International Scholars Journals

International Journal of Bacteriology and Mycology ISSN 2756-3669 Vol. 8 (4), pp. 001-007, April, 2019. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Morphological evaluation of olive plants propagated *in vitro* culture through axillary buds and somatic embryogenesis methods

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Accepted 16 March, 2019

The morphological fidelity of the olive plants propagated through axillary buds, microplants and somatic embryogenesis, somatic plants was evaluated. Thirty-two morphological traits were used to characterize the tissue culture propagated olive plants. The microplants showed very high phenotypic similarity compared to plants produced by conventional cutting propagation method. The somatic plants exhibited variant morphological stable phenotypes, among somaclonal population two variant phenotypes were studied: BOS (bush-olive somaclone) and COS (columnar-olive somaclone) . A wide range of plant traits were differently involved in somaclonal variation as plant height, canopy dimensions, leaf, inflorescence and fruit dimensions in respect to the putative control plants. The present study has established that the morphological stability of tissue culture-derived olive plants is strictly related with the *in vitro* propagation method used.

Key words: Tissue culture, phenotypic stability, somaclonal variation, *Olea europaea*.

INTRODUCTION

The olive tree is an economic and social resource for many Mediterranean countries in Italy in particular; it is part of the history and landscape of the country (Ciferri, 1950; Bartolini and Petruccelli, 2002). In recent years there is a renewed interest in this crop, which is now expanding in countries where it was hitherto unknown. The vegetative propagation is an integral feature of the olive production line and the first step for establishing new orchards and/or revitalising old ones. Propagation might be brought in tissue culture through axilliary buds or somatic embryogenesis methods to produce rapidly a large number of plants from selected genotypes and to meet increased demand for olive plants certified for both genetic fidelity and phytosanitary characteristics.

Micro propagation has been applied to the olive since the 1980s (Rugini, 1984; Fiorino and Leva 1986; Leva et al., 1995a; Grigoriadou et al., 2002). Many studies have been focused on induction of somatic embryogenesis in different olive cultivars (Rugini, 1988; Mencuccini and Rugini, 1993; Rugini and Caricato, 1995; Leva et al., 1995b; Peyvandi et al., 2001; Shibli et al., 2004). The commercial success in application of the axillary-buds or of the somatic embryogenesis methods to propagate olive cultivars depends mainly on the absence of somaclonal variants among plants produced. Somaclonal variation has been reported in a large number of plant species, vegetatively and sexually propagated (Hammer-schlag, 1992; Lamhamedi et al., 2000). Many reports have indicated the occurrence of somaclonal variation for morphological, biochemical and genetic traits in perennial plants derived from *in vitro* culture (Saieed et al., 1994; Brar and Jain, 1998; Etienne and Bertrand, 2003; Martins et al., 2004; Morcillo et al., 2006). This aspect is of para-mount importance for olive cultivars because the olive can be distinguished from other fruit tree species by its very long life span (hundreds of years), a long juvenile period for most, a broad biodiversity with the consequent variability in the fruit which influences quality aspects of the olive oil, including its aroma and taste (Roselli et al., 2003). In this context there is a need to evaluate in the field and on mature plants the performance of both micro-plants (derived from axillary-buds) and somatic plants (derived from somatic embryogenesis).

Despite the commercial importance of clonal fidelity of the olive plants produced by tissue culture, little has been published of field performance of microplants (Briccoli Bati et al., 2002; Leva et al., 2002b) and there is no information about mature somatic plants. The aim of this

study was to assess in the field the morphological fidelity of the regenerated olive plants, by axillary buds and somatic embryogenesis, through their vegetative growth and developmental-productive behaviour.

MATERIALS AND METHODS

Microplants (by axillary bud method)

The plant materials for the propagation by axillary buds were drawn from a single plant of the cv. Maurino clone M1B; the micro shoots were produced using *in vitro* protocol previously described (Leva et al., 1995a; Leva et al., 2004). The explants were *in vitro* subcultured for 12 subsequent subcultures. The microplants were acclimatized in the second half of 1996 and they were grown in pots until they were transplanted in an experimental orchard, 70 microplants and 20 cutting plants in 1998. At the time of planting the microplants were uniform: average of 45 cm in height, stem diameter of 4 mm, few ramifications and developed and spatially homogenous root system.

The cutting plants, used as control, were propagated from semi hardwood cuttings obtained from the same donor plant for tissue culture explants. The same training system (3 x 2 m rows spacing) and agronomic operations were implemented for both types of plants. All plants were 6 years old, 10 microplants and 10 cutting plants, randomly chosen, were evaluated during 2002 and 2003 years.

Somatic plants (by somatic embryogenesis method)

Plantlets were obtained from embryogenic tissue induced in de-embryonated immature cotyledon explants of the cv Frangivento using the protocol previously described; the embryogenic tissues were sub-cultured bimonthly on fresh medium for three years (Leva et al., 1995b) . The acclimated somatic plantlets were maintained in green house for one year and subsequently transferred to large pots and grown in open air from April 1994 till the end of 1997. During the development in pots among 43 somatic plants, some variant morphological phenotypes were detected (Leva et al., 2001).

After this preliminary phase aiming at describing and assessing the somaclonal population, all somatic plants were transplanted in the field in 1998. For this study two variant phenotype groups were considered: BOS (bush olive somaclone, 4 representative trees) and COS (columnar olive somaclone; 4 representative trees). Four replicates for each phenotype have been chosen in agreement with the replicate plants in a germplasm olive collection.

Microplants of the cv Frangivento (4 representative trees) obtained from explants derived from the same donor plant for induction of somatic embryogenesis and using the same *in vitro* protocol of Maurino microplants, were considered as Putative control (Pc). All plants observed were 8 years old and they were evaluated during 2002 and 2003 years.

On all micro, somatic and respective control plants normal management practices including fertilizer and pesticide applications were followed during the cultivation in the field. No irrigation or pruning manipulations were applied in order to avoid the influence on development of vegetative and reproductive organs.

Morphological analysis

Measurements of morphological traits were carried out during two growing seasons, 2002 and 2003. On microplants (Mp), somatic plants (BOS and COS) and respective controls (cutting plants Cp; Putative control Pc); the data, as mean values of the two years, have been reported. The number of morphological traits observed was 32 for somatic plants and among those 24 for microplants (Table 1).

The samples, 10 for vegetative characters, 50 for reproductive characters and leaves, were taken respectively from each BOS and COS somatic plant and Pc plants, and from 10 Mp and 10 Cp. The canopy spread was calculated as a circular projection of the canopy to the soil; the volume using the formula: $2/3\ r^2h$; where h = canopy height, r = canopy radius.

Analysis of variance was performed on average values of the two years and mean separations were done using the Tukey - test (P 0.01), employing ANOVA. The relationships between the Mp and Cp, BOS, COS groups and Pc were investigated by multivariate methods (cluster analysis). Cluster analysis were performed on microplants selecting among the variables those with statistically significant values and for somatic plants those variables with F ratio higher than 40.00.

The statistical analysis was performed using the Statgraphics plus statistical package (version 5.1 for Windows).

RESULTS

Microplants

During the field growth no differences were noted between the Mp and Cp regarding the vegetative traits, the development of the canopy and productive area. Even if the leaf area (LA) of the Mp was larger than the Cp, the shape (BL / W) did not show any variation (Table 2). The number of flowers per inflorescence (NF) was higher in Mp than Cp but it did not correlate with productivity of the fruiting shoot (NO, Table 3). Dimensional variations have been detected on the fruits (FL, FW); the Mp showed larger drupes and a higher yield than the Cp, very similar dry weight of the drupes, between the two types of plants, was observed (Table 3). Moreover the shape of drupes was equal, similarly the data obtained on the pit traits indicated that there was no variability for these characters between the two types of plants; the pit dimensions are not very sensitive to environmental factors such as the pulp of the drupes (Table 3).

The two-dimensional scatter diagram of two variables, yield production and leaf area (Figure 1) that, using one-way analysis of variance, showed statistical differences between the two types of plants (Tables 2 and 3), gave an accurate picture of the uniformity among the plants studied. The Mp and Cp showed very high similarity (same marker) in spite of the characters used for statistical analysis; only one “accession” of the M-plants group seems to form a separate group (Figure 1).

Somatic plants

Tables 4 and 5 report the BOS, COS and Pc values for each trait analysed and the results of the analysis of variance of the vegetative, inflorescence and fruit characters respectively. Under similar growth conditions most traits showed significant differences among BOS and COS and Putative-control. Among 32 characters observed 14 distinguish both BOS and COS from the Putative - control: HP, VSN, LA (Table 4) IL, FL, FW, FFW, FDW,

Table 1. Quantitative descriptors of olive plants propagated through tissue culture observed in the present study.

Characters and definition of the variables	
	Vegetative characters
1 HP	Plant height: measured in meter from the soil level to the highest point
2 CP	Canopy projection to the soil: measured at the two widest diameters in m^2
3 VP	Canopy volume in m^3
4 TA	Trunk area in cm^2
5 VSG	Vegetative shoot growth in cm
6 VSN	Node number of vegetative shoots
7 VSI	Internode length of vegetative shoots in cm
8 *FS	Number of feather shoots (lateral shoots developing from axillary buds formed in same year) on the vegetative shoots
9 *FG	Feather shoot growth in cm
10 *FN	Feather shoot node number
11 *FI	Internode length of feather shoots in cm
12 LBL	Leaf blade length in mm
13 LBW	Leaf blade width in mm
14 BL/W	Blade length/width
15 LA	Leaf area in mm^2
16 *LFW	Leaf Fresh weight in mg
17 *LDW	Leaf Dry weight in mg
18 *DW	Dry weight mg per 100 mm^2
	Inflorescence and fruit characters
19 *IL	Inflorescence length in mm
20 NF	Number of flowers per inflorescence
21 NO	Number of olive fruits per fruiting shoot
22 FL	Fruit length in mm
23 FW	Fruit width in mm
24 FL/W	Fruit length/width
25 FFW	Fruit fresh weight in g
26 FDW	Fruit dry weight in g
27 PL	Pit length in mm
28 PW	Pit width in mm
29 PL/W	Pit length/width
30 PFW	Pit weight in g
31 FFW/PW	Fruit weight/Pit weight
32 FY	Production weight in Kg

*no used for microplants.

PL, PW, PFW, FFW / PW (Table 5). In particular the heights of COS and BOS plants varied from 4.4 to 2.6 m respectively while the height of the Pc plants was 3.4 m. The BOS plants compared with the Pc showed differences in 19 traits, while 18 traits were different between COS and Pc, in especially most of them were related to reproductive traits (Table 5).

The morphological differences between BOS and COS were evident as they were related to the growth level and leaf, fruit and pit traits. The morphological variations on the somatic plants within the groups were not significant for all traits (data not shown). In BOS group the reduction of height, the increase of the feather shoot number, the reduction of the organ dimensions as leaves, inflorescences and fruits determined compact growth habit of the plants. The relations among BOS, COS plants and Pc are visualized in the Figure 2. As the variance analysis of morphological data, reported in Tables 4 and 5, the cluster analysis was quite able to separate and assign to the different groups the BOS, COS and Pc plants, in a way that had been expected.

DISCUSSION

The field performance of the cv Maurino microplants, verified by the analysis of the morphological and produc-

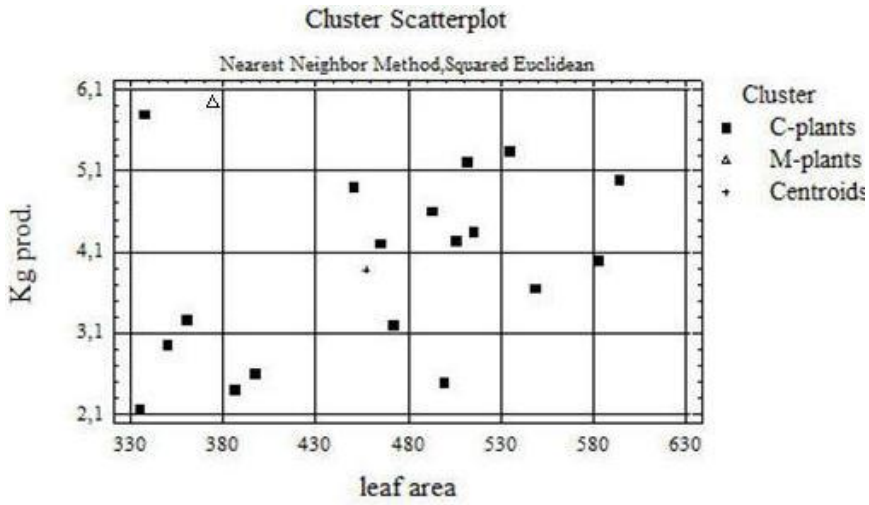

Figure 1. Scatter diagram and two clusters identified from two variable mean values for microplants (M-plants) and cutting-plants (C-plants).

Table 2. Comparison of vegetative trait means between microplants (Mp) and cutting propagated plants (Cp) cv Maurino.

Vegetative character	Cp	Mp
LBW cm	1.02 ± 0.04	1.24 ± 0.03*
LA cm^2	4.03 ± 0.21	5.16 ± 0.15*
VSI cm	2.27 ± 0.10	2.34 ± 0.10
BL/W	5.40 ± 0.2	5.30± 0.1
HP m	2.46 ± 0.04	2.49 ± 0.06
VP m^3	1.54 ± 0.10	1.64 ± 0.17
CP m^2	7.23 ± 0.33	7.48 ± 0.49
LBL cm	5.60 ± 0.10	6.53 ± 0.09*
TA cm^2	14.60 ± 1.02	15.40± 1.44
VSG cm	23.80 ± 1.50	25.80± 1.40

Each value is the average ± SE; the values followed by *are different from the control at the P 0.01 level of Tukey's Test.

Table 3. Inflorescence, fruit and productivity characters of the microplants (Mp) and cutting propagated plants (Cp) cv Maurino.

Inflorescence and fruit characters		
	Cp	Mp
FFW g	2.0 ± 0.03	2.1 ± 0.02
PL/W	2.2 ± 0.05	2.1 ± 0.02
FDW g	0.8 ± 0.01	0.82 ± 0.02
PW mm	5.5 ± 0.1	5.6 ± 0.1
FFW/PW	7.1 ± 0.1	7.2 ± 0.2
PL mm	12.2 ± 0.1	12.2 ± 0.1
PFW g	0.28± 0.01	0.29 ± 0.01
FL/FW	1.30 ± 0.01	1.30 ± 0.01
FW mm	13.40 ± 0.08	14.10 ± 0.15*
FY Kg	3.35 ± 0.50	4.40 ± 0.20*
NO	14.20 ± 1.50	16.10 ± 1.80
NF	12.80 ± 0.20	14.80 ± 0.40*
FL mm	17.40 ± 0.10	18.50 ± 0.20*

Each value is the average ± SE; the values followed by *are different from the control at the P 0.01 level of Tukey's Test.

tive traits, suggests that the propagation by axillary buds produced true-to-type plants. The data revealed no differences in growth habit, vegetative growth, canopy and trunk area; the leaves and drupes of microplants were slightly wider than the control plants but they still retained the characteristic shapes of the cultivar. The productivity of the fruiting shoots was similar despite the different number of flowers per inflore-scence; it is known that in olive the fruit set is low only 2 - 5% of the flowers (Gucci and Cantini, 2000) . The traits of the pit were equal. The vegetative and reproductive traits were the same in both Mp and Cp. The microplants showed a full flowering after two years of the field cultivation and there were not differences in time between the microplants and cutting plants (Leva et al., 2002). This fact confirms that that the axillary bud propagation does not affect the onset of production as reported for other cultivars (Bati et al., 2002). The data on the slight higher yield in microplants than Cp could be related to a different development of root system of the micro- plants in respect to the root system of the cutting-plants (A. Leva, personal communication); a study is in progress concerning this aspect.

Furthermore results are strictly related to the protocol used in *in vitro* culture. It is important to stress this aspect because as reported by Rani and Raina (2000) a micro propagation protocol should be released for commercial

Table 4. Comparison of means of vegetative traits among the BOS, COS groups and putative-control plants (cv Frangivento).

Vegetative characters	Putative control plants ± SE	BOS plants ± SE	COS plants ± SE	F- ratio
BL/W	6.1 5n.s	5.5 ± 0.6n.s	5.0 ± 0.3n.s	1.2
CP m^2	1.4 A	1.3 A	2.6 B	20.8
DW mg	26.4 ± 1.7n.s	32.8 ± 3.5n.s	28.8 ± 0.8n.s	1.03
FG cm	3.0 ± 0.9n.s	2.0 ± 0.5n.s	1.3 ± 0.4n.s	3.3
FI cm	1.2 ± 0.09n.s	0.9 ± 0.08n.s	0.9 ± 0.04n.s	1.0
FN	2.3 ± 0.4n.s	1.9 ± 0.1n.s	1.6 ± 0.3n.s	1.6
FS	1.5 B	6.3 A	1.4 B	7.8
HP m	3.4 B	2.6 C	4.4 A	73.5
LA mm^2	524.7 A	280.7 C	444.0 B	130.5
LBL mm	66.5 A	49.3 B	54.8 B	6.8
LBW mm	12.0 A	9.1 B	11.6 A	53.9
LDW mg	150.0 A	80.0 B	123.0 A	97.3
LFW mg	302.1 A	168.1 B	269.5 A	7.8
TA cm^2	43.4 ± 0.8n.s	60.3 ± 6.7n.s	74.3 ± 11.3n.s	3.3
VP m^3	4.7 B	4.5 B	11.2 A	10.8
VSG cm	31.7 B	38.5 B	50.0 A	23.7
VSI cm	1.9 B	2.0 B	2.7 A	43.7
VSN	16.2 B	19.1 A	18.5 A	5.7

related to 100 mm^2

Each value is the average ± SE; the values followed by the same letter are not different from the control at the P 0.01 level of Tukey's Test.

Table 5. Comparison of means of inflorescence and fruit characters among the BOS, COS groups and putative control plants (cv Frangivento).

Inflorescence and fruit characters	Putative control plants	BOS plants	COS plants	F- ratio
IL mm	32.3 B	21.2 C	35.8 A	146.4
NF	25.1 A	17.0 B	14.3 B	57.7
FL mm	1.9 B	1.7 C	2.5 A	384.3
FW mm	1.5 B	1.3 C	2.2 A	661.9
FL/W	1.2 A	1.2 A	1.1 B	33.5
FFW g	2.9 A	1.6 C	2.2 B	417.0
FDW g	1.6 A	0.9 C	1.3 B	409.9
PL mm	1.1B	0.9 C	1.2 A	162.6
PW mm	0.6 B	0.5 C	0.7 A	290.0
PL/W	1.8 B	1.8 A	1.7 B	5.2
PFW g	0.3 B	0.2 C	0.4 A	917.0
FFW/PW	10.3 A	8.5 B	5.8 C	297.0

The values followed by the same letter are not different from the control at the P 0.01 level of Tukey's Test.

purpose only when analyses on mature plants have established that the given protocol does not induce undesirable somaclonal variation. In our case we have morphological uniformity between the microplants tested and cutting plants; if there were some genetic variations they were not in relation to the development, the vegetative growth and productivity traits.

As for the performance of regenerated plants through so-matic embryogenesis, all information in literature, about perennials plants, was limited to the period of acclimatization of plantlets (Canas and Bebandis, 1988).

On juvenile period (Leva et al., 2001) or on 4 - 5 years

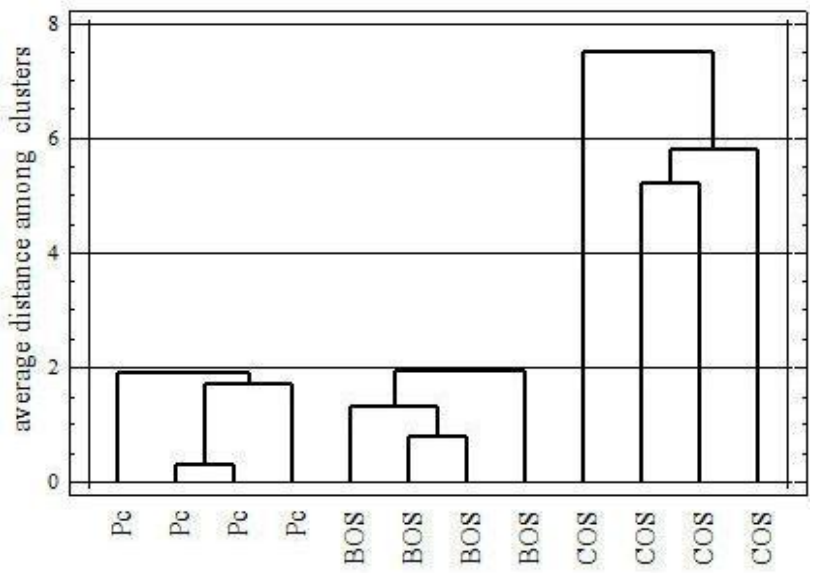

Figure 2. Dendrogram based on morphological traits of the BOS, COS groups and Putative control (Pc) according to a hierarchical clustering.

old juvenile trees of black spruce and white spruce (Tremblay et al., 1999).

In this study morphological variations of somatic plants respect to putative control were observed in mature 8 year old plants. Previous results (Leva et al., 1995b) reported normal feature without changes in performance on the same plants, after acclimatization, when they were six month old seedlings. When the plants were transferred in containers in the open air they began to show a different developmental behaviour (Leva et al., 2001). Under field conditions, the mature somatic plants retained their respective phenotypes and it has been possible to measure any morphological variation including those related to potential yield, inflorescences, fruits and their characteristics.

This demonstrates that the studies have to be conducted for a long period of time until flowering period in order to confirm the clonal fidelity of somatic plants. Deverno (1995) reported that the frequency of somaclonal variation increased with the duration of *in vitro* culture and Muller et al. (1990) found that the level of DNA polymorphism increased with the length of time in culture; similar results were found in tomato cell-population grown *in vitro* for more than two years (Bogani et al., 2001).

Moreover, studies carried out on frequency of somaclonal variation in plants of black spruce and white spruce derived from somatic embryogenesis demonstrated that the clone was the most important source of genetic instability instead of “time of maintenance in culture” (Tremblay et al., 1999).

In our study, we could not determine whether these two factors were directly involved in somaclonal variation or which one is relevant. The reported morphological analysis demonstrates that the study of phenotypes still represents an available way to identify the true-to type plants and/or variants. The phenotypic evaluation should not be ignored as a tool to assess the presence or not of somaclonal variation in regenerate plants, in particular in our case; it has been useful considering the long life cycle of Olea spp.

The reported data suggest that, particularly in long-term *in vitro* cultures of olive, new genotypes may become fixed. Keeping in mind the possibility that stable phenotypic variability may occur in somatic olive plants, somatic embryogenesis should not be used in commercial *in vitro* propagation, because this variation is undesirable for clonal propagation of olive tree and/ or *in vitro* germplasm collection.

ACKNOWLEDGEMENTS

The work was supported by Ministero delle Politiche Agricole e Forestali, Progetto: Ricerca ed Innovazione per l'Olivicoltura Meridionale. Programma di Sviluppo del Mezzogiorno.

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