Trees (2010) 24:723–732
DOI 10.1007/s00468-010-0442-9

ORIGINAL PAPER

Assessment of genetic stability of two micropropagated wild olive species using flow cytometry and microsatellite markers

Gina Brito · Tina Lopes · João Loureiro · Eleazar Rodriguez · Conceição Santos

Received: 5 August 2009 / Revised: 1 March 2010 / Accepted: 6 May 2010 / Published online: 25 May 2010

Abstract Micropropagated plants from two wild-olive species, *Olea maderensis* and *O. europaea* ssp. *europaea* var. *sylvestris* were screened for genetic stability. *O. maderensis* shoots were elongated/multiplied on OMG medium with zeatin (9.12 μM), and rooted on 1/2 OMG with NAA (3.22 μM). *O. europaea* var. *sylvestris* shoots were elongated/multiplied on OM medium with zeatin, and rooting was optimal after a hormonal shock (IBA 100 μM) followed by transfer to the same medium without growth regulators. In both species, acclimatization was successful and plants looked normal and morphologically identical to the donor field trees. Genetic variability was assessed at several stages of the micropropagation process using flow cytometry (FCM) and nuclear microsatellites (SSR). No changes in ploidy level were found among micropropagated plants, though small deviations, putatively due to the negative effects of cytosolic compounds on propidium iodide staining, between these and field plants were observed. In SSRs analyses, ten SSR markers were able to distinguish between genotypes. No mutations were found in these tested SSR loci among the donor tree and micropropagated plants, suggesting, for the tested markers, genetic uniformity throughout the process. The FCM and SSR results obtained do not exclude the occurrence of other changes in the nuclear genome but, considering the morphological stability of micropropagated plants, indicate that both protocols are suitable and efficient for large scale, true-to-type micropropagation of these two wild olive species.

Keywords In vitro culture · *Olea* sp. · Ploidy level · Somaclonal variation · SSR

Abbreviations

AFLP	Amplified fragment length polymorphism
FCM	Flow cytometry
IBA	Indole butyric acid
ISSRs	Intersimple sequence repeat
MS	Murashige and Skoog medium
NAA	α-Naphthalene acetic acid
OM	Olive medium
OMG	Modified olive medium
PI	Propidium iodide
RAPD	Random amplification of polymorphic DNA
RH	Relative humidity
SSR(s)	Simple sequence repeat(s)
WH	Without hormones
WPM	McCown woody plant medium

Introduction

The genus *Olea* includes approx. 40 extant taxa. The olive tree (*Olea europaea* L.) is well spread along the

Communicated by R. Hampp.

G. Brito (✉) · T. Lopes · J. Loureiro · E. Rodriguez · C. Santos
Laboratory of Biotechnology and Cytomics,
Department of Biology, CESAM, University of Aveiro,
Campus Universitário de Santiago, 3810-193 Aveiro, Portugal
e-mail: ginabrito@ua.pt

C. Santos
e-mail: csantos@ua.pt

Present Address:
J. Loureiro
Department of Life Sciences, Faculty of Sciences
and Technology, Centre for Functional Ecology,
University of Coimbra, Calçada Martins
de Freitas, 3001-455 Coimbra, Portugal

Mediterranean coast, comprising economically important cultivars and wild genotypes. In general, the olive trees have a wide range of adaptability, tolerating drought, poor soils and hot climates (Zacchini and De Agazio 2004).

Olea maderensis (Lowe) Rivas Mart. and Del Arco is an endemic and endangered species of the Madeira Archipelago (Macaronesia). Its few surviving individuals occur on nutrient-deficient rocky ground under semi-arid conditions (mostly in Porto Santo Island). In an attempt to fight the desertification process threatening Porto Santo Island, in the early twentieth century, trees of *O. europaea* L. ssp. *europaea* var. *sylvestris* Brot. (from now on referred as *O.* var. *sylvestris*) were introduced in some slopes of the Island (Brito et al. 2008) and represent nowadays, together with *O. maderensis*, interesting material for germplasm preservation. These two species present several morphological differences, and recently, *O. maderensis* was found to be tetraploid ($2n = 4x = 92$, nuclear DNA content = 5.97 pg/2*C*), while *O.* var. *sylvestris* is diploid ($2n = 2x = 46$, nuclear DNA content = 3.19 pg/2*C*) (Loureiro et al. 2007b; Brito et al. 2008).

The propagation of these species by traditional methods (e.g. own-rooted cuttings) has been rather inefficient and, as in other endangered or economically important species (e.g. Pinto et al. 2002; Zacchini and De Agazio 2004), in vitro programmes are convenient strategies for germplasm conservation. *O. europaea* commercial cultivars were already micropropagated from different explant sources (e.g. Rugini 1984; Canas and Benbadis 1988; Roussos and Pontikis 2002; García et al. 2002; Grigoriadou et al. 2002; Brito et al. 2003, Santos et al. 2003; Zacchini and De Agazio 2004), using mostly OM medium (Rugini 1984). While for *O. maderensis*, an efficient plant regeneration protocol was recently achieved using a modified OM medium enriched with Fe, Mg and Mn (Brito and Santos 2009), no information is still available for the micropropagation of *O.* var. *sylvestris.*

A major drawback of the micropropagation process is the putative occurrence of genetic variability (e.g. Rout et al. 2006). Thus, a successful true-to-type propagation of the plant material should be guaranteed (for reviews see, e.g. Kaeppler et al. 2000; Rout et al. 2006; Santos et al. 2007). Among the micropropagation methods, propagation by axillary bud stimulation is considered to bear low-risks of genetic instability (e.g. Rugini and Pesce 2006). Flow cytometry (FCM) and simple sequence repeat (SSR) markers are among the most commonly used methods to access somaclonal variation, allowing cheap, rapid and reliable analyses of the genetic variability at different genomic levels. FCM has been used to access clonal fidelity in several woody species (e.g. Conde et al. 2004; Pinto et al. 2004; Loureiro et al. 2005, 2007a), but never before in *Olea* species. SSRs are widely used for cultivar fingerprinting, paternity testing and genome mapping (e.g. Rallo et al. 2003; Brito et al. 2008). In olive, due to their desirable properties (reproducibility, level of polymorphism, information content and inheritance), SSRs became the markers of choice for cultivar identification (e.g. Rallo et al. 2000; Rathmacher et al. 2009). For assessing in vitro somaclonal variation, SSRs were previously used with success in some woody species (e.g. Rahman and Rajora 2001; Lopes et al. 2006) and only recently, this technique was used to evaluate the genetic stability of the micropropagation process of two olive species (Lopes et al. 2009). The authors reported no genetic changes between the donor tree and somatic embryos. In contrast, Peyvandi et al. (2009) found differences in the genetic pattern (using the RAPD technique) and physiological traits in micropropagated plants of an Iranian olive cultivar.

The aim of this work was to assess the genetic stability of micropropagated plants of the wild olive species, *O. maderensis* and *O.* var. *sylvestris*. For that we describe and compare two micropropagation protocols of *O.* var. *sylvestris* (first report) and of *O. maderensis* (after Brito and Santos 2009). The potential true-to-typeness of these protocols was evaluated using FCM and SSRs. Furthermore, the genetic analysis provides a basis for the conservation and multiplication of germplasm of these two wild olive species.

Materials and methods

Micropropagation protocols

Plant material and shoot proliferation

Cuttings were collected from adult field trees (>30 years old) belonging to two wild olive species, *O. maderensis* from Madeira Island and *O. europaea* ssp. *europaea* var. *sylvestris* from Porto Santo Island. Leaves were removed and cuttings treated according to Brito and Santos (2009). Disinfected cuttings comprising one or two axillary bud(s) were placed in groups of 4–5 explants per 300 ml glass flask (a total of 20 glass flasks per species) on the following induction media: (a) Olive medium (OM; Rugini 1984) and (b) OMG medium (Brito and Santos 2009). Both media were supplemented with 3% (w/v) sucrose, 0.7% (w/v) agar and pH adjusted to 5.8 (prior to autoclaving). Cultures were incubated for 1 month in a growth chamber with an average light intensity of 45 μmol m^{-2} s^{-1}, photoperiod of 16 h and constant temperature (22 ± 1°C) (Brito and Santos 2009). Shoot multiplication/elongation took place in the presence of 9.12 μM of zeatin. Sub-culturing was performed monthly and shoots were used as stock material for all experiments.

Due to different species responses to the induction media, shoot proliferation/elongation experiments were carried out using OM for *O.* var. *sylvestris* and OMG for *O. maderensis*. These experiments were performed for 5 weeks, under the same culture conditions used for stock material. Micropropagation efficiency was assessed by measuring growth parameters in three independent experiments (for 14 shoots in each species): shoot elongation, number of new leaves, number of new nodes, proliferation rate (number of new axillary shoots, considering that every new shoot could be used as a new explant for further subcultures), and *callus* diameter.

Rooting experiments

Apical segments with 2 or 3 nodes were selected, and cultivated on rooting media (Fig. 1). According to the available literature on the *Olea* genus (Grigoriadou et al. 2002; Roussos and Pontikis 2002; Mencuccini 2003; Zacchini and De Agazio 2004; Brito et al. 2007) several rooting strategies were tested in preliminary studies and the best conditions for each species were then selected for the micropropagation protocol: (a) *O. maderensis* shoots were incubated in the dark on 1/2 OMG with 3.22 μM NAA for 1 week, and subsequently transferred to 1/2 OMG medium without growth regulators (1/2 OMG_{WH}) to light conditions (Zacchini and De Agazio 2004); (b) *O.* var *sylvestris* shoots were exposed, under light conditions for 2 min in a 100 μM IBA followed by a transfer to 1/2 OM medium without growth regulators (1/2 OM_{WH}) (Brito et al. 2007). Experiments took place at the same growth conditions as those used for shoot proliferation. After 2 months, the number of rooted plantlets was recorded and expressed in terms of rooting percentage.

Plant acclimatization

In vitro plantlets (with 2–4 roots on average) were transferred to a sterilised mixture of peat:vermiculite (1:3) and watered with 1/2 OMG (for *O. maderensis*) or 1/2 OM (for *O.* var. *sylvestris*) medium (with no sucrose nor growth regulators). Plantlets were maintained in vitro for 2 weeks under the growth conditions described above. Then, plants were transferred for 1 month to a growth cabinet with progressive decreasing of relative humidity (down to 70% RH) and increasing light conditions (up to 70 μmol m^{-2} s^{-1}) and a photoperiod of 16 h. Plants were then transferred to a greenhouse (average of light intensity of 70 μmol m^{-2} s^{-1}) and after 3 months they were moved to an open greenhouse (200–400 μmol m^{-2} s^{-1}, at noon) in Porto Santo Island under the conditions described by Brito et al. (2009).

Genetic variability assessment

Plant material

To assess variation within clones of the same origin and between these and their donor trees, the following plant material was collected: (a) leaves from the field donor trees; (b) leaves from in vitro micropropagated plants (after 1 year of shoot subcultures) and (c) leaves from greenhouse acclimatized plants (after 3 months of acclimatization).

Flow cytometric analyses

Leaves from field donor trees were collected, maintained in moistened paper and analysed within a maximum period of 3 days. Leaves from the in vitro and acclimatized plants were collected and analysed immediately. In each case, only young healthy looking leaves were used for the flow cytometric analyses to avoid the possible interference of senescence-related secondary metabolites. Ploidy level was estimated according to Brito et al. (2008) using *Pisum sativum* cv. Ctirad as the internal standard ($2C = 9.09$ pg; Doležel et al. 1992). Briefly, nuclei were released by chopping with a razor blade half of a leaf of the olive plant and of *P. sativum*, in LB01 buffer (Doležel et al. 1989). Propidium iodide and RNase (both at 50 μg mL^{-1}) were added to the nuclear homogenates. After a 5 min incubation period, at least 5,000 nuclei were analysed in a Beckman-Coulter® EPICS-XL flow cytometer (Hialeah, FL, USA). The ratio between the mean fluorescence of G_0/G_1 nuclei of sample and standard was calculated as an indicator of the ploidy level of each sample. For each species and condition, 5–15 replicates were performed. The nuclear genome size of each type of plant material was estimated according to Brito et al. (2008).

SSR analyses

Leaves from donor trees and from in vitro and acclimatized plants were collected and stored at −80°C. DNA was extracted from 80 mg tissue with the DNeasy® Plant Mini Kit (QIAGEN™, Germany) according to the manufacturer's instructions. Ten microsatellites (Table 3) were selected as being the most polymorphic ones available for the genus (Sefc et al. 2000; De la Rosa et al. 2002). For SSR analyses, one of each set of two primers was labelled with a fluorescent dye (ABI dyes 6-FAM or HEX). Capillary electrophoresis was performed with an ABI Prism 310 Genetic Analyser (PE Applied Biosystems, USA) and the GeneScan internal size standard labelled with ROX (Applied Biosystems) was used. Presence (1) or absence (0) of alleles for each microsatellite marker was

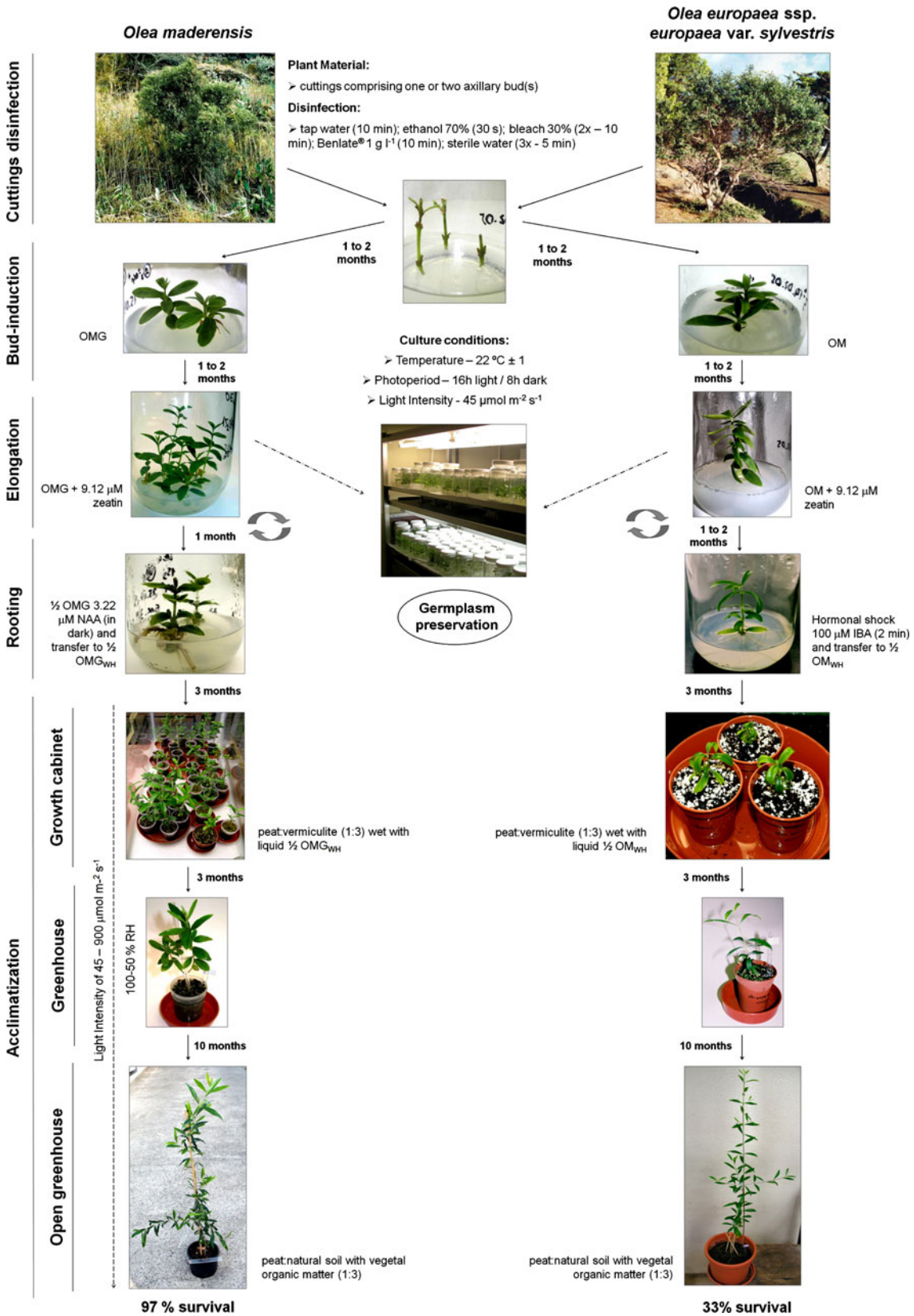

Fig. 1 Micropropagation protocols used in *Olea maderensis* and *Olea europaea* ssp. *europaea* var. *sylvestris*. *OM* olive medium (Rugini 1984), *OMG* modified OM (Brito and Santos 2009), *WH* without hormones, *HR* relative humidity, *IBA* indole butyric acid, *NAA* α-naphthalene acetic acid

recorded (Brito et al. 2008). For each species, five samples were performed per stage of the micropropagation process.

Statistical analyses

Differences in growth parameters and in the ratios of G_0/G_1 peaks were analysed using one-way ANOVA on Ranks (SigmaStat for Windows Version 3.1, SPSS Inc., Richmond, CA, USA). In both cases, either the Tukey test or the Dunn's methods (in the case of unequal group size) were used for pair-wise comparison.

Results

Micropropagation protocols

Olea var. *sylvestris* explant sprouting was successfully achieved only on OM medium, while in *O. maderensis* better results were obtained on OMG medium. Therefore, further quantitative analyses concerning micropropagation performance of both species were made on plants growing, respectively, on each media (Fig. 1). Both species presented high apical dominance, which was overcome with the use of basal segments, particularly in *O. maderensis* (Table 1). In *O.* var. *sylvestris*, the proliferation rate was low, but the formation of new nodes and shoot length, allowed shoot multiplication during subcultures (Table 1). In both species it was possible to maintain long-term cultures, up to 3 years for *O.* var. *sylvestris* and up to 5 years in *O. maderensis*. After 1 month, rooting rates for *O. maderensis* and *O.* var. *sylvestris* were on average 84 and 14%, respectively (Fig. 1).

Olea maderensis and *O.* var. *sylvestris* presented average survival rates in the greenhouse of 97 and 33%, respectively (Table 1; Fig. 1). After 10 months in an open greenhouse (Porto Santo Island), the survival rates were of 100% for both species. These plants looked healthy, presented green leaves and well developed internodes and no visible phenotypical variations (Fig. 1).

Assessment of genetic variability

Flow cytometric analyses

The FCM analysis of stained nuclei of both olive species resulted in highly reproducible histograms with well-defined G_0/G_1 peaks and CV values usually below 3.5% (Fig. 2), ensuring the reliability of the used protocol.

Different ploidy levels were confirmed between *O. maderensis* ($2n = 4x$) and *O.* var. *sylvestris* ($2n = 2x$). Genome size estimations indicate $2C = 6.11 \pm 0.07$ pg for *O. maderensis* and $2C = 3.23 \pm 0.02$ for *O.* var. *sylvestris*. The analysis of nuclear DNA content variability among the in vitro and acclimatized plants and the donor trees revealed small, but statistically significant differences in both species (Table 2). While in *O.* var. *sylvestris* there was a 2.5% decrease in nuclear DNA amount from the donor plants to the micropropagated material, in *O. maderensis*, ploidy was overall maintained, though, in acclimatized plants, a small difference (approx. 9%) in nuclear DNA content was observed.

SSR analyses

Amplification of the SSR loci involved the use of 10 primer pairs that produced 47 reproducible alleles that ranged from 120 to 256 bp in size (Table 3). Two loci, AJ279858 and AJ279865, resulted in a profile with only one band (allele) for *O.* var. *sylvestris*, representing homozygous individuals, whilst the other eight loci resulted in banding patterns with two bands (alleles) corresponding to heterozygous individuals of a diploid species. For this species, the electropherograms corresponding to the donor tree and micropropagation-derived plants are similar and show heterozygous individuals with two alleles of ca. 177 and 183 bp, respectively (Fig. 3). For *O. maderensis* microsatellite profiles presented from 2 to a maximum of 4 bands (alleles) which, as already shown above, concur with the fact that this is a tetraploid species. In this species, all electropherograms were similar showing heterozygous individuals with four alleles of ca. 160, 168, 185 and

Table 1 Shoot proliferation experiments in *Olea maderensis* and *O. europaea* ssp. *europaea* var. *sylvestris*

Species	Type of explant	Shoot length (cm)	No. of new leaves	No. of new nodes	No. of new shoots (proliferation rate)	*Callus* (Ø, cm)	Survival rate (%)
O. maderensis	Apical	1.21 ± 0.43*a*	4.00 ± 2.34*a*	2.71 ± 1.15*a*	1.29 ± 0.29*a*	0.53 ± 0.06*a*	97
	Basal	3.24 ± 0.61*b*	8.57 ± 2.53*b*	5.43 ± 0.95*b*	3.00 ± 0.38*b*	0.60 ± 0.06*a*	
O. var. *sylvestris*	Apical	0.41 ± 0.14	2.57 ± 1.00	1.43 ± 0.48	1.00 ± 0.00	0.74 ± 0.05	33

Data refers to growth parameters measured in 14 shoots during five weeks in three independent experiments. Values are given as mean and standard error of the mean

For each parameter mean values followed by the same letter are not statistically significant at $P < 0.05$

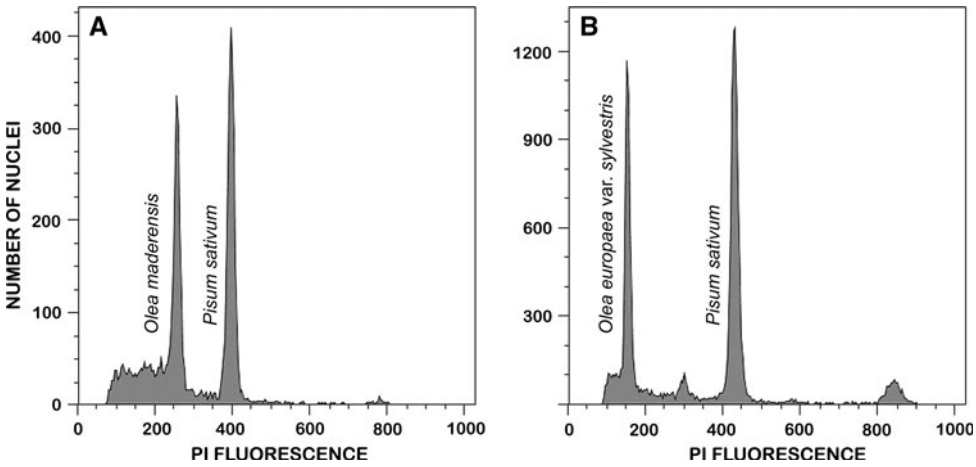

Fig. 2 Histograms of relative fluorescence intensity obtained after simultaneous analysis of nuclei isolated from leaves of *Pisum sativum* cv. Ctirad (internal reference standard) and leaves of the following donor trees: **a** *Olea maderensis*, **b** *O. europaea* ssp. *europaea* var. *sylvestris*. In each *histogram* a dominant peak corresponding to nuclei at the G_0/G_1 phase of the cell cycle and minor peaks corresponding to nuclei at the G_2 phase were detected for both sample and standard

Table 2 Genetic variability assessment of the micropropagation process in *Olea maderensis* and *O. europaea* ssp. *europaea* var. *sylvestris* using flow cytometry

	Ratio of G_0/G_1peaks		Mean nuclear DNA content (pg/2*C*)[a]	*n*
	Mean	SD		
O. maderensis				
Field donor trees	0.672	0.007	6.11*a*	9
In vitro plants	0.664	0.010	6.03*a*	6
Acclimatized plants	0.611	0.003	5.55*b*	5
O. var. *sylvestris*				
Field donor trees	0.355	0.003	3.23*a*	10
In vitro plants	0.347	0.002	3.15*b*	15
Acclimatized plants	0.349	0.002	3.17*b*	5

Values are given as the mean and standard deviation of the mean of the ratio of G_0/G_1peaks, as well as the mean nuclear DNA content (pg/2*C*). The number of replicates analysed in each case is also given

[a] For each species mean values followed by the same letter are not statistically significant at $P < 0.05$

Table 3 Allele size of the 10 microsatellite loci amplified in *Olea europaea* ssp. *europaea* var. *sylvestris* and *Olea maderensis* from Madeira Archipelago and their derived micropropagated plants

Locus	Allele size (bp)					
	Field donor trees		In vitro plants		Acclimatized plants	
	O. var. *sylvestris*	*O. maderensis*	*O.* var. *sylvestris*	*O. maderensis*	*O.* var. *sylvestris*	*O. maderensis*
AJ279854	236/250	205/236/256	236/250	205/236/256	236/250	205/236/256
AJ279856	197/203	192/195/199	197/203	192/195/199	197/203	192/195/199
AJ279858	139	121/136/142//152	139	121/136/142//152	139	121/136/142//152
AJ279859	165/190	170/176/186	165/190	170/176/186	165/190	170/176/186
AJ279865	144	121/133/163/168	144	121/133/163/168	144	121/133/163/168
AJ279867	177/183	160/168/185/193	177/183	160/168/185/193	177/183	160/168/185/193
AJ416320	210/212	191/209	210/212	191/209	210/212	191/209
AJ416321	211/215	195/198/207	211/215	195/198/207	211/215	195/198/207
AJ416322	120/138	135/138/140	120/138	135/138/140	120/138	135/138/140
AJ416323	184/191	183/187/191/193	184/191	183/187/191/193	184/191	183/187/191/193

193 bp, respectively (Fig. 3). Therefore, as all the primer pairs produced monomorphic amplification products either across all the in vitro and acclimatized plants derived from the two species or between them and their respective donor trees, data revealed that micropropagation induced no genetic changes for these specific markers (Fig. 3).

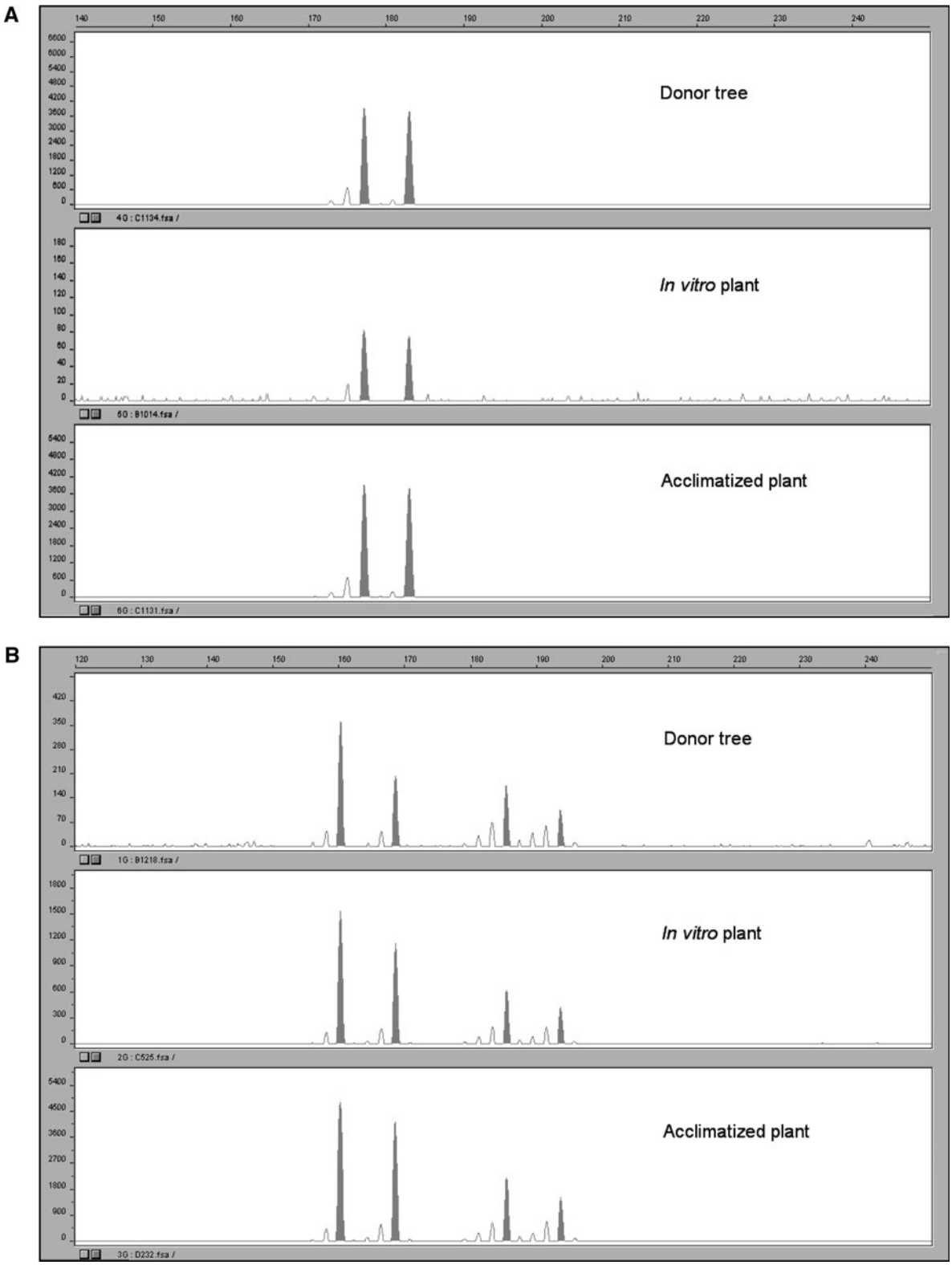

Fig. 3 **a** Amplification of ssrAJ279867 (HEX) locus in *O. europaea* ssp. *europaea* var. *sylvestris* (from *top* to *bottom*): donor tree, in vitro plant and an acclimatized plant. **b** Amplification of ssrAJ279867 (HEX) locus in *O. maderensis* (from *top* to *bottom*): donor tree, in vitro plant and an acclimatized plant. *Top scale* indicates fragment size in nucleotides. *Left scale* indicates fluorescence intensity measured in relative fluorescence units

Discussion

Micropropagation protocols

In the present work, two simple, reproducible, efficient and long-term in vitro protocols for regenerating two wild olive species, *O. maderensis* and *O. europaea* ssp. *europaea* var. *sylvestris* are described. Olive medium (OM), the most commonly used medium to micropropagate olive species, was also the most adequate medium to induce explant sprouting in *O.* var. *sylvestris*, suggesting that the macro and micronutrients combination developed by Rugini (1984) is well balanced for this species. Nevertheless, as *O. maderensis* explants did not proliferate on OM, we enriched this medium with Fe, Mg, Mn (OMG; Brito and Santos 2009) allowing the successful micropropagation of *O. maderensis*.

Data on growth parameters revealed that *O. maderensis* presented better performance in elongation (shoot length) and proliferation rates than *O.* var. *sylvestris*, but these comparisons must be regarded carefully as different media were used for each species. Apical dominance is recurrently reported for commercial cultivars of *O. europaea* and it is mostly associated with apical explants. The use of basal segments may overcome apical dominance, suggesting that explants deprived of the apical bud force the sprouting of axillary buds (Zacchini and De Agazio 2004).

Rooting is another crucial step of micropropagation. For both species we attained suitable rooting strategies. It is interesting to notice that each species had different rooting condition requirements. *O. maderensis* required NAA and dark incubation to achieve the highest rooting rates (84% after 1 month). Beneficial effects of rooting in the dark have already been reported in other olive species/cultivars (e.g. Roussos and Pontikis 2002; Mencuccini 2003; Zacchini and De Agazio 2004). This stimulatory effect of the dark may be due to a higher/faster metabolism of endogenous or exogenous auxins when compared to the light. Also, high light intensity may stimulate ABA production and other phenolic compounds that compromise rooting ability (Anderson 1984). On the other hand, in *O.* var *sylvestris*, only an auxin (IBA) shock for 2 min promoted rooting. IBA has been widely used as a root-inducing hormone in olive (e.g. Grigoriadou et al. 2002), but to our knowledge, this shock strategy has been seldom reported in olive micropropagation protocols (see Brito et al. 2007).

The acclimatization procedure was similar for both species and revealed to be equally successful. The initial acclimatization stage under in vitro conditions was crucial for plant survival of both species, as it facilitated plant adaptability to the ex vitro environment (for review see Pospíšilová et al. 1999). Acclimatized plants looked healthy and morphologically normal, having well hardened and developed stems and dark green leaves.

Finally, the different performance of these two species, may be due not only to external factors (medium composition) but, mostly, to endogenous factors, in particular genetic characteristics, such as the ploidy level. Positive effects of polyploidy on plant traits such as vegetative vigour, drought tolerance and pest resistance are well known in flowering plants (Osborn et al. 2003), and may also have played a significant role in this case. Furthermore, it is generally assumed that polyploid species may be more susceptible to somaclonal variation than diploid ones (e.g. Karp 1995). These variations are prone to occur in any micropropagation protocol and depend on the type of explant and environmental stressing conditions. Therefore, and to ensure a true-to-type propagation protocol, these micropropagated plants were evaluated for genetic stability.

Assessment of genetic variability

Changes in DNA ploidy level (polyploidization, aneuploidy or mixoploidy, for reviews see Karp 1995 and Kaeppler et al. 2000) and/or in DNA sequence are among the most frequent genetic variations found in in vitro cultures.

The analysis of ploidy level during the micropropagation stages showed, for each wild olive species, high homogeneity pattern among individuals of the same stage/population (field, micropropagated and acclimatized plants), supporting the suitability of the used protocol. These flow cytometric analyses revealed that no major ploidy changes occurred during the in vitro culture. However, the small differences in nuclear DNA amount (approx. 9.0%) found between acclimatized plants and both in vitro material and donor trees in *O. maderensis* do not exclude the possible occurrence of aneuploidy. Similar small DNA-PI fluorescence fluctuations have been observed in leaves of other woody species, in particular in older ones, and may be attributed to the presence of cytosolic compounds, and their interference with the DNA-PI staining (known as the "tannic acid effect", for details see Loureiro et al. 2006b). Therefore, acclimatized plants may have accumulated higher levels of cytosolic compounds, which decreased the PI fluorescence of isolated nuclei and resulted in a lower nuclear DNA content estimation. This interference has been also reported in similar true-to-typeness studies of other woody plant species (Pinto et al. 2004; Loureiro et al. 2005), and even in DNA studies of some olive cultivars (Loureiro et al. 2007b). The statistically significant differences detected in *O.* var. *sylvestris* were much smaller (approx. 2.5% between the field donor trees and in vitro plants; Table 2) and may be justified by the highly homogenous estimations obtained for each type of plant

material (SD < 0.003 independently of the number of replicates that varied between 5 and 15) in the different dates of analyses and correspondingly by possible technical related issues, such as instrumental drifts (known to account for up to 3% of FCM differences; Loureiro et al. 2006a, b).

Further clues for a genetic stability at the ploidy level come from the analyses of the nuclear microsatellite markers. Contrary to the diploid *O.* var. *sylvestris* (with 1–2 bands), *O. maderensis* microsatellite profiles presented from two to a maximum of four bands (alleles), which confirms the tetraploid nature of this species and supports FCM data and previous reports (Brito et al. 2008; Lopes et al. 2009). Moreover, in this study no genetic changes among all conditions were detected. The feasibility of these molecular markers, as well as RAPDs, RFLPs and AFLPs, to assess true-to-type micropropagation has long been certified, as endorsed by the increasing number of papers that have been published in recent years in several species. For instance, the absence of somaclonal variation in plant material micropropagated by stem cuttings was reported for the woody species such as *Cedrus libani* (Renau-Morata et al. 2005) and *Robinia pseudocacia* (Bindiya and Kanwar 2003) using RAPDs. Also Fernandes et al. (2008), using AFLP and SSRs in *Quercus suber* described the absence of molecular changes in embryogenic cultures. In contrast, the occurrence of somaclonal variation in micropropagation by stem cuttings, detected by SSRs and other molecular markers, has been reported for other woody species. As examples, genetic variation has been detected in *Eucalyptus tereticornis* using RAPDs and AFLPs (Tripathi et al. 2006), in *Populus tremuloides* using SSRs (Rahman and Rajora 2001) and in *Robinia ambigua* using ISSRs (Guo et al. 2006). To our knowledge, only four studies used molecular marker to ensure true-to-typeness in the genus *Olea*: for commercial cultivars of *O. europaea* var. *europaea*, García-Férriz et al. (2002) and Leva et al. (2002) used RAPDs and reported no somaclonal variation in plants regenerated by stem cuttings. However, using the same markers and type of explant, Peyvandi et al. (2009) detected somaclonal variation in micropropagated plants of a commercial olive cultivar. Finally, Lopes et al. (2009) using SSRs reported no changes between the donor trees and somatic embryos in two olive species. In general, SSRs and AFLPs are considered to be more reliable than RAPDs in the detection of polymorphisms, with SSRs being cheaper and easier to implement than AFLPs. Considering that microsatellite loci consist of sites of preferential mutation of the genome, the absence of mutation in the tested ten microsatellites, suggests that these micropropagation protocols lead to no or few mutation occurrence.

In conclusion, FCM and microsatellite analyses strongly suggest that in these two olive species, the micropropagation protocols here described may not induce genetic variability. However, considering the specific and restricted information given by any molecular markers, the putative occurrence of changes in other sites of the genome or aneuploidy cannot be totally excluded (Lopes et al. 2009). As far as we know, this is the first true-to-typeness screening using plant morphology, FCM and SSRs of micropropagated olives, and also the first report with respect to a complete micropropagation protocol (up to open greenhouse) of *O.* var. *sylvestris*. Since December 2006, micropropagated plants are being integrated in several sites of Porto Santo mountains, within a reforestation programme of Porto Santo Island (e.g. Brito et al. 2009). They are also being used in conservation programmes, mostly involving the endemic and endangered *O. maderensis*, a strong candidate for the priority sites of the Natura 2000 networking programme.

Acknowledgments Authors thank D.R.F. and S.R.E. from Madeira Autonomous Region and Porto Santo Town Hall. This work was supported by FCT POCTI/AGR/60672/2004; FCT/SFRH/BPD/6012/2001; FCT/SFRH/BPD/36601/2007; FCT/SFRH/BD/27467/2006.

References

Anderson WC (1984) Revised tissue culture medium for shoot multiplication of *Rhododendron*. J Am Soc Hort Sci 109:343–347

Bindiya K, Kanwar K (2003) Random amplified polymorphic DNA (RAPDs) markers for genetic analysis in micropropagated plants of *Robinia pseudoacacia* L. Euphytica 132:41–47

Brito G, Santos C (2009) Basal medium improvement for routine plant micropropagation of *Olea maderensis*: physiological comparative studies. Can J For Res 39:814–822

Brito G, Costa A, Fonseca H, Santos C (2003) Response of *Olea europaea* ssp. *maderensis* in vitro shoots to osmotic stress. Sci Hortic 97:411–417

Brito G, Jardim R, Coelho C, Santos C (2007) Micropropagation of a native species of Porto Santo Island as a strategy to combat desertification: wild olive as example. Silva Lusitana 15(2):229–247

Brito G, Loureiro J, Lopes T, Rodriguez E, Santos C (2008) Genetic characterization of olive trees from Madeira Archipelago using flow cytometry and microsatellite markers. Genet Resour Crop Evol 55:657–664. doi:10.1007/s10722-007-9268-8

Brito G, Costa A, Coelho C, Santos C (2009) Large-scale field acclimatization of *Olea maderensis* micropropagated plants: morphological and physiological survey. Trees 23:1019–1031. doi:10.1007/s00468-009-0344-x

Canas LA, Benbadis A (1988) In vitro plant regeneration from cotyledon fragments of the olive tree (*Olea europaea* L.). Plant Sci 54:65–74

Conde P, Loureiro J, Santos C (2004) Somatic embryogenesis and plant regeneration from leaves of *Ulmus minor* Mill. Plant Cell Rep 22:632–639

De la Rosa R, James CM, Tobutt KR (2002) Isolation and characterization of polymorphic microsatellites in olive (*Olea europaea* L.) and their transferability to other genera in the Oleaceae. Mol Ecol Notes 2:265–267

Doležel J, Binarová P, Lucretti S (1989) Analysis of nuclear DNA content in plant cells by flow cytometry. Biol Plant 31:113–120

Doležel J, Sgorbati S, Lucretti S (1992) Comparison of three DNA fluorochromes for flow cytometric estimation of nuclear DNA content in plants. Physiol Plant 85:625–631

Fernandes P, Rodriguez E, Pinto G, Roldán-Ruiz I, De Loose M, Santos C (2008) Cryopreservation of *Quercus suber* somatic embryos by encapsulation-dehydration and evaluation of genetic stability. Tree Physiol 28:1841–1850

García JL, Troncoso J, Sarmiento R, Troncoso A (2002) Influence of carbon source and concentration on the in vitro development of olive zygotic embryos and explants raised from them. Plant Cell Tissue Organ Cult 69:95–100

García-Férriz L, Ghorbel R, Ybarra M, Marì A, Belaj A, Trujillo I (2002) Micropropagation from adult olive trees. IV international symposium on olive growing. Acta Hort 586:879–882

Grigoriadou K, Vasilakakis M, Eleftheriou EP (2002) In vitro propagation of the Greek olive cultivar 'Chondrolia Chalkidikis'. Plant Cell Tissue Organ Cult 71:47–54

Guo W, Li Y, Gong L, Li F, Dong Y, Liu B (2006) Efficient micropropagation of *Robinia ambigua* var. *idahoensis* (Idaho Locust) and detection of genomic variation by ISSR markers. Plant Cell Tissue Organ Cult 84:343–351

Kaeppler SM, Kaeppler HF, Rhee Y (2000) Epigenetic aspects of somaclonal variation in plants. Plant Mol Biol 43:179–188

Karp A (1995) Somaclonal variation as a tool for crop improvement. Euphytica 85:295–302

Leva AR, Petruccelli R, Montagni G, Muleo R (2002) Field performance of micropropagated olive plants (cv. Maurino): morphological and molecular features. Acta Hortic 586:891–894

Lopes T, Pinto G, Loureiro J, Costa A, Santos C (2006) Determination of genetic stability in long-term somatic embryogenic cultures and derived plantlets of cork oak using microsatellite markers. Tree Physiol 26:1145–1152

Lopes T, Capelo A, Brito G, Loureiro J, Santos C (2009) Genetic variability analyses of the somatic embryogenesis induction process in *Olea* spp. using nuclear microsatellites. Trees 23:29–36

Loureiro J, Pinto G, Lopes T, Doležel J, Santos C (2005) Assessment of ploidy stability of the somatic embryogenesis process in *Quercus suber* L. using flow cytometry. Planta 221:815–822

Loureiro J, Rodriguez E, Doležel J, Santos C (2006a) Comparison of four nuclear isolation buffers for plant DNA flow cytometry. Ann Bot 98:679–689

Loureiro J, Rodriguez E, Doležel J, Santos C (2006b) Flow cytometric and microscopic analysis of the effect of tannic acid on plant nuclei and estimation of DNA content. Ann Bot 98:515–527

Loureiro J, Capelo A, Brito G, Rodriguez E, Silva S, Pinto G, Santos C (2007a) Micropropagation of *Juniperus phoenicea* from adult plant explants and analysis of ploidy stability using flow cytometry. Biol Plant 51:7–14

Loureiro J, Rodriguez E, Costa A, Santos C (2007b) Nuclear DNA content estimations in wild olive (*Olea europaea* L. ssp. *europaea* var. *sylvestris*) and Portuguese cultivars of *O. europaea* using flow cytometry. Genet Resour Crop Evol 54:21–25

Mencuccini M (2003) Effect of medium darkening on in vitro rooting capability and rooting seasonality of olive (*Olea europaea* L.) cultivars. Sci Hortic 97:129–139

Osborn TC, Pires JC, Birchler JA, Auger DL, Chen ZJ, Lee HS, Comai L, Madlung A, Doerge RW, Colot V, Martienssen RA (2003) Understanding mechanisms of novel gene expression in polyploids. Trends Genet 19:141–147

Peyvandi M, Noormohammadi Z, Banihashemi O, Farahani F, Majd A, Hosseini-Mazinani M, Sheidai M (2009) Molecular analysis of genetic stability in long-term micropropagated shoots of *Olea europaea* L. (cv. Dezful). Asian J Plant Sci 8:146–152

Pinto G, Valentim H, Costa A, Santos C (2002) Somatic embryogenesis in leaf callus from mature *Quercus suber* L. tree. In Vitro Cell Dev Biol 38:569–572

Pinto G, Loureiro J, Lopes T, Santos C (2004) Analysis of the genetic stability of *Eucalyptus globulus* Labill. somatic embryos by flow cytometry. Theor Appl Genet 109:580–587

Pospíšilová J, Tichá I, Kadleček P, Haisel D, Plzáková Š (1999) Acclimatization of micropropagated plants to ex vitro conditions. Biol Plant 42:481–497

Rahman MH, Rajora OP (2001) Microsatellite DNA somaclonal variation in micropropagated trembling aspen (*Populus tremuloides*). Plant Cell Rep 20:531–536

Rallo P, Dorado G, Martín A (2000) Development of simple sequence repeats (SSRs) in olive tree (*Olea europaea* L.). Theor Appl Genet 101:984–989

Rallo P, Tenzer I, Gessler C, Baldoni L, Dorado G, Martín A (2003) Transferability of olive microsatellite loci across the genus *Olea*. Theor Appl Genet 107:940–946

Rathmacher G, Niggemann M, Wypukol H, Gebhardt K, Ziegenhagen B, Bialozyt R (2009) Allelic ladders and reference genotypes for a rigorous standardization of poplar microsatellite data. Trees 23:573–583

Renau-Morata B, Nebauer SG, Arrillaga I, Segura J (2005) Assessments of somaclonal variation in micropropagated shoots of *Cedrus*: consequences of axillary bud breaking. Tree Genet Genomes 1:3–10

Roussos PA, Pontikis CA (2002) In vitro propagation of olive (*Olea europaea* L.) cv. Koroneiki. Plant Growth Regul 37:295–304

Rout GR, Mohapatra A, Jain SM (2006) Tissue culture of ornamental pot plant: a critical review on present scenario and future prospects. Biotechnol Adv 24(6):531–560

Rugini E (1984) In vitro propagation of some olive (*Olea europaea sativa* L.) cultivars with different root-ability, and medium development using analytical data from developing shoots and embryos. Sci Hortic 24:123–134

Rugini E, Pesce PG (2006) Genetic improvement of olive. Pomol Croat 12:43–74

Santos C, Brito G, Pinto G, Fonseca H (2003) In vitro plantlet regeneration of *Olea europaea* ssp. *maderensis*. Sci Hortic 97:83–87

Santos C, Loureiro J, Lopes T, Pinto G (2007) Genetic fidelity analyses of in vitro propagated cork oak (*Quercus suber* L.). In: Jain SM, Häggman H (eds) Protocols for micropropagation of woody trees and fruits, chap 7. Springer, Netherlands, pp 67–83

Sefc KM, Lopes MS, Mendonça D, Santos MR, Da Câmara Machado ML, Da Câmara Machado A (2000) Identification of microsatellite loci in olive (*Olea europaea* L.) and their characterization in Italian and Iberian olive trees. Mol Ecol 9:1171–1173

Tripathi SB, Mathish NV, Gurumurthi K (2006) Use of genetic markers in the management of micropropagated *Eucalyptus* germplasm. New Forest 31:361–372

Zacchini M, De Agazio M (2004) Micropropagation of a local olive cultivar for germplasm preservation. Biol Plant 48:589–592