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EFFECT OF *IN VITRO* PRESERVATION ON THE RAPD PROFILES IN "KORONAIKI" AND "KALAMATA" OLIVE CULTIVARS (*OLEA europaea* L.)

[27]

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ABSTRACT

The present work aimed to establish a protocol to minimize the potential effect of *in-vitro* preservation for 8 months of the two olive cultivars i.e Koronaiki and Kalamata. Three culture conditions (treatments) were chosen to investigate the most suitable one for minimizing somaclonal variations after the process of *in vitro* preservation. The conditions include MS medium plus mannitol, MS medium plus low temperature and MS medium plus naphthaline acetic acid. RAPD-based fingerprinting was employed to determine the effects of the different chemical constituents and other culture conditions on the extent of somaclonal variations after 8 months of *in vitro* preservation at the DNA level. Four arbitrary primers (OPA-04, -07, -14 and -16) were successfully utilized to achieve such goal. The obtained results showed that Mannitol has more pronounced effect than the other investigated preservation culture and conditions for decreasing DNA alterations.

Key words: Olive, Koronaiki, Kalamata, Preservation, DNA, Primers

INTRODUCTION

In vitro culture techniques are of a great interest for conserving plant germoplasm in different types of materials (protoplast) cells, tissues, apices, plantlets etc.) and for the use of plant genetic resoureces for plant genetic improvement, food, propagation and other agricultural programs.

Different methods have been used to assess the occurrence of somaconal variations after *in vitro* propagation or preservation. This information can be obtained by studying morphological traits and other growth characters. The development of randomly amplified polymorphic DNA (RAPD) by **Williams *et al* (1990)** generate ultimate potentialities in cultivars identification and discrimination **(Hassan *et al* 2002).** This approach have been adopted by many authors to determine the effects of different chemical constituents and other culture conditions on the extent

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of somaclonal variations during *in vitro* preservation at the DNA level. **Zhang *et al* (2000)** reported that 4-year observations conducted during 1993-96 on forty shoot tip-cultured plantlets of cherry cv. "Xesphye" revealed many RAPD variations. **Debabrata *et al* (2001)** used ancymidol as an alternative medium supplement to mannitol for slow-growth conservation of potato microplants *in vitro*. Ancymidol had a beneficial effect on culture viability after prolonged maintenance *in vitro*. Genetic stability of potato microplants conserved in ancymidol media was evaluated using RAPD fingerprints. Ancymidol did not induce any detectable genetic variations in genomic DNA as visualized by the absence of either any additional RAPD fragment or alterations in RAPD fragment patterns. **Soniya *et al* (2001)** examined genetic stability in tissue-cultured tomato plants by RAPD analysis. Picloram was used for the first time as alternative auxin, along with benzyladenine (BA) for callus induction in tomato. DNA samples from the mother plant and 11 randomly selected regenerated plants, obtained from a single callus, were subjected to RAPD analysis for the detection of putative somaclones. Six arbitrary decamer primers produced polymorphic amplification products. In this set of experiments, 15 non-parental bands were observed. **Hao *et al* (2002)** used RAPD analysis to detect DNA sequence variation in regenerated sweet orange (*Citrus sinensis*) plants derived from the embryogenic callus preserved for a long time. No difference in banding patterns was detected. **Helliot *et al* (2002)** used RAPD analysis to evaluate the genetic stability of *Prunus ferlenain* Plumina plants regenerated from cryopreserved apices. No genetic change was detected among the plantlets regenerated from frozen apices compared with the non-frozen material. This result suggested that the procedure used for *Prunus* cryopreservation could be used for long-term conservation. **Le *et al* (2002)** used *in vitro* cultures to built up a collection of Swiss potato genotypes, in order to store plant material in a disease-free environment and to supply, when it is necessary, healthy plants ready for large-scale distribution. At the same time, the identification of a range of varieties, also being achieved through molecular techniques (RAPD and AFLP), enables the conformity of the plant material, which has been stored and propagated to be confirmed. The objective of this work to minimize somaclonal variation effect *in vitro* preservation of Koronaiki and Kalamata olive cultivars.

MATERIAL AND METHODS

Two olive cultivars i.e Koronaiki and Kalamata were used in this investigation. Samples for each cultivar were kindly supplied by the Horticulture Research Institute, Agricultural Research Center, Giza, Egypt. After culturing on Murashige and Skoog media (MS) (1962) medium for Kalamata cv., and modified MS medium **(Fiorino and Leva 1986 and Charri *et al* 1999*)*** supplemented with glutamine (200mg/L), glycine(2mg/L), myo-inositol (100mg/L). for Koronaiki, cv., the obtained shootlet of the *in vitro* plantlets were aseptically microcutting (8-12mm long) and used as sources of the explants to serve in all of the preservation treatments for 8 months storage period as follow:

* Low temperature: the *in vitro* cultures were kept in refrigerator at 4^{o}C under dark conditions.

* Mannitol sugar [$CH_2(OH).(CHOH)_4 CH_2OH$] was used as an osmotic active compound applied to media using 15g/l.
* Naphthaline acetic acid (NAA) at 0.1mg/l.

The above mentioned chemical were added to the detrmined preservation media before adjusting Ph. 5.7. Then about 0.2 g of these preserved tissues were used for DNA extraction following the Dellaporta method (**Dellaporta *et al* 1983**) as follows:

About 0.1 gm (fresh weight) of plant tissues was ground to fine powder in liquid N_2 in a mortar. Before the tissue thawed, 1 ml extraction buffer (100 mM Tris-HCl pH 8.0, 50 mM EDTA and 0.5 M NaCl) and 0.2 ml 20% SDS were added. The mixture was incubated at 65 ^{O}C in water bath for 20 minutes. Then 1 ml of phenol, chloroform and isoamyl alcohol (25 : 24 : 1) was added. Centrifugation was performed at 10,000 rpm for 10 minutes. The supernatants of each sample were transferred separately to a new tubes, then 1 ml of chloroform and isoamyl (24 : 1) was added. Centrifugation was performed at 10,000 rpm for 10 minutes. The supernatants of each sample were transferred separately to a new tube, then 1 ml of isopropanol was added and then kept overnight in freezer. Centrifugation was performed at 10,000 rpm for 10 minutes. The resulted pellets containing DNA were re-suspended in 1 ml ethanol. Centrifugation was performed at 10,000 rpm for 2 minutes. The DNA pellets were re-suspended in 200 µl TE (10 mM Tris-HCl pH 8.0 and 1 mM EDTA) buffer. DNA was quantitated by spectrophotometer and gel electrophoresis.

A total of fifteen 10-*mer* random DNA oligonucleotide primers were independently used in the PCR reactions according to **Williams *et al* (1990)**. The primers are from Operon Kit (Operon Tech. Inc., USA). Only Five primers were generated reproducible polymorphism in the DNA profiles. Each experiment was repeated two times and only stable products were scored. The following are the code and sequences of these primers.

Primer code	Sequences
OPA-04	5`- AGT CAG CCA C -3`
OPA-07	5`- CAG CAC TGA C- 3`
OPA-14	5`- AGC ATG GCT C- 3`
OPA-16	5`- TCG GCG GTT C- 3`

Amplification was performed in 25 µl reaction volume containing the following:

* Primer	2.5 µl
* Template DNA	2.3 µl
* Sterile water	7.7 µl
* 2 x Ready Mix RED *Taq* PCR Reaction mix. It consists of the follwing:	12.5 µl
* 20 mM Tris-HCl (pH 83)	
* 100 mM KCl	
* 3 mM MgCl2	
* 0.002 % gelatin	
* 0.4 mM dNTBs mix (dATP, dCTP, dGTP and dTTP)	
* stabilizers	
* 60 units Taq DNA polymerase /ml.	

Each of the reaction mixtures was overlaid with a drop of light mineral oil per sample. Amplification was carried out in Perkin Elmer Gene Amp PCR thermocycler. The optimal condi-

tions for PCR amplification was as follows: an initial 4 minutes denaturation step at 95^{o}C followed by 35 cycles of 45 second at 94^{o}C, 1 minute at 37^{o}C and 2 minutes at 72^{o}C, with a final extension step at 72^{o}C for 12 minutes.

A volume of 10 µl of the RAPD products were electrophoresed in 1.4 % agarose gel. The gel was prepared by adding 1.4 g agarose to 100 ml of 1 X TBE (0.04 M Tris-acetate, 1 mM EDTA, pH 8), followed by boiling in water bath. Then 0.5 µg/ml ethidium bromide was added to the melted gel. The melted gel was poured in the tray of mini-gel apparatus and the comb was inserted immediately. The comb was removed when the gel become hardened. The electrophoresis buffer (1X TBE) was covered the gel. About 10 µl of DNA amplified product was loaded in each well and run at 60 V for about 45-75 minutes. The gels were visualized and photographed by gel documentation system (GelDoc BioRad 2000) under UV transilluminator.

RESULTS AND DISCUSSION

In this study RAPD-based fingerprinting was employed to determine the effects of different chemical constituents of media and other preservation conditions on the extent of somaclonal variation during *in vitro* preservation at the DNA level. Two new-imported olive cultivars Koronaiki and Kalamata were chosen to investigate the possible mutagenic effects (somaclonal variations) of the different tissue culture conditions in order to minimize it. Four arbitrary primers (OPA-04, -07, -14 and –16) were successfully utilized to achieve such goal. These primers succeeded to amplify reproducible DNA products. As described before modified MS medium was the most suitable one for micro-propagation of Koronaiki cultivar whereas, MS medium was optimal one for Kalamata cultivar. Three culture conditions (treatments) were also chosen to investigate the most suitable one for minimizing somaclonal variations after the process of *in vitro* preservation.

For both Koronaiki and Kalamata cultivars and for each tissue preservation conditions the amplification products generated by each primer were scored. Only clear and unambiguous DNA bands were included in the analysis. The shared bands between RAPD banding patterns of each tissue culture conditions and control as well as the altered ones were recorded and the percentage of alterations were determined.

Figure 1 demonstrates the effect of the tested media on the DNA banding patterns of the two preserved olive cultivars. Primer OPA-07 is the most effective than the three other primers for detecting alterations after the process of tissue preservation as compared with control samples. Many pronounced alterations were detected in the RAPD profile of the two olive cultivars that generated by this primer. The alterations involved are: the generation of novel bands, the disappearance of other bands and the change in band's intensity. Table (1) reveals that the alterations percentage scored by the primer 07 after 8 months *in vitro* preservation for Koronaiki explants on modified MS media plus mannitol, plus low temperature or plus naphthaline acetic acid were 44.44, 36.84 and 77.78 %, respectively. Similarly, the percentages of alterations scored in the RAPD profiles of the preserved Kalamata tissues on MS media plus mannitol, plus low

Figure 1. Effect of *in vitro* preservation on the RAPD profile of the two olive cultivars Koronaiki and Kalamata generated by the four OPA-04, -07, -14 and –16 primers.

Lane : M	DNA Marker		
Lane : 1	Control	Lane : 5	Control
Lane : 2	Modified MS media plus mannitol	Lane : 6	MS media plus mannitol
Lane : 3	Modified MS media plus low temperature	Lane : 7	MS media plus low temperature
Lane : 4	Modified MS media plus naphthaline acetic acid	Lane : 8	MS media plus naphthaline acetic acid

Table 1. The percentage of the altered bands detected by the OPA 04, 07, 14 and 16 primers after preservation of the Koronaiki olive cultivar for 8 months on any of Modified MS media plus (1) mannitol, (2) low temperature or (3) naphthaline acetic acid

	Total bands	Mono-morphic bands	Poly-morphic bands	% of altered bands
(1) Modified MS media plus mannitol				
Primer 04	14	14	0	0.00
Primer 07	18	10	8	44.44
Primer 14	8	8	0	0.00
Primer 16	19	19	0	0.00
Pooled effect	59	51	8	13.56
(2) Modified MS media plus low temperature				
Primer 04	14	14	0	0.00
Primer 07	19	12	7	36.84
Primer 14	9	8	1	11.11
Primer 16	19	17	2	10.53
Pooled effect	61	51	10	16.39
(3) Modified MS media plus naphthaline acetic acid				
Primer 04	14	14	0	0.00
Primer 07	18	4	14	77.78
Primer 14	9	7	2	22.22
Primer 16	19	17	2	10.53
Pooled effect	60	42	18	30.00

Table 2. Number and types of the amplified DNA bands as well as the percentage of the altered bands detected by the OPA 04, 07, 14 and 16 primers after preservation of the Kalamata olive cultivar for 8 months on any of MS media plus (1) mannitol, (2) low temperature or (3) naphthaline acetic acid

	Total bands	Mono-morphic bands	Poly-morphic bands	% of altered bands
(1) MS media plus mannitol				
Primer 04	8	8	0	0.00
Primer 07	21	19	2	9.52
Primer 14	8	7	1	12.50
Primer 16	13	12	1	7.69
Pooled effect	50	46	4	8.00
(2) MS media plus low temperature				
Primer 04	8	8	0	0.00
Primer 07	21	9	12	57.14
Primer 14	8	7	1	12.50
Primer 16	15	12	3	20.00
Pooled effect	52	36	16	30.77
(3) MS media plus naphthaline acetic acid				
Primer 04	10	8	2	20.00
Primer 07	20	6	14	70.00
Primer 14	9	7	2	22.22
Primer 16	15	12	3	20.00
Pooled effect	54	33	21	38.89

temperature or plus naphthaline acetic acid reached 9.52, 57.14 and 70.00 %, respectively (Table, 2).

Tables, (1) and (2) show that adding mannitol to MS medium had a pronounced effect for decreasing DNA alterations as compared to other investigated preservation conditions. The pooled effect on the Koronaiki olive cultivar detected by the four utilized primers reached the lowest value (13.56 %) after preservation on MS media plus mannitol whereas the highest pooled effect (30.00 %) observed after preservation on MS medium plus naphthaline acetic acid (Table 1). Similarly, Table (2) shows that the pooled effect on the Kalamata olive cultivar detected by the four utilized primers reached the lowest value(8.00 %) after preservation on MS medium plus mannitol whereas the highest pooled effect value (38.89%) exhibited after preservation on MS medium plus naphthaline acetic acid.

Callus is a potential source of genetic instability and represents an undesirable feature in clonal propagation protocols and *in vitro* preservation. After a basic tissue culture system has been developed for a given species, the problem of somaclonal variations has been emerged. This report presents a protocol to minimize the potential effect of the chemical constituents of the media used for micropropagation and *in-vitro* preservation of the two olive cultivars Koronaiki and Kalamata.

Detection of somaclonal variation requires a precise and simple method, which reveals changes at the gene level. To achieve such goal, RAPD analysis was employed to detect these changes at the DNA level. In the present study, four arbitrary primers (OPA-04, -07, -14 and –16) were succeeded to amplify reproducible DNA products.

The advantage of RAPD analysis in this study is that it covers the entire genome; therefore it provides sufficient information about structural changes that might be occurred inside the genome due to different chemicals involved in tissue culture media. In this regard, **Williams *et al* (1990)** showed that RAPD markers cover the entire genome, revealing coding or noncoding regions, repeated or single-copy sequences. Also, **Schnell *et al* (1995)** reported that the arbitrary nature of the primer resulted in amplified DNA products representing random samples of the entire genome.

The observed alterations in the RAPD profiles recorded after *in vitro* culture preservation of explants for Koronaiki and Kalamata olive cv. might be due to structural changes in the genomic DNA of these cultivars. This conclusion is in accordance with **Gozukirmizi *et al* (1992)** who used the primer-globin gene (–115 to –95 sequence) for fingerprinting barley somaclonal plants. Analysis of the amplified products revealed significant differences in the pattern of RAPDs from different regenerants. Similarly, **Brar and Jain, (1998)** showed that the genetic instability of *in vitro* cultured crops as revealed by the considerable variation in RAPD products might be due to the mutagenic effect of 2,4-D. Also, **Aly and Saker (1998)** reported that analysis of regenerated faba bean plants revealed a significant frequency of DNA polymorphism (i.e. somaclonal variation) among the somatic embryo-derived plants. **Badr *et al* (1998)** used RAPD technique to detect somaclonal variations in regenerated pea and kidney bean plants. **DeVerno *et al* (1999)** regenerate trees from six

white spruce (*Picea glauca*) embryogenic clones after cryopreservation for 3 and 4 years and evaluate genetic stability using RAPD fingerprints. Somaclonal variation was detected in some *in vitro* embryogenic cultures, 2 and 12 months after they were re-established following cryopreservation. The DNA fingerprints as revealed by RAPD have proved useful in detecting somaclonal variations among wheat plants regenerated from protoplasts (**Brown *et al* 1993**), micropropagated plants of *Populus eltoids* (**Rani *et al* 1995**) and sugarcane plants regenerated from embryogenic callus (**Taylor *et al* 1995**). The results of those authors indicated that RAPD technique is an efficient and effective tool for determining DNA alterations due to chemicals utilized for *in vitro* culture media.

This interpretation was reinforced by **Michelmore *et al* (1991)** who reported that polymorphism in RAPD profile results from base changes that alter primer-binding sites. Similarly, **Lu *et al* (1996)** revealed that polymorphism might be due to structural changes in the genomic DNA that alter the distance between two annealing sites, delete an existing site or insert a new one. Also, **Yang and Quiros (1993)** reported that, the intensity of DNA bands depends on the starting copy number of a particular DNA sequence within the genome. Therefore, the alterations in band's intensity could be interpreted on the basis of alterations of some DNA sequences.

In this study, the avoiding of long time for regeneration lead to minimizing of somaclonal variations. In this regard **Aly and Saker (1998)** reported that the relatively high frequency (30%) of somaclonal variation detected in regeneration of faba bean is due to the extended *in vitro* interval needed before achieving plant regeneration. Moreover, avoiding the use of 2,4-D in the utilized media counteracts the formation of high frequency of somaclonal variations in the regenerated olive cultivars. It could be also due to the mutagenic effect of 2,4-D (**Brar and Jain, 1998**), especially with explants subjected to long-term exposure (four months). The experimental material analyzed were the first group of regenerants obtained using this regeneration protocol and used to evaluate the regenerants.

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تأثير معاملات الحفظ داخل المعمل على التباين في التضاعف العشوائي لأجزاء من DNA لصنفي الزيتون الكروناكى والكلاماتا

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يهدف هذا البحث الى تقليل التباين الوراثى الناتج أثناء حفظ الأجزاء النباتية (المنفصلات النباتية) لصنفى الزيتون كروناكى وكلاماتا تحت ظروف المعمل خلال ثمانية أشهر. حيث أختيرت ثلاثة معاملات لتحديد أيها أكثر ملائمة وصلاحية لتحقيق هذا الهدف وكانت تلك المعاملات المختبرة هى الحفظ على بيئة الـ MS المعدلة لصنف الكروناكى وبيئة MS لصنف الكلاماتا المضاف اليهما مانيتول تحت ظروف الغرفة أوالحفظ المبرد على درجة حرارة 4°م أو نفثالين حمض الخليك تحت ظروف الغرفة وقد استخدمت البصمة الوراثية القائمة على دراسة التضاعف العشوائى المتباين لأجزاء من الحمض النووى DNA باستعمال أربعة بادئات هى

(OPA- 04, 07, 14 and -16) وللإستدلال على حدوث تباينات وراثية من عدمة وكذلك للحكم على معاملات تلك التباينات ...

* ويمكن أن نوجز أهم النتائج المنحصل عليها كالآتى :

1- البادئ OPA – 07 (,S'¯CAP CAC' TGA C-3') هو الأكثر فعالية وحساسية ومن ثم صلاحيته للحكم على حدوث ونسبة التباين الوراثى فى الأجزاء المختبرة من الحمض النووى DNA للعينات المحفوظة من صنف الزيتون الكروناكى والكلاماتا مقارنة بالبادئات الثلاثة الأخرى (OPA- 04, 07- 14 and 16) حيث لوحظت أعلى نسبة تباين مع هذا البادىء لصنفى الزيتون بغض النظر عن نوع بيئة وظروف الحفظ.

2- أوضحت النتائج أن أقل نسبة تباين أثناء الحفظ قد ارتيطت بالمعاملة (بيئة MS المضاف اليها المانيتول تحت ظروف الغرفة) وذلك لكلٍ من صنفى الزيتون تحت الدراسة وبغض النظر عن البادىء المستعمل للإستدلال على هذا التباين.

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