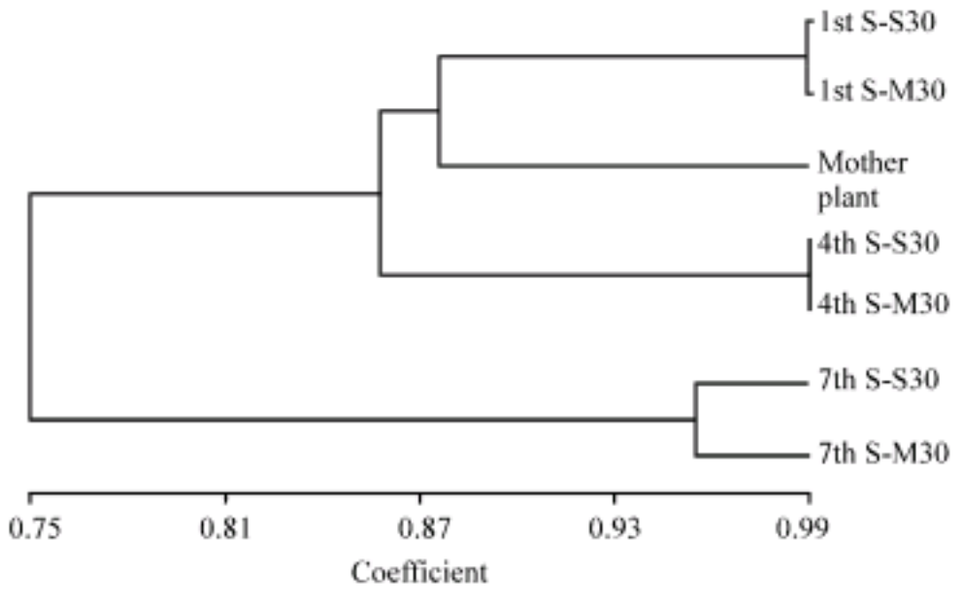

Fig. 2: UPGMA clustering of regenerated plants of first (1st), forth (4th) and seventh (7th) subcultures in two treatments sucrose 30 g L^{-1} (S30) and mannitol 30 g L^{-1} (M30) along with mother plant based on RAPD markers

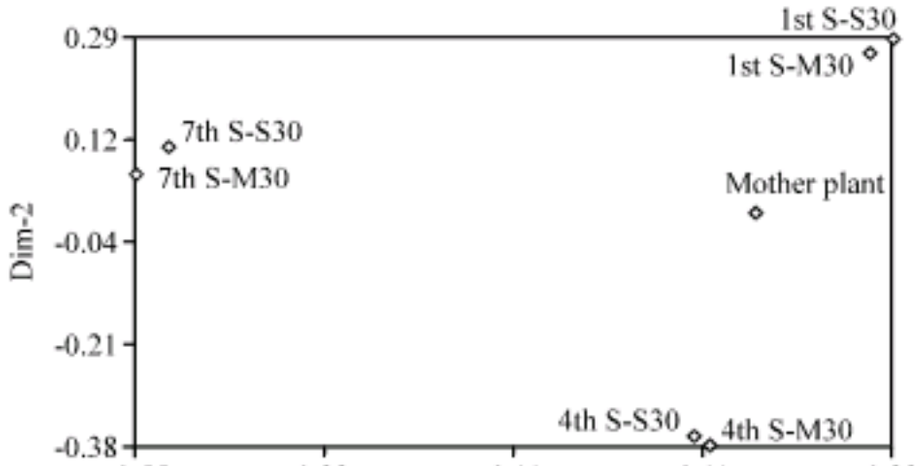

Fig. 3: PCO ordination of regenerated plants of first, 4th and 7th subcultures in two treatments sucrose 30 g L^{-1} (S30) and mannitol 30 g L^{-1} (M30) along with mother plant based on RAPD markers

Table 3: Simple matching similarity among regenerated plants of subcultures and mother trees

Subculture-carbohydrate	1st S-sucrose	1st S-mannitol	4th S-sucrose	4th S-mannitol	7th S-sucrose	7th S-mannitol	Mother plant
1st S-sucrose	1.00						
1st S-mannitol	0.99	1.00					
4th S-sucrose	0.86	0.85	1.00				
4th S-mannitol	0.86	0.85	0.99	1.00			
7th S-sucrose	0.75	0.74	0.77	0.78	1.00		
7th S-mannitol	0.73	0.70	0.77	0.78	0.95	1.00	
Mother plant	0.89	0.86	0.77	0.86	0.77	0.73	1.00

The stability of the groups was also confirmed by partitioning the variants of data sets using PCO (Fig. 3). The first and second Principal Components (PC) accounted for 51 and 29% of total variation, respectively. Generally, PCO plot supported the clustering results obtained.

DISCUSSION

In this study, 10 primers were analyzed for assessing genetic variability in micropropagated plantlets of the olive cultivar Dezful. In total 715 amplified products were produced, out of which, 41 amplified products exhibited polymorphism (34.2%). As mentioned before χ^2-test shows a significant difference in the number of RAPD bands among the subcultures studied and not between the two carbohydrate treatments used. Therefore, the genetic variation obtained among the regenerated plants is mainly due to the effect of time period of subcultures and not due to treatments.

The presence of a RAPD marker in two genotypes indicates a high level of sequence homology at that site (Williams *et al*., 1990). In the case, where the marker was present in one genotype but not the other, there is the certainly of sequences difference. Failure of amplification for regenerated plants in seventh subculture may be due to a single base change in DNA sequences. These changes could present in either the sequence of the primer binding site or changes which alter the size or prevent the successful amplification of a target DNA (e.g., insertions, deletions, inversions).

In present study, with increasing the number of subcultures, polymorphism increases. Similarity index value also shows distance between parent and micropropagated plants by increasing the number of subcultures. The micropropagated plants belonging to seventh subculture stand by far other explants in UPGMA cluster while micropropagated plants of first subculture have more similarity to mother trees (Fig. 3). This cluster also shows more similarity between explants grown in 2 treatments studied in each subculture. It has also been proved by analysis the number of bands in these two treatments.

As mentioned earlier, the factorial analysis performed for physiological characteristics shows a significant interaction indicating different effects of subcultures and treatments own these characters. For example, with increasing the number of subcultures number of nods in mannitol treatment increase, while the number of branches in sucrose treatment decrease. The reason for such interaction is that mannitol as the carbon source of medium improve growth rate. As regards the carbon source, it has been shown that the polyalcohol promoted significantly more growth than sucrose by increasing shoot length, pairs of leaves formed, breaking apical dominance, improves the general quality and uniformity of shoot cultures and reduces basal callus formation in some olive cultivars (Leva *et al*., 1994; Garcia *et al*., 2002). Mannitol the most widely distributed alditol, is present in over 100 higher plant species and it is one of the main soluble components in olive tissues and plays important roles acting as an osmoprotectant in salt and water stress tolerance and providing a carbon and energy source for sink tissue. In this way, membrane transport of mannitol is a major determinant of olive tree growth and productivity (Conde *et al*., 2007).

Molecular and physiological analyses on tissue culture regenerated plants in different species have shown the presence of genetic variability among the mother and regenerated plants (Hashmi *et al*., 1997; Zucchi *et al*., 2002; Modgil *et al*., 2005; Peredo *et al*., 2006). Leva *et al*. (2002) reported that micropropagation did not affect either the field performance or the genetic fidelity of micropropagated Italian olive cultivar (Maurino) which is in contrary to our results. It may be because of different cultivars studied or in vitro media used. Some of the studies showed that somoclonal variation is genotype-dependent. Therefore, the genotype of the plant can affect the amount of variability that occurs as a consequence of tissue culture *in vitro* (Hashmi *et al*., 1997; Loureiro *et al*., 2007).

In conclusion, the present study demonstrated that genetic differences occurred during micropropagation of olive *in vitro*. None of the micropropagated plants were exactly similar to the parental tree in terms of RAPD profile while two carbohydrate treatments studied did not show very genetic differences in each subculture. Therefore, other elements in tissue culture may involve in molecular variability produced by *in vitro* culture while physiological traits has been changed by different carbohydrate treatments.

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