Research Article

Assessment of genetic stability of olive *in vitro* propagated by RAPD marker

Maryam Peyvandi [1]*, Mahnaz Monsef[1], Mehdi Hosseini-Mazinani[2]

1. Department of Biology, Faculty of Sciences, Islamic Azad University, Tehran-North Branch, Iran
2. National Institute of Genetic Engineering & Biotechnology (NIGEB), Tehran, Iran

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Abstract

The effect of sub culturing frequencies and different cytokinins (benzyl amino purine (BAP), 2-isopentenyl adenine (2ip) on genetic stability of micropropagated shoots from olive plant were investigated by using physiological traits and Random Amplified Polymorphic DNA (RAPD) markers. Axillary buds of the olive (cv. Dezful) plants were cultured and subcultured in DKW medium, supplemented with BAP (4 mgl^{-1}) or 2-ip (4 mgl^{-1}). In different hormone media and subcultures there were not significant differences in shoot proliferation rate. To amplify DNA, 18 arbitrary decamer primers were screened, out of which 16 primers generated clear and reproducible bands. All RAPD profiles from the micropropagated plants were monomorphic. The treatments included four successive subcultures in two hormone treatments (2ip or BAP) (4 mgl^{-1}) The 16 primers produced a total of 213 (an average of 13.31 band per primer) scorable bands. The dendrogram constructed on the basis of jaccard's similarity matrix, followed by UPGMA based clustering analysis showed that micropropagated plants were genetically stable and similar to the mother plant.

***Keywords*:** Micropropagation, genetic stability, *Olea europaea* L., RAPD

Introduction

Olive (*Olea europaea L.*) is one of most important fruit tree with a great commercial value that cultivated in many countries such as Iran and Mediterranean countries (Rugini, 1984). Considering the development of olive cultivation programs and difficulties that an experienced in olive vegetative proliferation, propagation of plants through in vitro culture is considered to be the most effective method. In vitro culture techniques provide an alternative means of plants propagation and a tool for crop improvement, rapid clonal propagation and obtaining high number of elite genotypes and conservation germplasm of endangered species (Vasil, 1998).

Micropropagation has been applied to the olive since 1980s (Zacchini and De Agazio,2004; Mendoza-de Gyves et al., 2008; Peyvandi et al., 2009a, 2009b; Ansar et al., 2009; Haq et al., 2009).

In this study, proliferation of shoots through the stimulation of axillary buds has been achieved by different cytokinins. Cytokinin influenced the shoot elongation by their effect on cell division and cell expansion. Growth regulators such as cytokinin may lead to generated genetic instability in tissue culture, namely somaclonal variation (Larkin and Scowcroft, 1981).

Among the micropropagation methods, propagation by axillary bud simulation is considered to bear low-risks of genetic instability (Rugini and Pesce, 2006; Peyvandi et al., 2009 a, 2009b) and widely used in micro propagation systems.

In commercial propagation programs, the most crucial concern is to retain the genetic and physiological characteristic of the mother plants and it is compulsory to check regularly the clonal fidelity or genetic uniformity of micro propagated plantlets (khawale et al., 2006). The genetic variation rate depends on the genotype, the explant source, the number of subcultures and duration of the culture period and the composition of the culture medium (Smith, 1998). Assessment of the genetic stability of *in vitro* derived clones is an essential step in the application of biotechnology for micro propagation of true to type clones (Diaz et al., 2003).

Phenotypic identification, based on a description of the morphological and physiological traits, can be used but some changes induced by *in vitro* culture cannot be easily observed because the structural difference in the gene product does not

∗Corresponding author E-mail:
m_peyvandi@iau-tnb.ac.ir

always alter its biological activity enough to be noticeable in the phenotype (Jin et al., 2008). When this occurs, somaclonal variations can be detected using morphological, cytological, biochemical and molecular methods (Al- Zahim et. al., 1999; Zhao et.al., 2005; Joshi and Dhawan, 2007) among micropropagated plants in many Taxa. Molecular techniques, particularly Random Amplified Polymorphic DNA (RAPD) have been proposed to be appropriate powerful tools for identification of somaclonal variation and establish genetic stability (Rahman and Rajora, 2001; Bennic et al., 2004; Javanmardi et al., 2011).

In the present study, the influence of different hormones and the number of subculturing on genetic stability in the micropropagated olive plants, using RAPD markers, is evaluated.

Materials and Methods

Plant material and micropropagation

Young shoots of an Iranian olive cultivar 'cv. Dezful' (10-15 cm long) were collected from a five-year old plant grown greenhouse. Leaves were excised and sterilized with commercial bleach (20%) for 5 minutes then rinsed in sterile distilled water (3 times). Apical buds of sterile shoots were removed and shoots were cut into single nod segments. Uninodal explants were cultured in DKW medium (Driver and Kuniyuki Walnut, 1984) with two different hormone treatments (2ip 4 mg/l)or (BAP4 mg/l). The carbohydrate source in tissue culture medium was mannitol (30 g/l). pH was adjusted to 5.7-5.8 (prior to autoclaving) and subsequently autoclaved for 20 min at 120ºC.

To study the effect of the number of subculturing and different hormone treatments on genetic stability of the micropropagated olive plants, sterile *in vitro* shoots were used for plant tissue culture. Two nodal explants of sterile shoots were subcultured for 4 times with 45 days interval in the same/ different tissue culture medium (DKW) including 2ip or BAP. All samples were kept in a growth chamber with 16h light/8 h dark photoperiod and 24 ± 2 ºC. After each subculture, the number of nods and branches raised from each explant and number of leaves and the length of the internods were measured for further analyses.

Experiments followed a randomized complete block design. 4 explants per jar and 8 replications per treatment were tested. Analysis of variance was performed by General Linear Model procedure (SPSS ver.14) and differences among the treatments were evaluated by Duncan Test ($p \leq 0.05$).

Molecular analysis

DNA extraction and PCR amplification

Three explants were randomly collected from cultures, each of four successive subcultures, and from the parental genotype cv. Dezful. DNA was extracted from the leaves of these explants using the CTAB (Cetyl–Trimethyl–Ammonium Bromide) method, described by Murry and Thompson (1980) with modification by De la Rosa et al. (2002), using approximately 1 gr of fresh tissue from each explant was powdered.

DNA was qualified by electrophoresis (3 V/cm) 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 for PCR reactions.

PCR amplification for RAPD analysis was carried out in a total volume of 20 µl, containing 20–40 ng template DNA, 1x PCR buffer "complete"(670 mM Tris-HCL, pH 8.8, 160 mM (NH4)2SO4, 0.1% Tween-20, 25 mM MgCl2), 200 µM dNTPs, 1 µm 10-mer primers (Operon Technologies Alameda California) and 0.5 unit of Taq polymerase . The amplification reaction was performed in a (Touchgene Gradient) thermocycler. After an intial denaturation at 94 ºC for 5 minutes, followed by 35 cycles consisting of a denaturation step at 94 ºC for 1 min, annealing step at 35 ºC for 1 min, extension step at 72 ºC for 2 min and a final extension at 72 ºC for 10 min. The PCR amplified products were separated by electrophoresis on 2% agarose gels and stained with ethidium bromide. The representative samples from each culture and sub-culture, for obtaining more band resolution, were separated by electrophoresis on 6% polyacrylamide gels (Sambrook et al., 2001).

A DNA ladder (Mix, Gene RulerTM, Fermentase) was used as molecular weight marker and the gels were stained with silver staining (Sambrook et al., 2001).

Data Analysis

Only consistently reproducible, well-resolved fragments, in the size range of 200 bp to 2500 bp were scored from 3 repeats. The presence or absence of the RAPD markers in the micropropagated plantlets obtained from each culture and subculture stages as well as in the mother cultivar trees in different treatments were evaluated. Bands of equal molecular weight and mobility generated by the same primer were considered to be identical.

RAPD reproducible fragments were scored as 1 for presence and 0 for the absence of DNA band in each sample. 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).

Table 1. The mean number of branch nodes, leaves and length of internodes in culture, first, second, third and fourth subcultured shoots. Different letter columns indicate significant differences ($p \leq 0.05$).

Step of Culture	Hormone (4 mgl^{-1}) 2ip	Hormone (4 mgl^{-1}) BAP	Branch	Node number	Leaves number	Length of internode (cm)
Culture	+	-	1.76_{a}	4.13_{a}	8.75_{ab}	10.36_{ab}
	-	+	1.79_{a}	4.13_{a}	9.91_{a}	11.74_{a}
First Subculture	+	-	1.58_{a}	2.27_{bcde}	4.08_{cde}	5.96_{de}
	-	+	1.52_{a}	2.00_{bcde}	4.03_{cde}	5.82_{de}
Second Subculture	+	-	1.51_{a}	3.33_{abc}	6.03_{bc}	7.22_{bcd}
	-	+	1.62_{a}	2.95_{abcd}	5.52_{bcd}	6.48_{de}
Third Subculture	+	-	1.74_{a}	3.21_{ab}	5.97_{bcd}	5.71_{de}
	-	+	1.53_{a}	3.38_{ab}	6.31_{bc}	7.30_{bcd}
Forth Subculture	+	-	1.53_{a}	3.27_{abc}	6.00_{bcd}	6.82_{de}
	-	+	1.39_{b}	3.27_{abc}	6.2_{bc}	876_{bcd}

Table 2. Total number and size range of the amplified fragments generated by 16 random decamer primers in axillary shoots of olive plants (cv. Dezful).

Sr.#	Primer name	Primer sequence (5′-3′)	No. of amplified fragment	Size range (bp)
1	OPC-01	TTCGAGCCAG	11	580-2200
2	OPC-02	GTGAGGCGTC	12	330-1550
3	OPC-03	GGGGGTCTTT	8	780-2500
4	OPC-04	CCGCATCTAC	11	440-2500
5	OPC-05	GATGACCGCC	10	470-1750
6	OPC-06	GAACGGACTC	14	450-2600
7	OPC-08	TGGACCGGTG	20	310-2400
8	OPC-10	TGTCTGGGTG	17	450-2500
9	OPC-11	AAAGCTGCGG	13	370-2100
10	OPC-12	TGTCATCCCC	16	470-2500
11	OPC-13	AAGCCTCGTC	11	485-2400
12	OPC-14	TGCGTGCTTG	13	360-1860
13	OPC-16	CACACTCCAG	12	440-2650
14	OPC-17	TTCCCCCCAG	11	480-2300
15	OPC-18	TGAGTGGGTG	18	430-2400
16	OPC-20	ACTTCGCCAC	16	360-1645
Total			213	

Results

Micropropagation

The explants on DKW medium with different combinations of growth regulators started to sprout and elongate after 45 days of culturing. After this period the growth rate of the subcultured explants at different conditions were recorded. Analysis of variance (ANOVA) performed for comparing the effects of different growth factors in successive subcultures indicated that there was no significant difference ($p<0.05$) between the effects of these two hormones on the number of nodes, branches and the leaves in the regenerated plants (Table 1). In both hormone treatments, it was observed that the growth factors in the culture step were more than the other steps of subcultures.

RAPD analysis

Three sets of PCRs were carried out for RAPD fingerprinting of each sample. 16 out of the 18 arbitrary initially screened decamer primers, produced clear and scored bands. A total of 3195 bands (number of samples analyzed × number of scorable bands in all tested primers) were generated, which exhibited homogenous banding patterns with the RAPD markers.

All of the mentioned fragments were monomorphic, confirming the genetic stability of the micropropagated plants. The 16 primers used in this analysis yielded 213 scorable bands with an average of 13.31 bands per primer. The size of amplified products ranged from 200 bp to 2500 bp. Among the used primers OPC-08 produced the highest number, 20, of bands while primer OPC-03 produced the lowest number, 8, of bands (Table 2). The size of the monomorphic DNA fragments produced by these two primers ranged from 310 bp to 2400 bp and 780bp to 2500bp respectively. As an example, the pattern obtained for primers OPC-04 and OPC-20 are shown in Figures 1 and 2. Genetic similarities between the micropropagated plants and the mother plant were scored by comparing their RAPD profile for each primer and calculating the coefficient of genetic similarity.

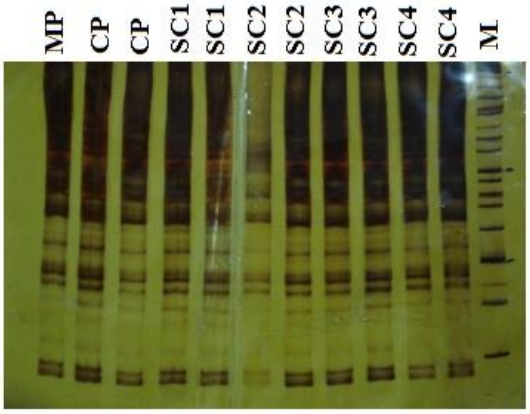

Figure 1. RAPD profile of micropropagated plants of *Olea europaea* L. generated by the primer OPC04 in the mother plant (MP), cultured (CP), and the subcultured microshoots in 2ip treatments. The abbreviations on top of each lane represent as, M: DNA ladder Mix, MP: the mother plant, SC1: the first subcultured plant, SC2: the second subcultured plant SC3: the third subcultured

plant, SC4: the forth subcultured plant; 2ip: 2-isopentenyl adenine, BAP: 6- Benzyl amino purine.

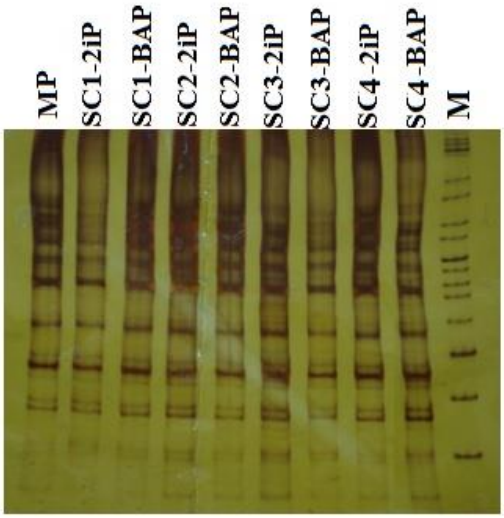

Figure 2. RAPD profile of micropropagated plants of *Olea europaea L.* generated by the primer OPC20 in the mother plant and subcultured microshoots. The abbreviations on top of each lane represent as, M: DNA ladder Mix, MP: the mother plant, SC1: the first subcultured plant, SC2: the second subculture plant SC3: the third subcultured plant, SC4: the forth subcultured plant; 2ip: 2- isopentenyl adenine, BAP: 6- Benzyl amino purine.

The dendrogram was constructed on the basis of jaccard's similarity matrix, followed by UPGMA (unweighted pair group mean average) based clustering analysis, which showed that the genotypes were grouped into single (in one major) cluster with the mother plant. The results indicate that plant regenerated in two different hormone treatments and in four successive subcultures had 100% similar to the mother plant.

Discussion

An important problem associated with plant propagation is the genetic stability among sub-clones derived from the original parents. The genetic integrity of the micrropropagated plants may lead to changes either in the phenotype or the genotype that can be determined with by different techniques. These variations are often undesirable, but still heritable (Brieman et al., 1987).

RAPD technique has been used to amplify regions of the genome of the plants regenerated from cultured cells, tissues or organs and useful for determination of genetic stability in the plantlets such as Ginger (Rout et al., 1998); Almond (Martins et al., 2004); Grape (Alizadeh and Kumar Singh, 2009); Date Palm (Kumaret al., 2010), Pistacia (Ehsanpour and Arab, 2009).

Of the 18 primers tested, 16 produced amplification products that were all monomorphic across the mother plant and plantlets (micro propagated plants). Similarity index value did not show distance between the parental and micropropagated plants even after increasing the number of subcultures at different hormone treatments. The results of the present study indicates that the genetic similarity between the parental plant and the *in vitro* propagated shoots from the subcultures in different hormone treatment were 100%.

Somaclonal variation in different plants have been widely studied (Rugini and Pesce, 2006). The presence or absence of variation depends very much on the source of explant and method of regeneration or on the source (callus, protoplast and cell) from them the shoots are regenerated (Larkin and Scowcroft, 1981). Micropropagation through axillary buds/organized meristems is generally considered to be a low risk method for genetic instability (Pierik, 1991; Martins et al., 2004) because the organized meristems are generally more resistant to genetic changes as compared to the unorganized calli under *in vitro* conditions (Shenoy and Vasil, 1992).

Present results showed no significant different ($p<0.05$) in physiological growth rate such as number of nodes, leaves, and branches as well as length of the shoots at all stages by different treatments. There are contrary reports on effects of two different hormonal treatment of BA and 2ip. While on olive, cultivar Kalamon (Dimassi-Theriou, 1994) showed that BA alone is more effective than 2ip and, Peyvandi (2009a) found that on olive cultivar Rowghani the 2ip was better than BAP. However our results here showed that there was not significant difference between application of two hormones (BAP,2ip).

Our finding are in agreement with Leva et al. (2002, 2009) who reported micropropagation did not affect morphological and genetic fidelity of tissue culture-derived olive plants. Lopes et al. (2009), using SSR method, reported no mutation between the donor trees and somatic embryos in two olive species. Also Brito et al. (2010) reported the genetic stability of two micropropagated wild olive species using flow cytometry and microsatellite markers.

According to our results, the number of subculturing and different hormonal treatments could not be affecting parameters to impose genetic instability. Also, the present study confirms the suitability of the micropropagation procedure in term of genetic stability and that the RAPD technique is sensitive enough for the somaclonal

variation analysis in the regenerated olive plants.

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