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BRIEF COMMUNICATION

# Monitoring of cultivar identity in micropropagated olive plants using RAPD and ISSR markers

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## Abstract

Randomly amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) markers were applied to assess the genetic stability of micropropagated olive (*Olea europaea* L. cv. Maurino) plants regenerated by axillary buds. Initial olive explants, isolated from one donor tree, were multiplied on Murashige and Skoog medium for 12 repeated subcultures. A total of 40 RAPD and 10 ISSR markers resulted in 301 distinct and reproducible band classes showing homogeneous RAPD and ISSR patterns. The amplification products revealed genetic stability among the micropropagated plants and between them and the donor plant. The results demonstrate the genetic stability of nine year old mature micropropagated olive plants cultured in field, and corroborated the fact that axillary multiplication is the safest mode for multiplication of true to type plants.

*Additional key words*: genetic stability, *Olea europaea*.

Commercial olive propagation has experienced little change for centuries, and is primarily accomplished by propagating trees *via* grafted-seedlings and more recently using softwood cuttings under mist (Fabbri *et al.* 2004). Multiplication by cuttings meets the current needs in the nursery sector but an increasing demand for plants requires a multiplication method that provides elite plant materials in a short period of time. Olive micropropagation results in the production of a substantial number of micropropagated plants from one or a few selected donor plants. In theory, one initial two-node microcutting can generate in excess of 200 000 micropropagated plants after 12 proliferation subcultures (Leva *et al.* 2004).

Plant tissue culture propagation can lead to phenotypic variation between *in vitro* derived plants and original stock material (D'Amato 1978). This variability is referred to as somaclonal variation (Larkin and Scowcroft 1981). Several studies have reported the genetic stability/instability of *in vitro* derived plants using morphological, biochemical and molecular analyses (Rani *et al*. 1995, Thomas *et al*. 2006, Samantaray and Maiti 2010, Mohanty *et al*. 2011).

Several studies have demonstrated different responses among species to the *in vitro* conditions and different responses may be also evident among cultivars of the same species (Rani and Raina 2000). The approach can be challenging when clonal uniformity is a goal of *in vitro* derived plants. The *in vitro* culture techniques can be ranked in order of high to low genetic stability as follows: micropropagation by preformed structures such as shoot tips and axillary buds; adventitiously derived shoots; somatic embryogenesis; and organogenesis from callus, cells and protoplasts (Scowcroft 1984). Micropropagation using axillary buds is considered a low risk method for genetic instability because the organized meristems are generally more resistant to genetic change relative to unorganized callus tissues (Karp 1995, Shenoy and Vasil 1992, Tyagi *et al*. 2010). Nevertheless, somaclonal variation was reported even when organized cultures, such as shoot buds, were used as explants for micropropagation (Bindiya and Kanwar 2003).

Many studies have reported the utility of molecular marker combinations to assess clonal fidelity in tissue culture plants. The combination of RAPD and ISSR markers, which amplify different regions of the genome, is a suitable tool to evaluate genetic similarities or

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*Abbreviations*: DP - donor plant; ISSR - inter simple sequence repeat; MPs - micropropagated plants; MS - Murashige and Skoog; PCR - polymerase chain reaction; RAPD - randomly amplified polymorphic DNA.

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dissimilarities in micropropagated plants (Martins *et al*. 2004). Palombi and Damiano (2002) recommended the application of more than one DNA amplification technique, in fact in plantlets *of Actinidia deliciosa,* RAPD and SSR markers exhibited different levels of polymorphism. Similar results have been obtained in *Coffea arabica* (Rani *et al*. 2000), *Camelia sinensis* (Devarumath *et al*. 2002), and *Musa* spp. (Tui *et al*. 2006).

Micropropagation by axillary buds has been successfully tested on many *Olea europaea* var. *sativa* cultivars (Rugini 1984, Leva *et al*. 2004, Varlato *et al*. 2009). Nevertheless, the genetic stability of micropropagated plants to the donor plant has been poorly studied. Therefore, in the present study, RAPD and ISSR analyses were applied to check the genetic fidelity of micropropagated olive plants cultured in the field conditions.

The donor plant (DP) for the initial explants was a 20-year-old *Olea europaea* L. cv. Maurino tree. The micropropagated plants (MPs) were raised following the axillary bud culture method described by Leva *et al*. (1994, 1995). In 1998, 72 MPs (16-month-old) were transferred to an experimental field. In 2006, a set of randomly chosen nine-year-old mature olive MPs (12 plants) were used to conduct RAPD and ISSR genetic analyses.

Total genomic DNA was extracted from fresh DP and MPs leaves using the *DNeasy* plant mini kit (*Qiagen*, Hilden, Germany). Quality and quantity of DNA preparations were verified by standard spectrophotometry (*ND-100* spectrophotometer, *NanoDrop Technologies*, Wilmington, DE, USA) and visualized on agarose gel. MPs and DP were tested for genetic stability using 40 RAPD and 10 ISSR primers (Table 1). RADP analysis was performed following the protocol of Bogani *et al.* (1992). Polymerase chain reaction (PCR) was conducted in a total reaction mixture of 0.025 $cm^3$, including 0.5 U *Taq* polymerase (*Amersham Biosciences*, Buckinghamshire, UK), 0.004 $cm^3$ 10× PCR buffer (100 mM Tris-HCl, 1.5 mM $MgCl_2$, 500 mM KCl), 0.2 mM each dNTPs, 0.2 μM each primer, and 50 ng DNA template. PCR conditions were as follows: initial denaturation at 94 °C for 1 min; 40 cycles at 95 °C for 30 s, 36 °C for 1 min and 72 °C for 2 min; and a final extension at 72 °C for 10 min. ISSR amplifications were conducted in a total reaction mixture of 0.025 $cm^3$ containing 50 μg genomic DNA template, 200 mM DNTPs, 1.25 μM each primer and 0.5 U *Taq* DNA polymerase with the suitable buffer. PCR conditions were as follows: initial denaturation at 94 °C for 5 min; 40 cycles at 94 °C for 1 min, 40 °C for 1 min and 72 °C for 1 min; and a final extension at 72 °C for 5 min. PCR amplifications for both RAPDs and ISSRs were performed in a *Perkin-Elmer 9600* (USA) thermocycler.

DNA molecular mass marker *VI* for RAPD and DNA molecular mass marker *VII* for ISSR (*Boehringer*, Mannheim, Germany) were used as size markers. The

Table 1. RAPD and ISSR primers used in the molecular analysis of MPs and DP olive plant.

| Primer | Sequence 5'-3' | Number of bands | Primer | Sequence 5'-3' | Number of bands |
|---|---|---|---|---|---|
| OPA-01 | CAGGCCCTTC | 10 | G-17 | ACGACCGACA | 4 |
| OPA-02 | TGCCGAGCT | 12 | G-18 | CCCTCATGTG | 10 |
| OPA-03 | AGTCAGCCAC | 5 | M-10 | ACAACTGGGG | 10 |
| OPA-04 | AATCGGGCTG | 6 | M-14 | TCGTGCGGGT | 6 |
| OPA-05 | AGGGGTCTTG | 11 | M-15 | CAGCGACTGT | 9 |
| OPA-07 | GAAACGGGTG | 12 | M-16 | AAGCGACCTG | 10 |
| OPA-09 | GGGTAACGCC | 9 | M17 | CATTGGGGAG | 3 |
| OPA-4 | AATCGGGCTG | 4 | M-18 | GGTGAGGTCA | 9 |
| OPP-02 | TCGGCACGCA | 12 | M-19 | GTCCGTACTG | 12 |
| OPP-06 | GTGGGCTGAC | 5 | AH-11 | TCCGCTGAGA | 8 |
| OPP-10 | TCCCGCCTAC | 6 | AH-29 | TGGTGACTGA | 9 |
| OPP-12 | AAGGGCGAGT | 5 | AH-30 | TGGTCACTGT | 8 |
| OPP-13 | GGGAGTGCCTC | 5 | AG-1 | TCCGAGTCTG | 9 |
| OPP-14 | CCAGCCGAAC | 9 | AI-2 | ATCGCACACT | 8 |
| OPP-15 | GGAAGCCAAC | 6 | AL-14 | TGGTGCACTC | 4 |
| OPP-18 | GGCTTGGCCT | 10 | AL-15 | GACACAGCCC | 9 |
| OPP-19 | GGGAAGGACA | 8 | CD-11 | TGGCCAGTGA | 6 |
| OPF-8 | CTCTGCCTGA | 3 | CD-12 | TCCGAGTCTG | 7 |
| OPF-15 | GACCGCTTGT | 1 | 1281 | AACGCGCAAC | 12 |
| 40148 | TGGCCCCGGT | 5 | 1283 | GCGATCCCCA | 5 |
| ISSR1 | $(GT)_7YR$ | 4 | ISSR6 | $(GT)_6AY$ | 8 |
| ISSR2 | (CT)8RA | 6 | ISSR7 | $(CA)_6RY$ | 3 |
| ISSR3 | GGC (GA)8 | 5 | ISSR8 | $(CA)_6RG$ | 7 |
| ISSR4 | $CAA(GA)_5$ | 3 | ISSR9 | $(CT)_9RG$ | 4 |
| ISSR5 | $(TG)_6YR$ | 4 | ISSR10 | $(GT)_6RG$ | 2 |

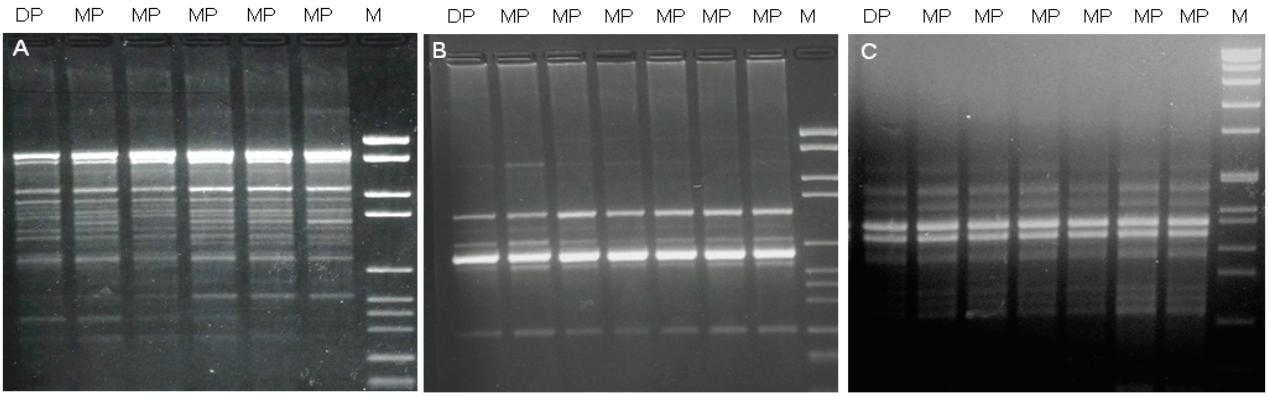


Fig. 1. Analysis of genetic stability of the MP and DP plants by RAPD and ISSR markers. RAPD profiles generated by the primer 1281 (*A*) and by the primer 40148 (*B*); M - molecular mass marker *VI*. ISSR profiles generated by the primer ISSR3 (*C*); M - molecular mass marker *VII*.

amplification products were visualized following electrophoresis in a 2 % (m/v) agarose gel (*Gibco BRL Life Technologies*, USA), and staining with ethidium bromide. PCRs were repeated at least twice to establish reproducibility; and fragments of low visual intensity were considered ambiguous and not scored. For detecting any genetic change, all RAPD and ISSR results were compared among all MPs and between MPs and the DP plant.

The 40 RAPD primers generated 301 scorable band classes, ranging in size from 2 300 to 400 bp. The number of primer bands varied from 1 (OPF-15) to 12 (OPA-02, OPP-02, M19, 1281), with an average of 7.5 bands per primer (Table 1). The RAPD primer amplification products were monomorphic in MPs and DP. The 10 ISSR primers produced 46 reproducible fragments ranging in size from 6 000 to 850 bp. The number of bands varied from 2 (ISSR10) to 8 (ISSR6), with an average of 4.6 bands per ISSR primer (Table 1). ISSR monomorphic bands were detected within MPs and between MPs and DP samples. Representative samples of the RAPD and ISSR profiles (Fig. 1) detected in MPs and DP showed genetic stability of all DNA samples.

In this study, the choice of two combined molecular markers to assess the genetic stability produced by micropropagation agreed with the results of several other studies in unrelated taxa (Oliveira *et al*. 1999, Sarmento *et al*. 2005, Das *et al.* 2010, Xing *et al*. 2010).

The reported molecular results corroborated the Leva *et al*. (2003) study on phenotypic stability of *Olea europaea* cv. Maurino micropropagated plants. Additionally, the screening of genetic stability in MPs plants was made over several years through a subset of the RAPD primers, included in this study, and based on our earlier report (Leva *et al*. 2002). Herein, RAPD analysis performed on the same nine-year-old trees confirmed the same stable RAPD patterns as was obtained on two year-old MP plants (Leva *et al*. 2002). Moreover, when the reproducibility is strictly controlled, the RAPD system still seems to be the most rapid and inexpensive for testing the genetic stability of olive plants produced by *in vitro* tissue cultures.

Our study clearly indicates that the *in vitro* propagated cv. Maurino, a common commercial olive cultivar, is genetically stable and the RAPD assays show no lack of reproducibility. Two molecular markers demonstrated the complete genetic similarity of micropropagated plants to donor plant. These results are strictly related to the respective protocol and medium conditions used in the micropropagation of olive plants.

To the best of our knowledge, no extensive study on molecular markers in nine year-old micropropagated olive plants has been made before showing successful amplifications with no intraclonal polymorphism detected.

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