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Assessment of Genetic Stability of Micropropagated Olive (*Olea europaea* L.) Cultivars Using RAPD Marker

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Abstract: Olive (*Olea europaea* L.) cultivars are multiplied by grafting, suckers and cuttings. *In vitro* propagation may be a good alternative for olive multiplication. The aim of the work was to evaluate genetic fidelity of three different micropropagated olive cultivars, compared with the donor plants by RAPD-PCR assay. The response of three olive cultivars ('Koroneiki', 'Picual' and 'Manzanillo') to *in vitro* multiplication was studied by examining different types and concentrations of both 6-Benzylaminopurine (BAP) and 6-(γ,γ-Dimethylallylamino) purine (2ip). The effect of genotypes was obvious; 'Manzanillo' showed better performance compared with the other cultivars. On the other hand, 5 ppm BAP record the highest mean shoot number (MSN) and mean shoot length (MSL) compared with 2ip. Also, the best mean leaf number (MLN) was obtained when cultured on MS medium supplemented with 5 ppm BAP. Random amplified polymorphic DNA (RAPD) analysis was performed to evaluate the genetic stability of the micropropated plants compared with the donor plants. A total number of six decamer RAPD primers gave 39 distinct and reproducible bands ranging from 90 to 1500 bp. UPGMA dendrogram depend on Jaccard's coefficient illustrating that olive plants regenerated *in vitro* had high similarity with the mother plants.
Key words: Tissue culture, genetic fidelity, molecular marker, genetic distance.

Introduction

Olive (*Olea europaea* L.) is one of the most important fruit trees in the Mediterranean basin [1-3]. Olive trees were usually multiplied by grafting, suckers and cuttings. Micropropagation is a powerful method which allows propagation of pathogen-free cultivars under controlled conditions. Through the last two decades, many approaches have been made towards the optimization of olive micropropagation [4]. Micropropagation techniques allow alternate methods of plants rapid clonal multiplication and a tool for the germplasm conservation [5-8] and genetic improvement [9]. Meanwhile, the process of *in vitro* multiplication is affected by many factors [10, 11].

Occurrence of genetic changes is a dangerous trouble in micropropagation of crop species because of their undesirable traits[12]. The variations produced during tissue culture techniques are generally induced by single gene mutations and chromosomal rearrangements. The *in vitro* changes in plants may be reflected in the banding profiles developed by different molecular marker methods [13, 14]. DNA markers have been applied to characterize and verify the origin, stability of clones and plants propagated from culture[15]. DNA-based markers such as RAPD (Random amplified fragment length polymorphisms) allow an efficient method for screening changes and *in vitro* caused mutations because these molecular markers are not affected by environmental

conditions and implementing more reliable and reproducible results [16]. The aim of the work was to evaluate genetic stability of three micropropagated olive cultivars compared with the donor plants by RAPD-PCR assay.

Materials and Methods

Plant materials and explants preparation

The current research was carried out during 2015/16 seasons at the laboratory of Pomology Department, Faculty of Agriculture, Cairo University and Biotechnology Lab., Pomology Dept., and Genetic Engineering and Biotechnology Division, Genetics and Cytology Department, National Research Centre.

Active spring shoots were collected from mature olive trees of cultivars namely 'Koroneiki', 'Picual' and 'Manzanillo', (grown at olive collection farm, Faculty of Agriculture, Cairo University, Giza, Egypt) during the summer season. After removing of leaves; the shoots were cut into nodal segments with 2-3 cm with buds. The surface sterilization performed with 10% Sodium hypochlorite (NaOCl) for 10 min. followed by Mercury chloride ($HgCl_2$) at 1000 ppm for 5 min then washed for 5 min with sterile distillated water.

Starting stage

Nodal segments of the selected cultivars cultured on MS media [17]. Two types of cytokinins were used 6-Benzylaminopurine (BAP) and 6-(γ,γ-Dimethylallylamino) purine (2ip) at concentration of 2.5 or 5 ppm. All media were supplemented with 30 g/L mannitol and 6 g agar/L and autoclaved at 121°C for 15 min. Four explants were cultured on 50 ml of semi-solid medium and maintained in the growth chamber at 23-25°C in 16h photoperiod with 40-60μmol m^{-2} s^{-1} provided by cool-white fluorescent lamps.

Multiplication stage

After three weeks the sprouted buds were transferred to fresh media of the same composition and the sub-culture was performed every four weeks during this stage. The following parameters were recorded: mean number of shoots per explants (MSN), mean shoot length (MSL) and mean number of leaves per shoot (MLN).

Experimental design and data analysis

This study followed the randomized complete design with three replicates, analysis of variance were performed according to Snedecor and Cochran,[18] means of the treatments were compared by Least Significant Difference L.S.D.[19] at significance level of 0.05 data analysis performed by MSTAT-C statistical package software.[20]

Extraction of genomic DNA

0.5 g of fresh young olive leaves of the studied cultivars ('Koroneiki', 'Picual' and 'Manzanillo') collected from both *in vitro* regenerated (the third sub-culture) and the mother plants (25-year-old-trees) and were soaked in liquid nitrogen for DNA extraction using the 2% Cetyltrimethylammonium bromide (CTAB) procedure as described by Murry and Thompson[21] with modification by De la Rosa et al [22].

RAPD profiles

Random amplified polymorphic DNA (RAPD) analysis of *in vitro* propagated and the mother plants were performed using random decamer primers. Six RAPD primers used in the analysis are summarized in the supplementary Table (1) [23]. Each 25-μl amplification reaction containing 1X PCR reaction buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTPs mix, 0.4 μM of each primer set, 1.5 U *Taq* DNA polymerase and adjusted to 25 μl using double distilled water (ddH_2O). Amplifications were carried out in DNA a thermo cycler (Biometra, Germany) with the following temperature profiles: 1 initial denaturation step at 94°C for 5 min, followed by 35 cycles at 94°C for 30 sec, 36°C for 1 min and 72°C for 1 min, and a final extension cycle at 72°C for 10 min.

Table 1. List of RAPD-PCR decamer primers used in this study.

Primer name	Sequence ('5 to 3')
Primer-1	GTTTCGCTCC
Primer-2	AACGCGCAAC
Primer-3	CCCGTCAGCA
Primer-4	GGACGGCGTT
Primer-5	AAGCCCGAGG
Primer-6	AAGGCGGCAG

Amplification product analysis

The amplified DNA (15 µl) for all samples was electrophoresed on 1% agarose containing Ethidium bromide (0.5 µg/ml) in 1X TBE buffer (89 mMTris-HCl, 89 mM Boric acid, 2.5 mM EDTA, pH 8.3) at 75 constant volt, and determine with UV transilluminator. The size of each fragment was estimated with reference to a size marker of 1 Kbp DNA ladder (BioRoN, Germany). The gels were analyzed by UVI Geltec version 12.4, 1999-2005 (USA).

Data analysis

A matrix for RAPD was generated by scoring reproducible bands as (1) for their presence and as (0) for their absence across the cultivars. Genetic similarity coefficients were computed following[24] as under;

$$\text{similarity} = \frac{2N_{x\,y}}{N_x + N_y}$$

where 'Nx' and 'Ny' are the number of bands present in cultivars 'x' and 'y', respectively; and Nxy are the number of bands shared by the cultivars 'x' and 'y'.

The data were subsequently used to construct a dendrogram using the un-weighted pair group method of arithmetic averages (UPGMA) [25] employing sequential, agglomerative hierarchic and non-overlapping clustering (SAHN). All the computations were carried out using the software NTSYS-PC (Numerical Taxonomy and Multivariate Analysis System), version 2.1.[26] Correlation coefficients were calculated using similarity coefficients obtained from RAPD-PCR.

RESULTS AND DISCUSSION

Effect of the different treatments on shoot multiplication during the proliferation stage

The studied olive cultivars showed significant differences in mean shoots number (MSN), mean shoot length (MSL) and mean leaf number (MLN) at the multiplication stage depending on cytokinine type and concentration. Data presented in Table (2) and Fig.(1) showed that, increasing the cytokinin concentration in the proliferation medium increased the MSN and the highest number of shoots (2.76 A and 2.22 B) obtained in the presence of 5 and 2.5 ppm BAP, respectively. In addition, 'Manzanillo' cultivar showed the maximum number of shoots (3.41 A), followed 'Koroneiki' (2.07 B) then 'Picual' (1.15 C). Data presented in Table (3) showed that the highest MSL was recorded in cv. 'Manzanillo' compared with the other two cultivars. There was a slight difference between the used cytokinine two types regarding the shoot length; 2ip at 5 ppm showed the highest MSL compared with the BAP. Furthermore, the maximum MLN produced in 'Koroneiki' (9.36A), followed by 'Manzanillo' (8.49 B). Finally, 'Picual' gave the lowest MLN (2.45C) (Table 4).Besides, 5 ppm BAP produced the highest MLN, while there were not significant differences among the other cytokinin concentrations.

Table 2. Mean values of shoot number (MSN) of three olive cultivars on MS media supplemented with different concentrations of BAP or 2ip.

Cytokinin	Concentration (ppm)	Cultivars			
		Koroneiki	Manzanillo	Picual	Mean
BAP	2.5	2.0 de	3.66 b	1.00 f	**2.22 B**
BAP	5	2.1 cd	5.00 a	1.20 f	**2.76 A**
2ip	2.5	2.0 de	2.33 cd	1.00 f	**1.77 C**
2ip	5	2.2 cd	2.66 c	1.40 ef	**2.08 BC**
Mean		**2.07 B**	**3.41 A**	**1.15 C**	

Values followed by the same letter are not significantly different according to *Duncan's* multiple range test ($P < 0.05$).

Table 3. Mean values of shoot lengths (MSL) (cm) of three olive cultivars on MS media supplemented with different concentrations of BAP or 2ip.

Cytokinin	Concentration (ppm)	Cultivars			
		Koroneiki	Manzanillo	Picual	Mean
BAP	2.5	4.00 d	4.66 ab	1.63 e	4.02 A
BAP	5	3.58 d	7.00 a	2.00 e	4.19 A
2ip	2.5	6.5 ab	5.00 c	1.20 e	4.08 A
2ip	5	5.53 bc	6.00 b	1.70 e	4.41 A
Mean		**4.76 B**	**6.11 A**	**1.63C**	

Values followed by the same letter are not significantly different according to *Duncan's* multiple range test ($P < 0.05$).

Table 4. Mean values of leaf number (MLN) of three olive cultivars on MS media supplemented with different concentrations of BAP or 2ip.

Cytokinin	Concentration (ppm)	Cultivars			
		Koroneiki	Manzanillo	Picual	Mean
BAP	2.5	9.33 ab	8.98 bc	2.00 f	6.77 A
BAP	5	9.50 bc	10.33 a	2.46 ef	7.28 A
2ip	2.5	9.42 ab	6.66 c	2.10 f	6.06 B
2ip	5	9.66 ab	8.00 d	3.26 e	6.97 A
Mean		**9.36 A**	**8.49 B**	**2.45 C**	

Values followed by the same letter are not significantly different according to *Duncan's* multiple range test ($P < 0.05$).

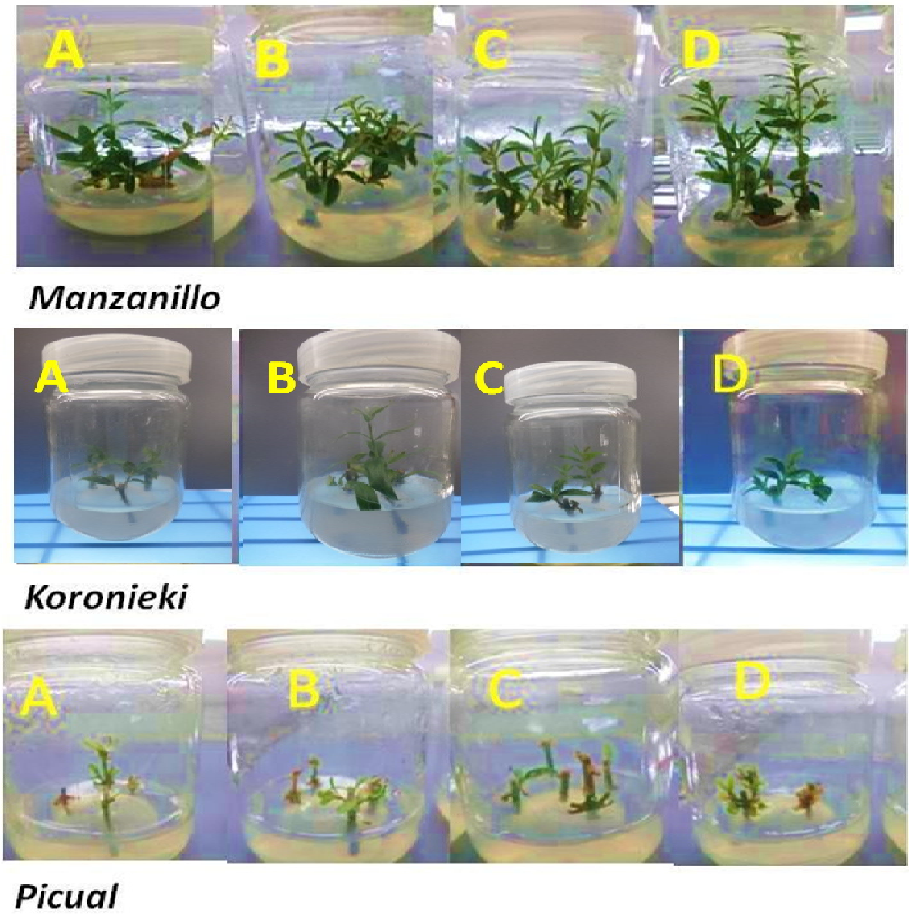

Figure 1. Micropropagation of three different olive cultivars on MS media supplemented with different concentrations of cytokinin. (A) 2.5 ppm BAP, (B) 5 ppm BAP, (C) 2.5 ppm 2ip, (D) 5 ppm 2ip.

Micropropagation is a reliable technique applies for large-scale plant multiplication. The media composition represents an important factor for olives high multiplication rate[27]. Pervious studied showed that olive micropropagation depending on the genotype[28], media mineral composition and growth regulators [29, 30]. Our results showed that, cytokinins play essential role for *in vitro* olive cultivars proliferation. In this study, 'Manzanillo' cultivar recorded the highest MSN and MSL, while 'Koroneiki' showed the maximum MLN. On the contrary, 'Picual' cv. gave the lowest MSN, MLS and MLN. Additionally, BAP has proven to be the most efficient cytokinine in multiplication medium with different olive cultivars. In most cases, using synthetic cytokinins such as kinetin and BAP in media did show a proper performance in proliferation of olive explants. Grigoriadou *et al*., [31] found that BAP at the different concentrations was better than 2ip. Also, Dimassi-Theriou[4] reported that BAP was effective during shoot multiplication of 'Kalamon' cultivar. Rostami and Shahsavar[32] reported that increasing BAP concentration in the media significantly increased shoots number, length and leaf number of olive cultivar 'Mission' cultured on MS media.

RAPD analysis and cluster analysis

Six decamer RAPD primers were used to study genetic fidelity of the three different olive cultivars regenerated *in vitro* and the mother plants (Fig 2 and Table 5). A total of 39 reproducible bands, ranging from 90 bp (Primers1 and 2) to 1500 bp (Primer-5) were detected using the six RAPD primers. The number of bands per primer varied from three (Primer-2) to 11 (Primer-3). Thirteen bands out of the 39 reproducible bands were polymorphic (33.3%) and 26 fragments were monomorphic (66.7%). The primer-3 scored the highest polymorphism with 54.5% (Table 5). On the contrary; primer-5 gave the lowest polymorphism (14.3%). Three out of the 39 markers were cultivar-specific (Table 5). On the other hand, 'Koroneiki' (T) propagated *in vitro* gave three markers with molecular sizes 710, 400 and 1000 bp using primers 1, 3 and 4, respectively (Table 5).

Table 5. RAPD-PCR amplified bands, polymorphic, monomorphic and unique bands of the parental and micropropagated three olive cultivars using six primers.

Primer name	Size range of the scorable bands (bp)	Total bands	No. of monomorphic bands	No. of polymorphic bands	% Polymorphism	Unique bands	Molecular size of markers
Primer 1	90-710	4	3	1	25	1	710
Primer 2	90-230	3	3	0	0	0	0
Primer 3	150-952	11	5	6	54.5	1	400
Primer 4	160-1250	8	4	4	50	1	1000
Primer 5	91-1500	7	6	1	14.3	0	0
Primer 6	110-830	6	5	1	16.7	0	0
Total	**90-1500**	**39**	**26 (66.7%)**	**13**	**(33.3%)**	**3 (7.7%)**	

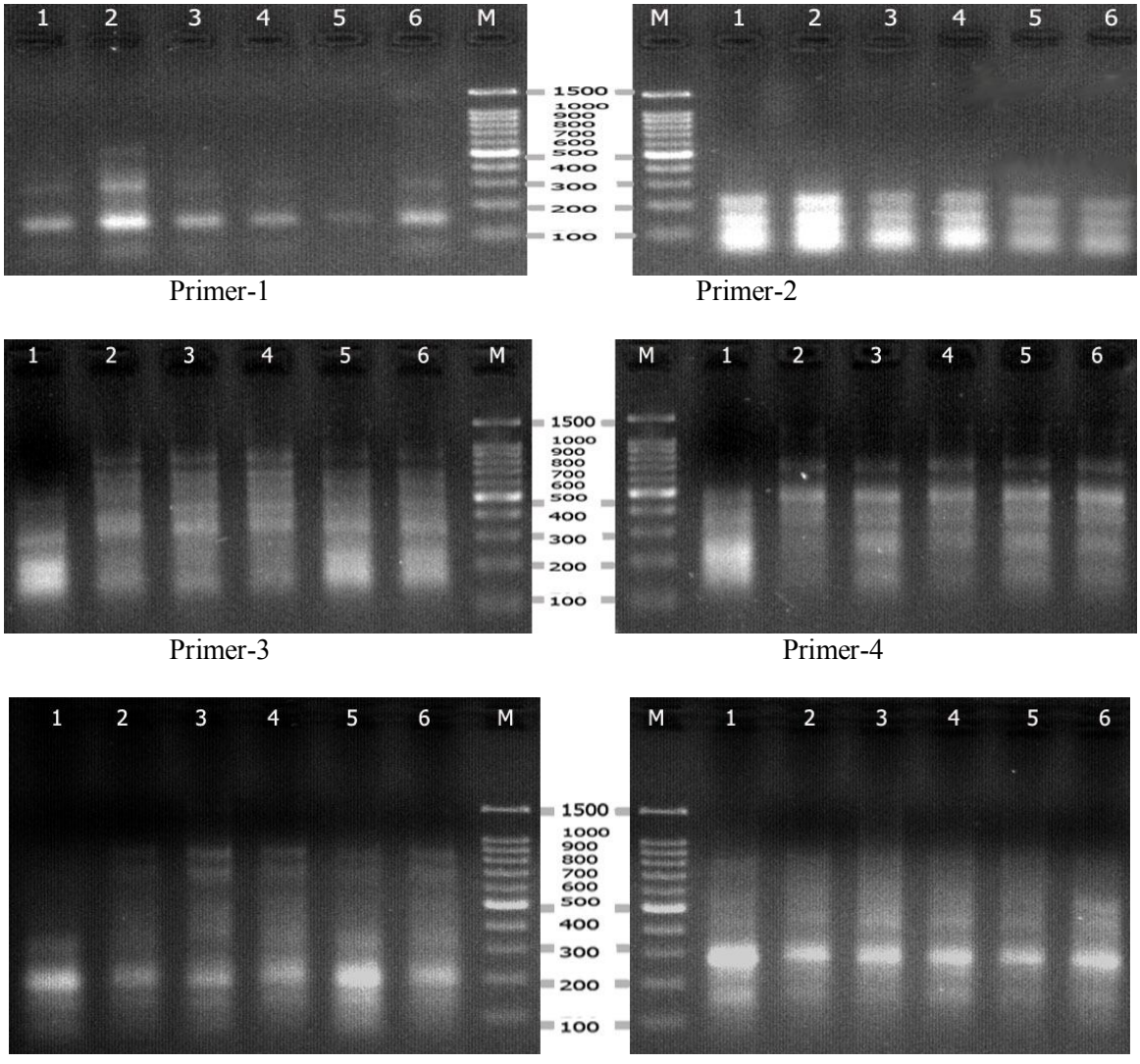

Figure 2. RAPD-PCR amplification patterns of three olive cultivars micropropagated *in vitro* and the donor plants using six primers. Lane M= DNA ladder 100 bp. Lanes 1, 3 and 5= the donor plants of 'Koroneiki', 'Picual' and 'Manzanillo' cultivars, respectively. Lanes 2, 4 and 6 = *in vitro* vegetative propagation of Koroneiki', 'Picual' and 'Manzanillo' cultivars, respectively.

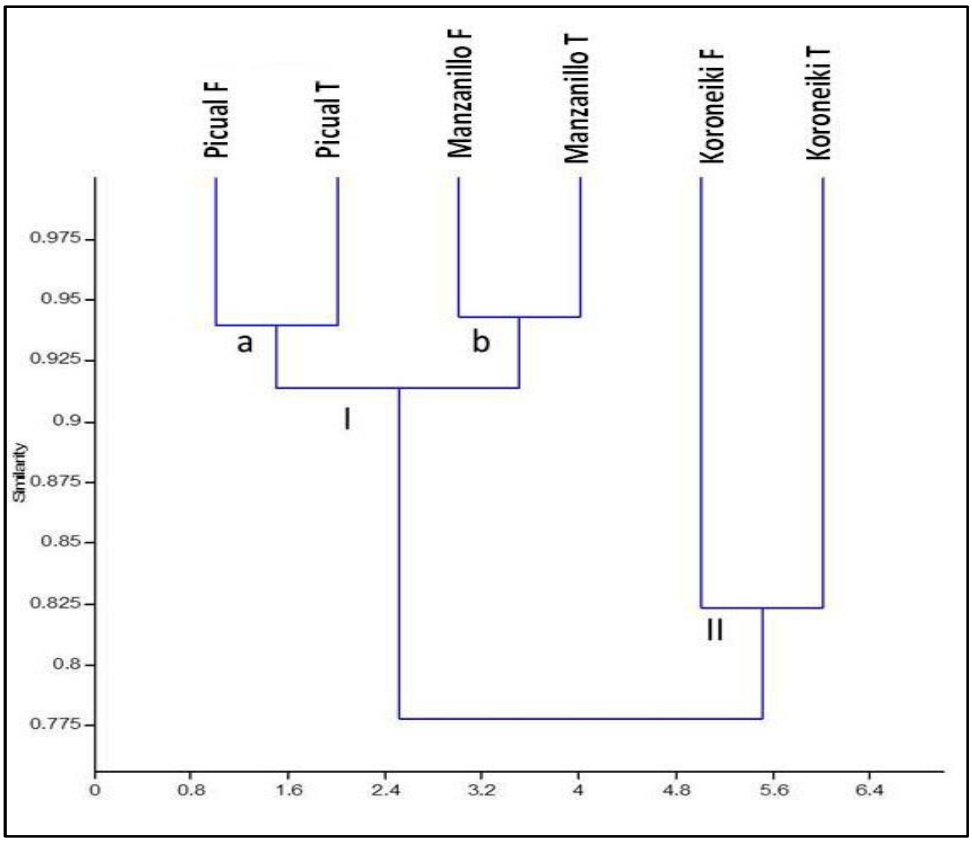

Figure 3. Dendrogram of three olives cultivars multiplied *in vitro* (T) and the mother plants (F) depend on RAPD-PCR by UPGMA cluster analysis from the similarity matrix obtained by Nei's genetic distance.

The first group (I): Composed of two sub-groups: Sub-group (a): (0.94 similarity) consisted of both Picual (F) and Picual (T) cultivars. Sub-group (b): (0.94 similarity) contained on two cultivars 'Manzanillo' (F) and Manzanillo (T). The second group (II): (0.82 similarity) involved into 'Koroneiki' cultivar grown *in vivo* (F) and propagated *in vitro* (T) (Table 6 and Fig 3). On the other hand, the primers used in this study revealed highly similarity between 'Manzanillo' (F) and 'Picual' (F) (0.94), also between 'Manzanillo' (T) and 'Picual' (T) which could reflex the common ancestor of 'Manzanillo' and 'Picual' cultivars (Table 6).

Table 6. Similarity index among the three olives cultivars micropropagated *in vitro* (T) and the donor plants (F) estimated by RAPD-PCR.

Cultivars	Koroneiki F	Koroneiki T	Picual F	Picual T	Manzanillo F	Manzanillo T
Koroneiki F	1.00					
Koroneiki T	0.82	1.00				
Picual F	0.79	0.81	1.00			
Picual T	0.74	0.76	0.94	1.00		
Manzanillo F	0.79	0.81	0.94	0.89	1.00	
Manzanillo T	0.75	0.77	0.89	0.94	0.94	1.00

A crucial problem linked with olive plants propagation *in vitro* by tissue culture technique is the genetic fidelity among sub-clones produced from the mother plants. The genetic integrity of the micropropagated plants can lead to variations in the olive cultivars. These changes are often undesirable, but still heritable[1]. RAPD assay has been applied to amplify regions of the genome of the olive plants micropropagated *in vitro* and beneficial

for determination of the genetic stability in the different fruit trees[18, 33-35]. Our results showed that RAPD analysis revealed that the donor plants and its clonal derivatives could be grouped together in a single group.

Cluster analyses appeared highly similarity among the parental and micropropagated plants. There are a number of findings in the literature which report similar results for detection of the genetic stability using molecular markers in various crops such as *Solanum aculeatissimum* [36] and *O. europaea* [1,2,37]. Bhatia *et al*., [38] evaluated the genetic stability *in vitro* regenerated and mother plants of gerbera using ISSR and RAPD markers which appeared 100% similarity. Our findings were in an agreement with Leva *et al*., [39] reported that micropropagation of olive by tissue culture did not influence of morphological characterization and the genetic fidelity. According to our results, we also showed that the RAPD analysis is sensitive enough for detection of the genetic stability in olive cultivars the parental and micropropagated plants.

Conclusion

The results indicate that, the olive plants propagated *in vitro* had highly similarity of the donor plants. The RAPD technique is a sensitive and an easy assay for detection of the genetic fidelity in the *in vitro* regenerated olive plants.

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