PLANTA PERSICA
An International Journal of Comparative Plant Physiology
Volume 1, Number 1, Autumn 2017

# Molecular analysis of genetic stability in nano Iron treated olive microshoots

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Received: 2017/01/18; Accepted after revision: 2017/10/16

Abbreviations: EDTA: Ethylene diamine tetra acetic acid; SSR: Simple Sequence Repeat; 2Ip: 6-(γ,γ-dimethylallylamine) purine

**Abstract**
Plant tissue culture may generate genetic variability. Media components and increases in the number of subcultures may increase the likelihood of somaclonal variation. Although Fe-EDTA was found to be a suitable form of iron in the medium, the effects of nano-Fe and Fe-EDTA were compared on growth and somaclonal variation of micropropagated shoots of two Iranian olive cultivars during three continues cultures. Single nodes of Rowghani and Mari cultivars were cultured in DKW medium with 4mgl$^{-1}$ 2iP. In both cultivars, the highest number of growth factors was obtained in normal DKW medium. In the nano Fe treatment, the growth factors were significantly decreased with increasing culture numbers. The Genetic stability of microshoots was investigated using SSR techniques. *In vitro* plants were screened using six microsattelite markers. In total, 14 alleles were detected with a mean number of 2.33 alleles per locus. In the Rowghani cultivar, the heterozygosity deficiency was found in all six examined markers, whereas in the Mari cultivar it was found in two markers only. The phenogram showed variability between the two cultivars. However, during continuous cultures, the percentage of the similarity between treated shoots, was 100% in each cultivar.

***Keywords*:** Fe, Nano Fe, Olive, Somaclonal variation, SSR marker.

## Introduction

Olive (*Olea europaea* L.) plays an important role in the economies of the Mediterranean countries (Liphschitz et al. 1991). Protocols for *in vitro* propagation of the olive cultivars were reported many years ago (Rugini 1984). This technique allows high quality production and rapid growth of plants which are pathogen free on the surface and also in the vascular system (Rugini and Pesce 2006).

The occurrence of somaclonal variation is a matter of great concern for any micropropagation system (Modgil et al. 2005, Peredo et al. 2006, Zucchi et al. 2002). Studies of somaclonal variation have been taken as a tool to control the production of genetically identical plants and to achieve genetic variability in which would enable genetic improvement for breeders (Leva et al., 2012).

The evaluation of plants with genetic alterations is relatively simple if it is associated with phenotypic changes. Unfortunately, it is not possible to verify the genetic changes from the observation of the phenotype nor the biochemical components are visible by instrumental examinations (Rugini and Pesce, 2006). The recent development of molecular biology allows evaluation of the genetic variability in olive trees by using different methods, including RFLPs, RAPDs ISSR, AFLPs, SNIPs, microsatellites (Belaj et al., 2003; Leva et al., 2012; Noormohammadi et al., 2014).


Published by Golestan University

Different factors are involved in inducing variation such as genotype, method of *in vitro* propagation, type of tissue, explant source, media components, and the duration of the culture cycle (Pierik 1997, Sheidai et al., 2008).

Iron has key roles in plant biology (Taiz and Zieger, 2011). It is the metal used at the active site of many redox enzymes dealing with photosynthesis, cellular respiration and oxidation and reduction in plants and animals (Robin et al. 2008; Zancan et al. 2008). The most conspicuous prolongation of culture vitality was found to occur at 40 µM Fe: 100 µMol $Na_2EDTA$.

Nano particles have high reactivity because of more specific surface area, more density of reactive areas, or increased reactivity of these areas on the particle surfaces 100 nm (Roco, 2003; Nel et al., 2006). There are studies that show nanoparticles can have effects on plants which are either beneficial (growth and development of seedling) or non-beneficial (preventing root growth) (e.g., Zhu et al. 2008).

We aimed to study the effect of the chelated iron and nano iron on growth and genetic stability of micropropagated shoots of two Iranian olive cultivars during three continues subcultures.

## Materials and Methods

Experimental studies reported in here were conducted in the National Institute of Genetic Engineering and Biotechnology (NIGEB), Iran.

### *Plant material and culture media*

Six year old Iranian olive cultivars (Mari and Rowghani) were maintained as maternal plants at the greenhouse of NIGEB. Actively growing shoots were sterilized, using 0.1% Sodium hypochlorite solution. Single node explants were cultured in DKW medium(Driver and Kuniyuki 1984) supplemented with 4 $mgl^{-1}$ 2ip.Two different treatments of Iron (Fe EDTA) and nano Fe were used as Fe source in tissue culture medium. The pH was adjusted to 5.8 before agar addition (%6) and autoclaving. *In vitro* shoots (4 -5 nodes) raised from explants were used for further experiments after a 60 day treatment. Two nodal explants of sterile shoots were sub-cultured with 45 days interval period in the same tissue culture medium. All samples were kept in a growth chamber with 16h light/8h dark photoperiod and 24±2˚C. After each subculture, the number of nodes and branches raised from each explants were measured. Analysis of variance (ANOVA) was performed, using the SPSS software (ver. 16).

### *DNA extraction and PCR*

Leaves of three replicates from maternal plants and *in vitro* shoots with different Fe treatments were randomly collected during different culture periods. DNA was extracted using the CTAB method, described by Murry and Thompson (1980) with modification by De la Rosa et al. (2002).

### *SSR analysis*

The *in vitro* shoots were analyzed by six high polymorphic simple sequence repeat (SSR) markers of ssrOeUADCA09 (D9) (Sefc et al. 2000), ssrOeUADCA18 (D18) (Sefc et al. 2000), UDO99-43(U43) (Cipriani et al. 2002), GAPU71B (G71B) (Carriero et al., 2002), GAPU101 (G101) (Carriero et al. 2002), GAPU103A(G 103) (Carriero et al. 2002) using forward primers carrying VIC, FAM, PET or NED label at their 5′-end (Table 2).

Genotyping was carried out in CNR-Institute of Plant Genetics, Perugia Italy**.** PCR products were checked by electrophoresis on 2% agarose gels and then loaded on an ABI 3130 Genetic Analyzer (Applied Biosystems-Hitachi), using the internal GeneScanTM-500 LIZ Size Standard (Applied Biosystems), Output data were analyzed by Gene Mapper 3.7 (Applied Biosystems-Hitachi).The data content of SSR was evaluated by expected heterozygosity (H), according to the formula $H=1-\Sigma\ p_i^2$ where *pi*is the frequency of the *i*th allele at the SSR locus studied. Discrimination power (D) was also calculated according to the formula as above, where *pi* represents the frequency of the *i*th genotype (Bracci et al., 2009).

Grouping of the genotypes was determined using the unweighted paired group average (UPGMA) method.

## Results

Microshoots growth (number of nodes, new shoots per explants, stem length) of *Olea europaea* L. cv. Rowghani and cv. Mari was high up to four nodes and two new shoots, depending on Fe source and culture period. Results showed that Nano Fe had a negative effect on shoot proliferation. The explants of Mari could not survive at the first step of subculture in the DKW medium supplemented with nano Fe. In cv. Rowghani, the highest number of nodes, new shoots and stem length were obtained in normal DKW medium. In nano Fe treatment, with increasing culture numbers, the growth factors were significantly decreased (Table 1). However, nano Fe showed an effect on growth, so that leaves were chlorosis, stems length were shorter, and the nodes numbers were fewer compared with the control.

**Table 1.** Means of growth parameters (±SE) in Fe and nano Fe traetments.

| cultivar | Culture period | treatment | Shoot/explant | Nodes no. | Stem length(cm) |
|---|---|---|---|---|---|
| Rowghani | culture | Fe | 2±0.00 | 1.91±0.28 | 1.00±0.00 |
| | | Nano Fe | 2±0.00 | 2.66±0.43 | 0.75±0.08 |
| | First subculture | Fe | 2±0.00 | 3.33±0.25 | 1.06±0.17 |
| | | Nano Fe | 2±0.00 | 2.33±0.18 | 0.95±0.15 |
| | Second subculture | Fe | 2±0.00 | 3.08±0.22 | 1.20±0.08 |
| | | Nano Fe | 1±0.00 | 1.33±0.18 | 0.65±0.15 |
| Mari | culture | Fe | 2±0.00 | 2.33±0.88 | 0.88±0.12 |
| | | Nano Fe | 1±0.00 | 1.50±0.9 | 0.62±0.06 |
| | First subculture | Fe | 1.30±0.00 | 1.00±0.13 | 1.47±0.09 |
| | Second subculture | Fe | 1.11±0.02 | 1.11±0.14 | 1.61±0.09 |

**Table 2.** Discrimination of two cultivars in different Fe treatments and four steps of culture, based on the profile of alleles.

| Cultivar | Culture Step | Fe treatment | D9 | D18 | G71B | G101 | G103 | U43 |
|---|---|---|---|---|---|---|---|---|
| M | M | - | 190/190 | 171/179 | 127/127 | 197/197 | 136/136 | 174/176 |
| M | C | Fe | 190/190 | 171/179 | 127/127 | 197/197 | 136/136 | 174/176 |
| M | C | Nano Fe | 190/190 | 171/179 | 127/127 | 197/197 | 136/136 | 174/176 |
| M | SC1 | Fe | 190/190 | 171/179 | 127/127 | 197/197 | 136/136 | 174/176 |
| M | SC2 | Fe | 190/190 | 171/179 | 127/127 | 197/197 | 136/136 | 174/176 |
| R | M | - | 190/206 | 171/179 | 124/127 | 195/197 | 150/186 | 174/178 |
| R | C | Fe | 190/206 | 171/179 | 124/127 | 195/197 | 150/186 | 174/178 |
| R | C | Nano Fe | 190/206 | 171/179 | 124/127 | 195/197 | 150/186 | 174/178 |
| R | SC1 | Fe | 190/206 | 171/179 | 124/127 | 195/197 | 150/186 | 174/178 |
| R | SC1 | Nano Fe | 190/206 | 171/179 | 124/127 | 195/197 | 150/186 | 174/178 |
| R | SC2 | Fe | 190/206 | 171/179 | 124/127 | 195/197 | 150/186 | 174/178 |
| R | SC2 | Nano Fe | 190/206 | 171/179 | 124/127 | 195/197 | 150/186 | 174/178 |
| Total Number of allels | | | 2 | 2 | 2 | 2 | 3 | 3 |

Abbreviations: Cultivars: R= Rowghani, M= Mari; Culture step: M= Mother plant, C= culture, SC1= first subculture, SC2= second subculture

DNA samples of each treatments and cultures steps were genotyped using SSR loci. It was expected to discern the differences among treatments in terms of the length of SSR loci. Six SSR primers were used as marker. There were 14 alleles considering all six markers and the average number of alleles was 2.33. The range of

alleles size (bp) for each primer is shown in table 2. Among the populations (two cultivars Mari and Rowghani) G103 and U43 included three alleles, and D9, D18, G71B, and G101 included two alleles. In Rowghani cultivar, the heterozygosity deficiency was found in all six markers examined but in Mari cultivar it was found in U43 and D18 markers only (Table 2).

As a result, the similarity between treatments during continuous subcultures was 100%. The results indicated that there was not any contrast between treatments and cultures, in terms of loci's length (bp), in each cultivar. However, out of six markers, D9, G71B, G101, G103 and U43 were able to distinguish the two cultivars from each other. Data obtained from six SSR markers were used for elucidating the genetic relationships among the two olive cultivars in different treatments and in each step of culture by constructing the UPGMA clustering method (Figure 1). Both cultivars are in the separate cluster, but in each cultivar all samples from treatments and cultures are in the same cluster.

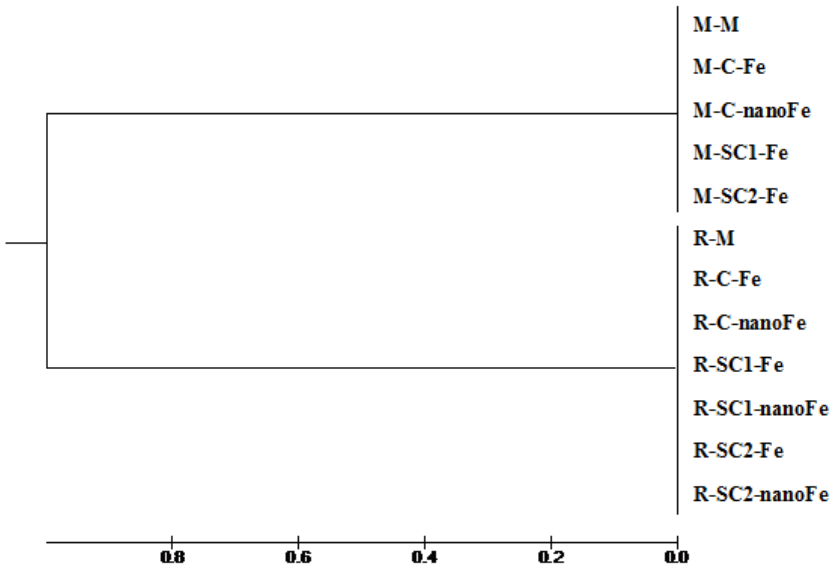


**Figure 1.** Dendrogram of two cultivars, including Fe treatments and cultures step, using UPGMA. Abbreviations: Same as Table 2

## Discussion

Studies have revealed that SSR method is a useful technique to identify the olive cultivars (Alba et al., 2009; Noormohammadi et al., 2007). However, the co-dominant nature of microsatellite markers permitted the discrimination of a higher number of genotypes.

Although nano Fe decreased the growth factors of olive microshoots especially in Mary cultivar, but it was not effective in inducing genetic variability. In different subculture periods there was no somaclonal variation between microshoots and donor plants. Many reports have been presented based on the lack of genetic diversity in *in vitro* olive plants (Kaeppler et al., 2000; Brito et al., 2010; Kanwar and Bindiya, 2003; Peyvandi et al., 2013). Genetic uniformity was reported within donor plants and *in vitro* plants which were obtained via somatic embryogenesis (Lopes et al. 2009). In contrast to our results, few studies have been reported on inducing somaclonal variation (Farahani et al., 2011; Noormohammadi et al., 20014). The most common factors affecting somaclonal variation are genotype, explants source, in vitro period and cultivation conditions (Sheidai et al., 2008). Peyvandi et al. (2009) demonstrated that the type of carbohydrates in the medium did not have any effects on the genetic variability but with increasing subcultures number, somaclonal variation in the microshoots was also increased. Therefore the observed genetic variation

among the regenerated plants is mainly due to the effect of the time period of subcultures and not due to the treatments. It has been reported that somaclonal variation among somatic embryo-regenerated plants are more common than that of nodes regenerated plants (Peyvandi et al., 2010). In order to increase the genetic variation in a species, it is a common technique to expose the embryos or the embryogenic masses to ionizing radiations, grow them in fungal filtered cultures, or filtered toxins of some pathogens or other selective pressures that induce changes or allow the selection of changing material (Rugini and Pesce 2006).

In conclusion, it is well known that increasing the numbers of subculture increases the likelihood of somaclonal variation, so the number of subcultures in microproagation protocols should be kept to a minimum. Our results indicated that after the second subculture the plants are completely similar to the mother plant.

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