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Somaclonal Variation Among Somatic-Embryo Derived Plants of *Olea europaea L* "*cv. Kroneiki*"

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Abstract

Frequency of somaclonal variation in embryogenic calli and plants of *Olea europaea L cv.Kroneiki* derived from somatic embryogenesis were examined by randomly amplified polymorphic DNA (RAPD) analysis. Radicle and cotyledon (proximal part) of mature zygotic embryo were cultured in OMc medium supplemented with IBA (5 mgL^{-1}) and 2-ip (0.5 mgL^{-1}). Somatic embryogenesis was induced in calli of radicles (55%) and proximal part of cotyledons (8%) on OM medium without any hormones. After culturing the embryogenic calli on OM medium in the presence of BAP (2 mgL^{-1}), regenerated plants were obtained. DNA sample from the leave of the mother plants, seedlings (germinated from zygotic embryo), plantlets (regenerated from somatic embryo), embryogenic calli, non-embryogenic calli and zygotic embryos were subjected to RAPD analysis. 20 arbitrary decamer primers produced polymorphic amplification products. These primers gave a total of 315 reproducible fragments from which 221 (71%) were useful as polymorphic bands with an average of twelve RAPD marks obtained for each primer. A dandogram, based on the unweighted pair group mean average (UPGMA) method of cluster analysis, were constructed using a similarity matrix derived from the RAPD amplification generated by all primers. The estimation of genetic similarity coefficient based on RAPD band sharing data indicated that regenerated plants were less than 75% similar to mother plants.

Keywords: Olive; RAPD markers; Somatic embryogenesis; Somaclonal variation

Introduction

Olive tree (*Olea europaea* L.) has a high economic value and many countries such as Iran and Mediterranean countries use its oil and canned fruits [1]. Micropropagation protocols have already been established in *Olea europaea*, but only for a small number of cultivars and using different explant sources:

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for example, nodal segment cuttings [2-5], cotyledons [6] embryo [7, 8] or micro grafting [9].

Direct [10] and indirect organogenesis [6] have also been described for some olive cultivars. Also, somatic embryogenesis in olives has been suggested as an efficient and alternative method for the *in vitro* propagation of this species [11]. However, somatic embryogenesis has been achieved so far in a few commercial cultivars, remaining other extremely recalcitrant to the process, which emphasizes the importance of the genotype as a factor highly conditioning the somatic embryogenesis process.

In *Olea europaea* , somatic embryogenesis has been induced mostly in juvenile material from cotyledonary tissues [12,13,14], immature zygotic embryos [15,13, 16,17] and mature embryo callus [12,1,18,19,20]. Only Rugini and Caricato (1995) succeeded in inducing somatic embryogenesis from leaf petiole tissues of adult plants using a method which they designated " double regenerating system " and which requires previous rejuvenation by *in vitro* shoot culture acquired by recurrent regeneration increased the regeneration capacity in these tissues [11]. More recently, Shibli et al. (2001) and Beneli et al. (2001) [21,22] obtained somatic embryos from rejuvenated material and Capelo et al. (2003) have also succeeded in inducing in direct somatic embryogenesis from leaf and petiole tissues not previously rejuvenated from a mature tree [23].

One of the main goals of this micro propagation technique is to achieve a successful true-to-type propagation of the plant material to be cloned. Clonal fidelity is a major consideration in commercial micro propagation using *in vitro* tissue culture methods [24].

It is known that in many cases, a consistent proportion of the regenerated plants differ from the original parental type when submitted to tissue-culture techniques. This phenomenon is called "somaclonal variation" [25]. Although such variation may provide useful source of genetic variability for crop improvement, it is undesirable in plant propagation and genetic transformation. A better understand for somaclonal variation is necessary to assess the potential usefulness of the tissue – culture techniques [26], and also to develop methods whereby the material can be easily and rapidly screened to reveal any genetic difference from non tissue culture – derived controls [27,28,29].

Genetic variation depends of the plant species, the genotype involved, the type of explant, and the culture media and conditions [30, 31]. These alterations may involve genomic, chromosomal and gene mutation [32], activation of transposable elements, or changes in methylation pattern [33].

Karyological analysis of plant can very often reveal significant chromosomal changes [30] but can not reveal alterations in individual genes. Procedures such as isozyme analysis provide a relatively convenient method for examining biochemical changes although they are usually limited by the number of available markers [27]. A precise determination of changes in a particular gene sequence resulting from tissue culture can be obtained by RFLP analysis. However, this method is time consuming and very expensive, and the results are limited only to gene sequences used as probes [27].

The development of the polymerase chain reaction (PCR) has been one of the major technical advances in molecular biology in recent years. RAPD known as random amplification polymorphic DNA or arbitrarily PCR (RT-PCR) allows the amplification of discrete fragment of the genome without the previous knowledge of their sequences. These fragments can be used as genetic marker to determine differences among individuals at the DNA level [34, 35, 36, 37, 29, 38, 31].

We have employed random amplified polymorphic DNA (RAPD) method for the analysis of genetic stability among accessions. Randomly amplified polymorphic DNA (RAPD) has been used widely in genetic analysis of *Olea europaea L*. [39, 40, 41] and identifying olive cultivars [42].

The aim of the present experiments is to determine the frequency of somaclonal variation of embryogenic calli in olive *cv. Kroneiki*. To our knowledge, this is the first assessment of genetic variation at DNA level in somatic embryogenesis of *Olea europaea cv. Kroneiki*.

Materials and Methods

Plant Source

Kroneiki cultivar, well-known for its quality of oil production, was used. Fruits were collected in the middle of October from agricultural Department Garden of Golestsn Province, after harvesting, mesocarp was removed and seeds were dried after washing in room temperature.

Isolation and Culture of Different Part of Embryo

Just before beginning to culture, hard endocarp of seeds were mechanically broken and surface of seeds was sterilized with Ethanol 70 % (1 min) and calcium hypochlorite 5% (20 min) and washed with sterile distilled water (5 times) .Sterilized seeds were placed on sterilized glass petri dishes with two sterilized filter paper and 5 ml sterilized water added to each petri

dishes. The petri dishes were maintained about 48 h in dark and in 24 ± 2°C and then embryos were isolated from endosperm with scalpel. Radicle and proximal parts of cotyledon were separately cultured in each Petri dish (five explants in each petri dish).

To obtain embryogenic calli, radicle and proximal parts of cotyledon were separately cultured on OMc medium [7] supplemented with indole -3-butyric acid (IBA, 5 mgL^{-1}) and 2-iso pentenyladenine (2-ip, 0.5 mgL^{-1}) and 2% (w/v) sucrose. The pH was adjusted to 5.8 before adding 0.7% (w/v) agar.

For callus maintenance, cultures were subcultured every 3 weeks on OM medium [2] without hormones. After about 8 weeks (3 subcultures), somatic embryos were cultured on OM medium supplement with Benzylaminopurine (BAP, 2 mgL^{-1}). In this media the concentration of organic compounds (myo-inositol, glycine, thiamine HCl, pyridoxine HCl, nicotinic acid, biotin and folic acid) were 10 times higher than the one suggested by Rugini (1984)[2].

For induced, seedlings, whole zygotic embryo were cultured on OM medium without any hormones, and 3% (w/v) sucrose, solidified with 0.7% (w/v) agar. After 3 weeks, seedlings germinated from zygotic embryo.

The experiments were conducted in a culture room with a temperature of 24±2 °C and 16h light/8h dark photo period after about 60 days, when no somatic embryogenesis was observed in calli (non-embryogenic calli), they were isolated for DNA extraction.

Experiments followed a randomized completed design with at least 30 replicates. Analysis of variance was performed with ANOVA (SPSS ver. 14.0).

DNA Extraction

DNA extracted from the leaves of the mother plant, seedling, plantlet (germinated from somatic embryo), embryogenic calli, non-embryogenic calli and zygotic embryo with trice repetitious following acetryltrime thylammonium bromide (CTAB) protocol was described by Murry and Thompson (1980) [43] with modification by De la Rosa *et al.* (2002) [44]. Approximately 1 gr of fresh tissue from each explants were powdered in liquid nitrogen. 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 ngL^{-1} for PCR reactions.

RAPD

RAPD analysis for each sample, with three replicate was performed as described by Williams *et al.* (1990) [34]. A total of 20 arbitrary decamer primers (Operon Technologies Alameda California) were screened for RAPD amplification in the olive *Kroneiki cv*. Genome DNA. A single primer was used in each PCR reaction, which was carried out in a total volume of 20µl containing of 20 or 40 ng template DNA, 2 µl of 10x PCR buffer (10 mM Tris-Hcl pH 8.8, 250 mM Kcl_2), 0.6 µl $MgCl_2$ (50 mM), 0.4 µl of 10 mM dNTPs and 0.5 µL of Taq polymerase (5v). PCR amplification was performed on a Techne Gradiant under the following conditions: A hot start at 95°C for 5 min, followed by 35 cycles of three steps: Denaturation at 94°C for 1 min, annealing at 33°C for 1 min, extension at 72°C for 1 min, and a final extension for 7 min at 72°C. The PCR amplified products including all of samples were separated by electrophoresis on 2% agaros gels due to examine RAPD profiles of repeated samples. The representative samples from samples for obtaining more band resolution were separated by electrophoresis on 6% polyacrylamide gels [45]. A DNA ladder (1000 bp, Gene Ruler™, Fermentase) was used as molecular weight marker and the gels were stained with silver staining [45].

Data Analysis

Only consistently reproducible, well-resolved fragments, in the size range of 200 bp to 2000 bp were scored after three times repetitions as the present or absent for RAPD markers in each samples. 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 sample. Genetic similarities between samples were measured by the Jaccard [46] and Simple Matching similarity coefficient and the similarity matrix obtained was used to construct dandrogram using the UPGMA (Unweighted Paired Group with Arithmetic Average).

Results

1) Somatic Embryogenesis

Twenty one old-day calli obtained from Radicle and proximal parts of cotyledon produced somatic embryos after third subcultured on OM medium without any hormones. The results revealed that the rates of somatic embryogenesis from radicle and proximal parts of cotyledon were 55% and 8% respectively. The results of analyzing the variance indicated that differences between means of somatic embryogenesis in calli of different parts were significant.

2) RAPD

In order to test the reproducibility of the technique, triplicate samples were run for all of samples. These assays were complete re- runs, i.e. all procedures from DNA extraction up to PCR amplification took place on different occasions for each of triplicates. All sets of triplicates returned exactly the same banding patterns.

Somaclonal variation was examined among of mother plants, plantlets (regenerated from somatic embryo), seedlings (germinated from zygotic embryo), embryogenic calli, non-embryogenic calli and zygotic embryos by RAPD using 20 random primers. Among the 20 primers used, 18 have shown polymorphism between accessions. Each primer generated a unique set of amplification products ranging in size from 200 bp to 2000 bp (Fig. 1). These primers gave a total of 315 reproducible fragment from which 221(71%) were useful as polymorphic bands with an average of twelve RAPD marks obtained for each primer. The number of band for each primer varied, among the primer used. OPC-10 and OPC-11 produced the highest number of total band (23), while OPC-4 produced the lowest number of total band (8). The band patterns of each accession were heterotologous with mother plant. Although not all accessions, gave unique products with every primer. The highest number of polymorphic band (19) was observed in the primer OPC-11, while the lowest number (3) was observed in the primer OPC-12 (Table 1).

Genetic similarity between accessions and the mother plant and between them was scored by comparing their RAPD profile for each of above primers and calculating the coefficient of genetic similarity as Jaccard (1908) [46].

The genetic similarity between the mother plant and seedlings were 63% to 74%, with plantlets were 72% to 77 %(the highest value of similarity), with embryogenic calli 69% to 73%, with non-embryogenic calli 68 % to 75% and with zygotic embryos 65% to 70% (the lowest value of similarity) (Table 2).

A dandogram, based on the unweighted pair group mean average (UPGMA) method of cluster analysis, were constructed using a similarity matrix derived from the RAPD amplification generated by all of primers (Fig. 2). In general one major cluster is formed. The cluster is comprised of three sub- cluster. The first sub-cluster comprised of two sub-clusters, one of them was non-embryogenic calli and embryogenic calli, other one mother plant and plantlet. The second sub-cluster was zygote embryos and seedlings are placed in the third sub-cluster. The result demonstrated the genetic instability of accessions from the mother plant.

Discussion

The results of somatic embryogenesis revealed that embryogensis in calli of radicle was much more than calli of proximal parts of cotyledon which indicated the potential of embryogenesis decreased from the radicle to the proximal parts of embryo. The existence of a decreasing gradient the potential of regeneration from apex to proximal of mature olive embryos and more somatic embryogenesis from radicle to cotyledon parts of mature embryos have been reported [19].

The fate of the cells in tissue and their differentiation potential in to other tissue are different. This potential is

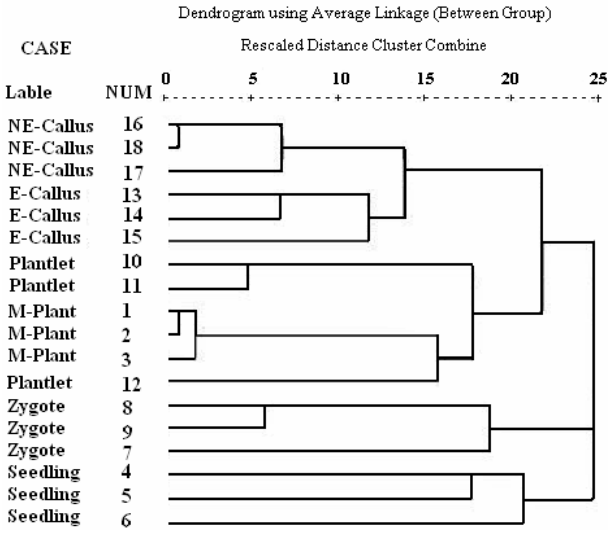

Figure 1. UPGMA clustering of seedling, plantlet and embryogenic calli(E-Callus), non-embryogenic calli (NE-Callus) and zygotic embryo with mother plant(M-Plant) based on data of 18 primers (OPC-1, OPC-2, OPC-3, OPC-4, OPC-5, OPC-6, OPC-7, OC-8, OPC-9, OPC-10, OPC-11, OPC-12, OPC-14, OPC-15, OPC-16, OPC-18. OPC-19, OPC-20).

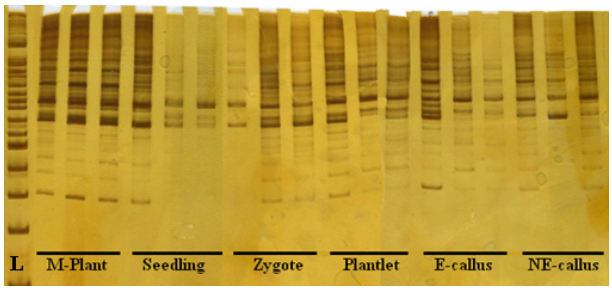

Figure 2. RAPD profile of primer OPC- 11 of samples: Size marker(L), Mother Plant, Seedling, Zygote, Plantlet, Embryogenic callus, Non-Embryogenic callus.

Table 1. Polymorphic number of RAPD bands in 18 RAPD primers studied in samples

Sample	OPC 01	OPC 02	OPC 03	OPC 04	OPC 05	OPC 06	OPC 07	OPC 08	OPC 09	OPC 10	OPC 11	OPC 12	OPC 14	OPC 15	OPC 16	OPC 18	OPC 19	OPC 20
M-plant*	0	0	0	0	0	0	0	1	0	1	4	0	-	1	2	1	3	0
M- plant	0	0	0	0	3	1	2	1	1	1	2	0	2	1	0	0	1	0
M- plant	0	0	0	0	3	2	2	4	1	0	5	0	1	1	1	2	2	0
Seedling	1	1	4	4	4	3	4	3	5	5	3	0	0	1	3	3	0	4
Seedling	3	4	4	3	0	6	1	3	0	7	9	0	2	2	3	5	1	0
Seedling	1	2	1	5	4	7	2	4	10	4	8	-	4	1	3	2	2	1
Zygote	2	-	2	0	0	5	3	6	2	1	3	1	1	3	3	1	0	0
Zygote	3	0	2	1	0	2	11	0	3	8	7	1	4	1	0	2	4	1
Zygote	3	0	1	1	0	4	8	5	5	6	10	1	4	2	-	8	4	2
plantlet	2	0	1	0	4	5	3	3	2	2	7	1	1	3	0	1	-	2
Plantlet	1	0	1	0	1	4	3	5	7	2	8	0	2	1	0	1	-	3
plantlet	1	2	1	5	6	3	0	8	7	7	5	3	0	1	4	2	-	1
E callus*	2	2	1	2	1	3	2	3	6	7	9	0	3	1	1	1	3	2
E. callus	1	2	1	2	3	2	3	1	2	6	5	0	3	1	2	0	3	3
E.callus	1	0	0	0	1	2	1	2	2	3	2	0	4	-	4	1	2	1
NE.callus*	0	2	0	1	0	3	7	3	0	2	3	0	1	1	2	2	2	2
NE.callus	1	2	0	0	-	3	1	2	2	5	5	0	0	0	0	2	0	0
NE. callus	0	0	0	2	0	2	6	1	0	5	2	0	2	0	2	0	0	0

* E. callus: Embryonic callus, NE. callus:Non Embryonic callus, M-Plant: Mother Plant

Table 2. Matrix of similarity coefficient based on RAPD profile for all of the primers

Sample	M- plant	M-plant	M- plant	seedling	seedling	Seedling	zygote	zygote	zygote	Plantlet	Plantlet	Plantlet	E. callus	E. callus	E. callus	NE. callus	NE. callus	NE. callus
M-plant*																		
M- plant	91.7																	
M- plant	89.2	90.8																
Seedling	70.8	74.8	73.0															
Seedling	65.6	66.8	68.4	72.8														
Seedling	63.9	64.5	64.3	69.5	70.6													
Zygote	65.8	65.2	69.2	63.9	63.8	63.4												
Zygote	68.7	68.7	70.2	65.1	62.6	65.7	72.7											
Zygote	67.9	66.2	70.6	62.7	64.9	65.6	73.1	86.6										
plantlet	74.9	72.3	74.4	69.2	69.1	69.8	71.3	72.9	71.9									
Plantlet	75.0	73.7	76.5	69.3	67.3	69.9	69.4	73.0	71.4	86.9								
plantlet	76.0	77.3	76.8	74.4	70.3	69.2	70.0	70.3	71.3	73.9	75.9							
E callus*	69.6	69.0	72.2	66.7	65.4	68.4	66.8	70.7	72.8	69.9	70.0	78.4						
E. callus	70.3	70.9	73.6	68.5	65.4	65.5	66.2	72.7	71.2	67.6	68.3	76.2	85.6					
E.callus	69.3	70.0	72.7	70.8	64.4	63.9	65.2	64.6	65.6	67.3	67.4	72.8	78.2	83.4				
NE.callus*	71.3	71.3	75.3	73.5	68.2	65.4	69.1	68.3	69.3	72.4	68.8	78.5	78.1	79.8	82.4			
NE.callus	70.0	68.2	72.1	69.5	67.5	64.1	65.9	67.0	66.3	68.6	68.1	76.6	78.8	80.5	78.4	85.1		
NE. callus	70.9	70.3	74.3	70.5	65.3	62.0	68.0	68.4	68.3	70.1	68.3	76.3	75.3	78.1	79.4	92.1	85.6	

* E. callus: Embryonic callus, NE. callus:Non Embryonic callus, M-Plant: Mother Plant

induced by external conditions such as different kinds of media, growth regulators, photoperiods, and etc [47, 48]. Subculture interval also has a great impact on maintence of embryogenic callus [49].

Somatic embryos were regenerated on free hormone of OM medium. Somatic embryos usually develop in to small plants, comparable to the seedlings on culture medium lacking plant growth regulators [50], for this reason regenerated plants were subcultured in the media supplemented with BAP.

Results indicated that the genetic similarity between the mother plant and seedlings were 63% to 74%. Studies in some medicinal plant species like *Bupleurum falcutum* [51] have shown variation between plants obtained from seeds.

RAPD analysis has shown polymorphism between accessions. The number of bands in each sample was varied for each primer. Analysis of the coefficient of genetic similarity among the different accessions indicated that all of them had varied degree of genetic difference from the mother plant as well as themselves. The dandrogram, based on UPGMA method of cluster analysis demonstrated the genetic instability of accessions from the mother plant. Genetic variability is common feature of plant cells that have undergone a tissue culture protocol. This genetic variability could be pre-existing in the cells of the donor plant or could be originated in the culture process, mainly in the callus phase [52]. Eshraghi (2005) reported all callus-derived plantlets have varied degree of genetic different from mother plant as well as themselves in Date palm [53]. Temel (2008) also reported genetic differences between mature embryo and calli derived from them in barely [54].

Molecular markers are believed to be reliable in monitoring variability at the DNA level in plants. RAPD technique was used by several research groups to examine genetic variability and it has been found to be very efficient and reliable [55, 37]. The variation 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. 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 can induced varied amount of genetic changes in different regenerated plants [48]. On the other hand, the observed small variation in DNA may have occurred *denovo* during the period of differentiated cell proliferation between the culturing of the explant and tissue regeneration [56].

The lack of somaclonal variation in somatic embryogenesis-derived plant material has been reported for other woody species including Norway spruce [57], *Eucalyptus globules* [58], *Quercus suber* [59], *Ulmus minor* [60] ,*Vitis vinifera* [61]. In contrast, the occurrence of somaclonal variation in somatic embryogenesis, detected by RAPD and other molecular markers has been reported by several authors for other woody species such as *Quercus robur* [62, 63, 64] and *pinus roxburghii, sylvestris* [65, 66].

As far as it is known, and despite its importance, no variability / stability studies based on molecular markers have been done on embryogenesis derived material in Olea spp. [20, 21, 67]. Only Garcia – ferriza *et al.*, (2002) described an efficient ture- to – type micro propagation (by stem cuttings) protocol for *O. europaea*, which was confirmed by RAPD analysis [40]. Lopes *et al.*, (2009) reported no mutation were found at the SSR loci at any of successive developmental stage from pre- embryogenic masses to somatic embryos on *O. maderensis* and *O. europaea* derived from petiols and leaf explants[68].

The analysis of genetic variability/stability during the somatic process (or other micropropagation processes) may be a helpful tool to predict if any detected genetic instability could be initiated at an early stage of callus formation / proliferation and thus contribute to the under standing of different rates of genetic instability among genotypes [68] .

As stated by Leroy *et al.*, (2000) [69] it is likely that the number of genomic lesions will be greater in calli with increasing of cellular proliferation.

The results pointed out that genetic variation may take place during the callus step; such variation may, indeed, results from changes in either the hormones composition of the media used, or cell dedifferentiated step.

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