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Micropropagation of olive tree Olea europaea L. 'Oueslati'

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Abstract: The present study was undertaken to contribute to the continuous search for efficient in vitro protocols for the micropropagation of olive trees, with special focus on the Oueslati cultivar. It investigates the main factors involved in the micropropagation process, namely medium composition, growth regulators, rooting, and acclimatization conditions. It demonstrates that under particular conditions, the Oueslati cultivar displays a high capacity to proliferate. During the multiplication phase, the multiplication medium, which is a modification of Rugini medium with zeatin (1 and 2 mg L^{-1}), yielded the highest multiplication rates, with an average of more than 9. Furthermore, almost 91% of the proliferated shoots rooted in 40 days when their basal parts were dipped in an IBA solution and transferred to an agar medium enriched with the same growth regulator. The acclimatization of the in vitro-derived plantlets was carried out under greenhouse conditions, which yielded a success rate of about 88%. The findings from the analyses of the anatomical and physiological changes during the acclimatization step, particularly in terms of photosynthetic activity and transpiration, indicated that the micropropagated plantlets acquired good acclimatization and total autotrophic capacity after 4 months of ex vitro conditions.

Key words: Acclimatization, in vitro culture, medium formulation, photosynthesis, transpiration, zeatin

Abbreviations: BAP: benzylaminopurine, IBA: indole butyric acid, DKW: Driver and Kuniyuki walnut medium, MM: multiplication medium, MS: Murashige and Skoog; NAA: naphthalene acetic acid, OM: olive medium, RH: relative humidity, RM: rooting medium, SE: standard error, STB: shoot with terminal bud, SWTB: shoot without terminal bud.

Introduction

The olive tree is a valued, long-lived evergreen tree that has been grown in the Mediterranean region from time immemorial. The conventional method of olive tree propagation is based on vegetative multiplication using cuttings, grafting, or suckers. Although occasionally yielding satisfactory results, these methods have been frequently criticized as being very slow and inefficient for some highly valued cultivars, particularly the Oueslati cultivar, one of the main Tunisian olive genotypes cultivated in the region of Oueslatia (Kairouan, central Tunisia) (Trigui and Msallem 2002). This cultivar is commonly propagated by 2 techniques, namely cutting and grafting, whose applications have often been reported to be highly challenging (Khabou et al. 1999; Khabou and Drira 2000; Kammoun-Gargouri et al. 2009). It is partly for this reason that the culture of this cultivar remains almost exclusively limited to its area of origin, i.e. the Tunisian region of Oueslatia.

In fact, several studies have been conducted with the aim of developing alternative micropropagation

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methods that can help to overcome the limitations associated with the traditional techniques currently used for the propagation of olive trees. One of the driving forces behind this growing interest is the challenging attempt to increase the number of plants produced while decreasing the interval of plant production time. Special attention has recently been given to micropropagation methods as viable alternatives. In fact, micropropagation techniques have been quite useful for producing olive tree cultivars of high quality and valuable genotypes (Briccoli-Bati and Lombardo 1995; Garcia-Ferriz et al. 2002; Zuccherelli and Zuccherelli 2002; Sghir et al. 2005).

In this context, previous studies have often reported that the composition of the micropropagation medium represents an important factor for the achievement of high multiplication rates (George 1993; Revilla et al. 1996; Şaş Sertkaya and Çınar 1999). Furthermore, several studies have been conducted to evaluate the effect of alternative medium compositions or culture conditions (Revilla et al. 1996; Lambardi et al. 2006) on the propagation of explants taken from adult plants or vegetative explants cultured in vitro. Olive medium (OM), proposed by Rugini (1984), has been reported to be efficient for the micropropagation of a wide array of olive cultivars (Rugini 1984; Rama and Pontikis 1990; Chaari-Rkhis et al. 1999, 2002, 2003; Zuccherelli and Zuccherelli 2002).

Research conducted on the effect of the culture medium on the in vitro propagation of olive also indicates that the use of mannitol (as a carbon source) and zeatin (as a growth regulator) in culture media is essential to achieve satisfactory results in shoot proliferation (Rugini 1984; Fiorino and Leva 1986; Micheli et al. 2009). However, Revilla et al. (1996) achieved micropropagation from mature olive trees using a Driver and Kuniyuki walnut (DKW) medium (Driver and Kuniyuki 1984) containing sucrose as well as benzylaminopurine (BAP) and indole butyric acid (IBA) as phytohormones. Similarly, Peixe et al. (2007) proposed coconut water and BAP to replace zeatin in olive micropropagation.

As far as the Tunisian context is concerned, research on the micropropagation of native olive genotypes started in 1997 using adult and young vegetative material as initial explants (Chaari-Rkhis et al. 1999, 2002, 2003; Maalej et al. 2002, 2006). Reports indicate that the multiplication or proliferation phase of the micropropagation has succeeded with some, but not all, Tunisian varieties, on the grounds that in vitro shoot production of olive is widely dependent upon cultivar and medium formulation (Cozza et al. 1997; Chaari-Rkhis et al. 1999, 2002, 2003; Brahadda et al. 2003).

Several other studies have, on the other hand, demonstrated that naphthalene acetic acid (NAA) (Rama and Pontikis 1990) and IBA (Chaari-Rkhis et al. 2002; Zuccherelli and Zuccherelli 2002; Brahadda et al. 2003) have significant effects on the in vitro root formation of olive plants. Similarly, the addition of polyamines to the medium has also been reported to induce in vitro root elongation (Rugini et al. 1990; Mencuccini 1995; Zuccherelli and Zuccherelli 2002; Nas 2004).

Another equally important factor that has often been emphasized in the literature in terms of its effects on propagation is acclimatization. Several plantlet morphological structures and physiological parameters have been studied because of their potential to change abruptly during the first days of ex vitro culture, which is likely to affect their eventual survival rates (Pospisilova et al. 1999). Likewise, the density and morphology of stomata and the photosynthesis rate are affected by the relative humidity (RH) and light conditions during the acclimatization phase (Pospisilova et al. 1998, 1999).

Despite this significant flow of data, only a few studies have so far been carried out with the aim of accelerating the propagation time of olive plants, and the requirements that govern their in vitro development are not yet fully known or elucidated. Accordingly, the present study was undertaken to gather further information that might contribute to the development of an in vitro protocol for the rapid and efficient micropropagation of olive plants, with special focus on the Oueslati cultivar, to help overcome the inadequacies of the current conventional propagation methods.

Materials and methods

Plant material and culture conditions

The in vitro micropropagation performed in the current study was carried out on olive plant material taken from mature, 50-year-old, healthy olive trees (*Olea europaea* L. 'Oueslati') cultivated in

the orchards of the region of Oueslatia (Kairouan, central Tunisia). Apical shoots (15-20 cm long) were collected during the summer and cut into uninodal segments (from 1.0 to 2.0 cm in length), which were then thoroughly washed under running tap water for at least 30 min, surface sterilized under a laminar flow hood by being dipped and constantly agitated for 8 min in commercial bleach containing sodium hypochlorite (12%), and finally washed 3 times with sterile distilled water.

The nodal segments, from which the leaves were partially eliminated, were cultured in OM (Rugini 1984) for culture initiation. This medium, as well as the ones employed in the experiments described below, contained 30 g L^{-1} of mannitol (Aldrich Chemistry, Steinheim, Germany) and 8 g L^{-1} of agar (Scharlau Microbiology, Barcelona, Spain). All media were adjusted to a pH of 6.0 before being autoclaved at 120 °C for 20 min. In all experiments, 70-mL glass tubes (2.5 cm in diameter, 20 cm in height) were employed, with 20 mL of agar medium. The cultures were maintained in a growth chamber at 25 ± 1 °C and under cool white fluorescent lamps (45 µmol m^{-2} s^{-1}) with a 16 h photoperiod. After 10 weeks of culture, the explants developed into shoots, 3.0-5.0 cm in length, which were then divided into nodal explants with 1 pair of buds and subcultured twice under the same culture and environmental conditions for 90 days each. Finally, the proliferated shoots were cut into 1-cm-long leafy uninodal portions with 1 pair of buds and used for Experiment 1.

Experiment 1: shoot proliferation

This experiment was undertaken to explore and evaluate the effect of medium composition on the shoot proliferation of the explants. Three basic nutrient formulations were used, namely OM (Rugini 1984), Murashige and Skoog (MS; Murashige and Skoog 1962), and a new medium called multiplication medium (MM; Table 1) that consists of a 1:1 combination of OM and MS macronutrients together with OM micronutrients and vitamins. All media contained 2 mg L^{-1} of glycine, 200 mg L^{-1} of glutamine, and 100 mg L^{-1} of myo-inositol. These proliferation media were supplemented with different doses of zeatin (0, 1, 2, and 4 mg L^{-1}), among the most cited cytokinins for the micropropagation of olive trees (Rugini 1984; Rama and Pontikis 1990; Chaari-Rkhis et al. 2002; Micheli et al. 2009), and 30 g L^{-1} of mannitol, among the most commonly applied carbon sources for the in vitro elongation of olive plants (Leva et al. 1994). The leafy uninodal explants described above were transferred individually into glass tubes containing 20 mL of the proliferation media mentioned above. The experiments were performed in 3 replications of 24 explants, each incubated in a growth room under the temperature and light conditions described above. After 90 days, the cultures were assessed for shoot length and node number. Two other replications of this same experiment were performed after the sixth and tenth subcultures.

Table 1. Multiplication medium (MM) composition.

Compound	Quantity (mg L^{-1})
NH_4NO_3	1031
KNO_3	1500
$CaCl_2$ $2H_2O$	440
$MgSO_4$ $7H_2O$	935
KH_2PO_4	255
$Ca(NO_3)_2$ $4H_2O$	300
KCl	250
$FeSO_4$ $7H_2O$	27.80
Na_2-EDTA	37.20
H_3BO_3	12.4
$MnSO_4$ $4H_2O$	22.3
$ZnSO_4$ $7H_2O$	14.3
KI	0.83
Na_2MoO_4 $2H_2O$	0.25
$CuSO_4$ $5H_2O$	0.25
$CoCl_2$ $5H_2O$	0.025
Glutamine	200
Glycine	2
Myo-inositol	100
Thiamine	0.5
Pyridoxine	0.5
Nicotinic Acid	5
Biotin	0.05
Folic Acid	0.5

Experiment 2: shoot rooting

The aim of this experimental step was to explore and evaluate shoot response under certain rooting conditions pertaining essentially to growth regulators. Proliferated shoots that grew up to 3-4 cm and had 3-4 nodes were employed, and shoot

response was assayed and assessed with respect to 2 types of shoot rooting conditions, namely shoot with terminal bud (STB) and shoot without terminal bud (SWTB). More succinctly, the following 2 rooting conditions were compared.

Condition 1: A single STB or SWTB was transferred to a glass tube containing 20 mL of rooting medium (RM) composed of half-concentration MS nutrients and supplemented with IBA (1 mg L^{-1}), thiamine (1 mg L^{-1}), mannitol (30 g L^{-1}), and agar (8 g L^{-1}).

Condition 2: Before putting a STB or SWTB into a glass tube containing the same RM as in Condition 1, its bottom was aseptically dipped for 4-5 s in a solution containing 2000 ppm IBA.

In all cases, the basal parts of the cultures were covered with aluminum foil to provide darkness, and were then maintained under growth room conditions in a 16-h photoperiod at 25 ± 1 °C, with a light intensity of 25 µmol m^{-2} s^{-1}. For each rooting condition, 3 replications of 15 shoots each (STB or SWTB) were used. Observations of rooted shoots and roots per plantlet were recorded after 40 days of culture.

Experiment 3: plantlet acclimatization

This experimental step was undertaken with the aim of exploring and evaluating the micropropagated plantlets in terms of their adaptation and survival potential with respect to the acclimatization environment. The micropropagated plantlets were carefully taken out of the glass tubes and their roots were thoroughly washed to remove any adhering agar medium. They were then transplanted into plastic pots (7 cm in diameter and 8 cm in height) containing a compost, peat moss, and peat mixture (1:1:1 v:v:v). The potted plantlets were irrigated once a week with a nutrient solution (10-20 mL) composed of half-concentrations of MS macro- and micronutrients, and were placed in a greenhouse in which the RH was adjusted to 80% with the temperature maintained between 20 and 25 °C.

For the first week following transplantation, the plantlets were covered with transparent polythene to maintain the temperature and high humidity. The survival and acclimatization parameters were recorded 30 days after the beginning of this experimental step, which involved 45 in vitro-derived plantlets divided into 3 lots (replications) of 15 plants. Moreover, the net photosynthesis (µmol CO_2 m^{-2} s^{-1}) and transpiration (mmol H_2O m^{-2} s^{-1}) rates of the already formed in vitro leaves were measured 30, 90, and 180 days after the beginning of acclimatization using an LCi photosynthesis apparatus (ADC Bioscientific Ltd., England). At least 30 measurements were made for both parameters. The number of trichomes, which are protective structures on the leaves, was also computed by taking leaf trichome imprints on the self-adhesive bands (Boujnah 1997). These ribbons were then stuck onto the blades and observed using a photonic microscope (Leitz) connected to a computer. The diameter measurements were performed using UTHSCSA ImageTool Version 3.0. At least 30 counts were taken for the number and diameter of the trichomes on each leaf side.

Statistical analyses

All experiments were conducted in 3 replicates using a randomized complete block design. The statistical analyses were performed using SPSS 11.0 (Chicago, IL, USA). The results are expressed as mean ± standard error (SE). Duncan's multiple range test was used to determine the significance of differences among the compared mean values at a level of confidence of 0.05.

Results

Experiment 1: shoot proliferation

The findings revealed that, after 3 months of culture, the nodal explants underwent relatively different trends and levels of proliferation. Table 2 shows that these variations depended on the medium formulation and zeatin concentration. For instance, average values of 6.8, 8.1, and 6.5 cm for shoot length and 6.5, 9.5, and 10.2 for new nodes were recorded for the MM containing 1, 2, and 4 mg L^{-1} of zeatin, respectively.

Seeing that the highest multiplication rates were obtained with the MM, only growth rates achieved with this medium for shoots sprouting from the uninodal portions, particularly in terms of node number, were reported. The findings indicated that while the explants showed relatively low growth

Table 2. Mean values of shoot lengths (cm) and node numbers registered on the tested media (OM, MS, and MM) supplemented with 0, 1, 2, or 4 mg L^{-1} of zeatin after 90 days of culture.

Medium	Zeatin (mg L^{-1})	Shoot Length (cm)	Node Number
OM	0	0.5 ± 0.5 a	0.6 ± 0.5 a
	1	3.6 ± 1.4 c	3.1 ± 1.1 bc
	2	5.1 ± 1.8 d	5.5 ± 1.9 d
	4	5.1 ± 1.5 d	6.8 ± 2.4 d
MS	0	0.5 ± 0.4 a	0.8 ± 0.5 a
	1	1.5 ± 0.9 b	1.8 ± 1.1 b
	2	2.9 ± 1.4 b	2.2 ± 0.9.1 b
	4	2.4 ± 1.9 b	4.1 ± 1.6 c
MM	0	0.6 ± 0.5 a	0.7 ± 0.5 a
	1	6.8 ± 2.1 e	6.5 ± 1.9 d
	2	8.1 ± 1.8 f	9.5 ± 2.1 e
	4	6.5 ± 2.5 e	10.2 ± 2.6 e

The results are expressed as mean ± SE; in each column, values followed by the same letter are not significantly different according to Duncan's multiple range test ($P < 0.05$).

during the first 30 days of culture on media enriched with 1, 2, or 4 mg L^{-1} of zeatin, their growth curves took on an exponential form in the second month, indicating that maximum growth was attained during this period. Finally, the growth rate was observed to stabilize after about 70 days of culture and to become evidently low after 90 days (Figure 1).

Experiment 2: shoot rooting

The findings revealed that, for both types of shoots (STB or SWTB), and in terms of rooting percentage and root number per plantlet, Condition 2, which involved double IBA treatment (in the RM and in the dipping of the basal part of the shoots into a solution containing the growth regulator), was better than Condition 1 (with the IBA in the RM only). In fact, the shooting response attained its highest level with Condition 2 after 40 days of culturing, reaching up to 90.9% for the STB but 81.8% for the SWTB (Table 3). However, no significant differences were observed between the 2 types of shoots and conditions in terms of root number per plantlet.

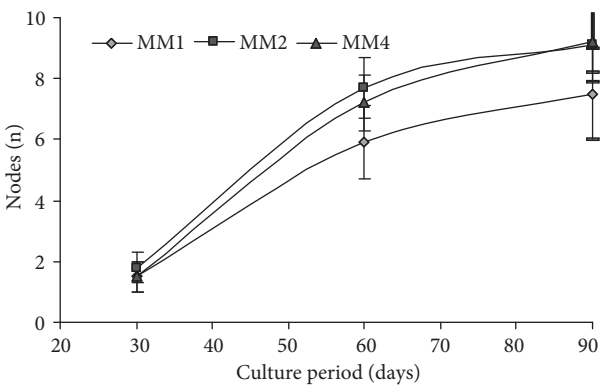

Figure 1. Mean values of node number per shoots registered during the 90 days of proliferation on MM enriched by 1 mg L^{-1} (MM1), 2 mg L^{-1} (MM2), and 4 mg L^{-1} (MM4) of zeatin. The values are expressed as means ± SE.

The follow-up of periodic root growth in Condition 2 revealed that, in STB, roots began to appear from the first 10 days of culture (5.9%) and that the maximum rates of root emergence were recorded after 20 days, with a rooting percentage of 72.7%. The rooting percentages recorded in the same period for SWTB, on the other hand, were lower, reaching only 63.6% (Figure 2).

Experiment 3: plantlet acclimatization

The findings revealed that after the first month of acclimatization, 88% of the plantlets transferred to the greenhouse survived, but with a slow rate of growth. After a relative period of adaptation to the acclimatization environment, however, the growth rates started to accelerate. In fact, the plantlets became taller and new nodes appeared after the first

Table 3. Mean values of rooting rates (%) and of root numbers (n) per rooted shoot after 40 days in the tested rooting conditions (1 and 2) of shoots without terminal bud (SWTB) and shoots with terminal bud (STB).

	Rooting Condition			
	1		2	
	Rooting (%)	Root/Plant (n)	Rooting (%)	Root/Plant (n)
SWTB	50.00 ± 7.7 a	4.33 ± 0.9 a	81.8 ± 4.2 b	9.66 ± 0.9 b
STB	45.45 ± 5.2 a	4.40 ± 1.1 a	90.9 ± 2.2 b	9.70 ± 1.2 b

The values are expressed as mean ± SE; in each column, values followed by the same letter are not significantly different according to Duncan's multiple range test ($P < 0.05$).

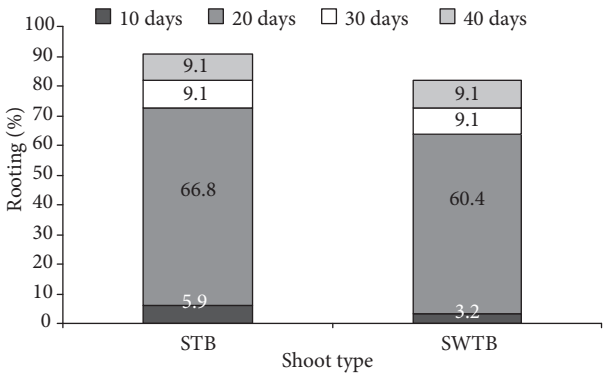

Figure 2. Rooting percentage after 10, 20, 30, and 40 days of Condition 2 (double IBA treatment) for STB or SWTB.

successive 30 days. At 180 days after the beginning of the acclimatization step, the mean value of the plantlets' length was 8.9 cm and the mean number of nodes was 5.8 (Table 4).

Moreover, trichomes were found both on the upper and lower faces of the leaves of the micropropagated plantlets, with a progressive increase in their number, particularly on the lower side, during acclimatization. In fact, while the average density of trichomes on the lower side was 7.3 mm^{-2} at the beginning of the ex vitro conditions, it increased to 28.6 mm^{-2} after 6 months. During the same period, however, the average density of trichomes on the upper face went up from 1 to 6 mm^{-2} (Figure 3). Furthermore, an increase in terms of the diameter of the trichomes, particularly those on the lower side of the leaves, was observed during the acclimatization phase (Figure 4).

As far as photosynthesis was concerned, its net value was observed to be relatively high in the early days of transfer to the greenhouse (4.8 μmol CO_2 m^{-2} s^{-1}), then to decrease into lower values 30 days after transfer, and finally to undergo a rapid and significant increase that

Table 4. Mean values of plant length (cm) and node numbers (n) during the acclimatization.

Days in Acclimatization	Plant Length (cm)	Node/Plantlet (n)
0 (Transplanting)	4.1 ± 1.1 a	3.2 ± 0.9 a
30	4.8 ± 1.1 ab	3.9 ± 0.8 ab
60	5.9 ± 0.9 b	4.1 ± 0.8 b
90	7.6 ± 0.9 c	4.9 ± 0.9 bc
180	8.9 ± 0.8 c	5.8 ± 1.0 c

Values followed by the same letter are not significantly different according to Duncan's multiple range test ($P < 0.05$).

reached 7.4 µmol CO_2 m^{-2} s^{-1} 180 days after transfer (Figure 5). Unlike photosynthesis, transpiration was observed to increase remarkably from the early days of plantlet transfer to reach a high value (5.15 mmol H_2O m^{-2} s^{-1}) 15 days after transplanting. It was only after 120 days of acclimatization that transpiration showed a significant fall, which was taken to mean the end of the acclimatization phase, particularly because a parallel highly active photosynthesis process was observed.

Discussion

In spite of the continuous attempts to delineate a definite culture medium composition for the efficient in vitro proliferation of olive tree species (Rugini 1984; Rama and Pontikis 1990; Chaari-Rkhis et al. 2002; Zuccherelli and Zuccherelli 2002; Peixe et al. 2007), it seems that different olive tree cultivars require different basic medium formulations. In fact, the results presented in the current study indicate that the best propagation performances attained for the Oueslati cultivar were recorded using MM composed of a half-concentration of macronutrients, micronutrients, and vitamins of OM mixed with a half-concentration of MS macronutrients. Compared to OM, MM is richer in total nitrogen, especially in nitrate form. In addition to having a stimulating effect on in vitro organogenesis (George 1993), particularly in olive plants (Rugini 1984; Cozza et al. 1997), the nitrogen form may have significant effects on multiplication rates. In fact, similar high proliferation results were previously reported in the literature when some modifications were introduced, namely the reduction of NH_4NO_3 and the increments of KNO_3 concentrations (Rama and Pontikis 1990; Briccoli-Bati and Lombardo 1995). It is worth noting, however, that the levels of calcium and magnesium that were used in the MM were lower than those used in the OM. This suggests that the reduction did not have marked effects on the growth rates, particularly because the OM is already very rich in these 2 nutrients and, hence, the use of only half of the OM's Ca and Mg quantities was sufficient to achieve good results.

With regards to growth regulators, the findings of the present study showed that at least 1 mg L^{-1} of zeatin was necessary for inducing proliferation activity. In fact, several studies have previously described zeatin as a central cytokinin required for the micropropagation of olive plants (Rama and Pontikis 1990; Rugini 1990; Chaari-Rkhis et al. 2002, 2003; Zuccherelli and Zuccherelli 2002; Micheli et al. 2009). While several previous studies reported on the use of this cytokinin at very high concentrations in olive (Fiorino and Leva 1986; Rama and Pontikis 1990; Briccoli-Bati and Lombardo 1995), good proliferation responses were recorded for the Oueslati cultivar when zeatin concentrations varied from 1 to 4 mg L^{-1} after 90 days of culture.

Furthermore, satisfactory results in terms of the rooting of both types of proliferated shoots, i.e. with and without terminal buds, were achieved on agar media containing IBA, into which the shoots were transferred after the immersion of their basal part

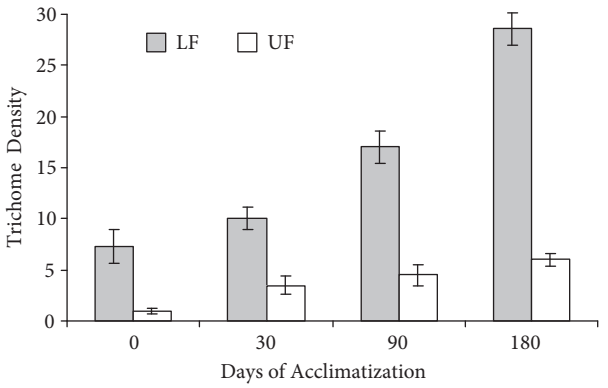

Figure 3. Trichome density on the upper face (UF) and lower face (LF) of the leaves during acclimatization. The values are expressed as means ± SE.

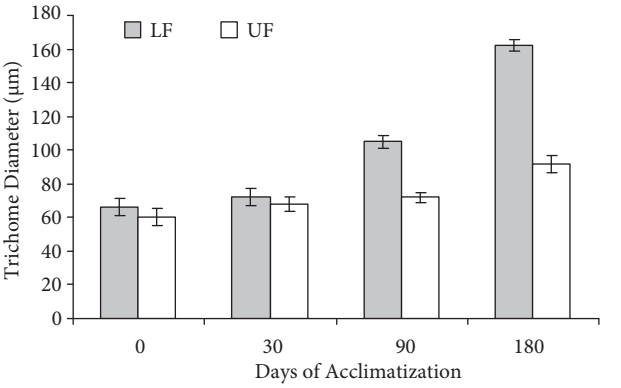

Figure 4. Trichome diameter on the lower face (LF) and upper face (UF) of the leaves during acclimatization. The values are expressed as means ± SE.

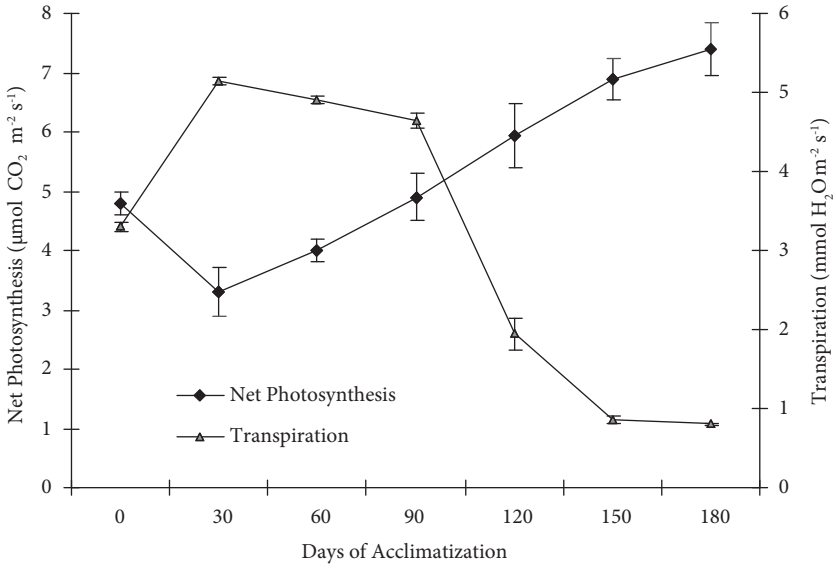

Figure 5. Mean values of net photosynthesis and transpiration during acclimatization. The values are expressed as means ± SE.

for a few seconds in a solution containing the same growth regulators. In fact, a number of studies have previously reported on the rooting of leafy cuttings from the Oueslati cultivar (Khabou et al. 1999), and their results indicated that response depended on the season of cutting. While Khabou et al. (1999) reported a rooting average of 24% attained after 60 days, Kammoun-Gargouri et al. (2009) reported a poor rooting of 4% for the same cultivar. In the case of the present study, 90% of the shoots were rooting in 40 days, with an average of 9.7 roots per plant. The results of the present work confirm the beneficial effect of IBA on the in vitro rooting of proliferated shoots of olive plants. In fact, IBA is one of the most important growth regulators commonly used for olive plants propagated by cutting or in vitro rooting; it is sometimes used together with NAA (Rugini 1990).

Several previous studies reported that the in vitro rooting of olive varied from 25% to 100%, depending on the cultivar, auxin type, and concentration. Other reports indicated that the preservation of the shoot bases in darkness enhanced the success of rooting and helped avoid instances of deformation at the roots. Such treatment may be carried out by covering the base of the culture, either by adding a coloring agent or by activated charcoal in the rooting media (Mencuccini 1995). The rooting response has also been reported to increase when the whole culture vessel is placed in darkness at the beginning of the rooting process (Chaari-Rkhis et al. 2002). This seems to be attributed to the enhanced peroxide activity, which is generally implicated in the rooting process (Zryd 1998). Likewise, the experimental assays performed in the current study involved the covering of the basal part of the culture tube with aluminum foil, a procedure that might have contributed to the good rooting responses attained.

The acclimatization of in vitro-derived plantlets is an intricate and delicate step. The findings of the present study show that although the net photosynthesis rates achieved by the plantlets during the first days of transfer were relatively low, those rates were noted to undergo a significant increase before and during the acclimatization phase. This result seems to be in disagreement with the commonly known fact that photosynthesis under in vitro conditions is usually very low (Serret et al. 2001). Transpiration, on the other hand, was noted to be intensified. The reduction observed for the net photosynthesis rates after the ex vitro transfer was previously noted for other species, such as tobacco and chile ancho pepper (Pospisilova et al. 1999; Estrada-Luna et al. 2001). This behavior could be attributed to the stress following transplantation, which might have temporarily distorted the

photosynthetic system. In fact, after a few days, the vigor and activity of photosynthesis were restored to values similar to those recorded for olive trees in the field (Boujnah 1997).

Transpiration, on the other hand, increased at the beginning of the acclimatization step and then tended to decrease after a few days to reach very low values after 180 days. Similar results were reported in previous studies (Pospisilova et al. 1999; Estrada-Luna et al. 2001) on micropropagated plantlets of tobacco and chile ancho pepper, which showed that transpiration increased after the ex vitro transfer, reached values that were much higher than those recorded during in vitro culture, and then gradually decreased to reach normal values a few days later. Furthermore, a comparison between the transpiration rates obtained in the current study and those observed for olive trees cultivated in the field (Boujnah 1997) showed that in vitro-derived plants transpired much more, particularly during the first days of acclimatization. In fact, an apparent decrease of these rates occurred in about the third month of transfer and was paralleled by a high increase in the photosynthesis rates. Taken together, these observations suggest that the acclimatization phase reached its end. The increase in the transpiration rates in micropropagated plants after the ex vitro transfer could be attributed to the late development of the cuticle (Pospisilova et al. 1999). Nevertheless, this could also be in direct relationship with the degree of stomatal opening. In fact, the cuticle is implicated in the regulation of water loss and, probably, with the number of trichomes. Concerning the trichomes, the results of the present study showed an increase in their number and size during acclimatization. A similar observation was also demonstrated in a previous study by Lambardi et al. (2006) on Italian and Spanish olive tree cultivars.

Although these changes were noted to have simultaneous effects on the diameter and density of the trichomes on both sides of the leaves, the trichomes were larger and more numerous on the lower faces of the leaves, presumably because of the presence of stomata. Their presence allowed for a more elevated coverage of the foliar area and, consequently, better stomatal protection that limited transpiration and, eventually, led to the good acclimatization potential of the plantlets. Similar results were reported in a previous study conducted on the Silvan cultivar of blackberry (Donnelly et al. 1986).

In summary, the findings of the present study are encouraging and can, therefore, open new potential pathways for the micropropagation of olive plants. It is for this reason that further research on acclimatization is currently under way in our laboratories to make this step easier and faster for application and to explore the potential use of tissue culture techniques in the nursery practices and environments.

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References

Boujnah D (1997) Variations morphologiques, anatomiques et écophysiologiques en rapport avec la résistance à la sécheresse chez l'olivier *Olea europaea* L. Thèse de Doctorat d'Etat. Faculté Landbouwkundige, Université de Gent, Belgique.

Brahadda N, Abousalim A, Walali Loudiyi DE (2003) Effet du milieu de culture sur le microbouturage de l'olivier (*Olea europeae* L.) cv. Picholine Marocaine. Biotechnologie, Agronomie, Société et Environnement 7: 177-182.

Briccoli-Bati C, Lombardo N (1995) Propagazione *in vitro* della cv Nocellara etnea. In: Proceedings of Congrès sur l'oléiculture méditerranéenne: état et perspective de la culture et de la recherche. Rende, Consenza, Italy, pp. 249-256.

Chaari-Rkhis A, Chelly-Chaabouni A, Maalej M, Drira N (2002) Meski olive variety propagated by tissue culture. Acta Horticulturae 586: 871-874.

Chaari-Rkhis A, Maalej M, Drira N (2003) Micropropagation des variétés Tunisiennes d'olivier. Synthèses des résultats préliminaires. Olivae 95: 19-24.

Chaari-Rkhis A, Trigui A, Drira N (1999) Micropropagation of Tunisian cultivars of olive tree: preliminary results. Acta Horticulturae 474: 79-81.

Cozza R, Turco D, Batti CB, Bitonti MB (1997) Influence of growth medium on mineral composition and leaf histology in micropropagated plantlets of *Olea europaea*. Plant Cell Tissue and Organ Culture 51: 215-223.

Donnelly DJ, Skelton FE, Daudeny HA (1986) External leaf features of tissue-cultured Silvan blackberry. Hort Science 21: 306-308

Driver JA, Kuniyuki AH (1984) In vitro propagation of Paradox walnut rootstock. Hort Science 19: 507-509

Estrada-Luna AA, Davies F Jr, Egilla JN (2001) Physiological changes and growth of micropropagated chile ancho pepper plantlets during acclimatization and post-acclimatization. Plant Cell Tissue and Organ Culture 66: 17-24.

Fiorino P, Leva AR (1986) Investigation on the micropropagation of the olive (*Olea europaea* L.): influence of some mineral elements on the proliferation. Olea 17: 101-104.

Garcia-Ferriz L, Ghorbel R, Ybarra M, Mari A, Belaj A, Trujillo I (2002) Micropropagation from adult olive trees. Acta Horticulturae 586: 879-882.

George EF (1993) Plant Propagation by Tissue Culture, Vol 1. Exegetics Ltd., England.

Kammoun-Gargouri L, Gouiaa M, Gargouri K, Skhiri F, Msallem M (2009) Study of semi-hardwood cutting of the olive tree cultivar Oueslati. In: Abstracts of Olivebioteq 2009, Sfax, Tunisia, p. 104.

Khabou W, Drira N (2000) Rhizogenesis variation of Tunisian olive tree varieties semi-hardwood cuttings. Olivae 84: 47-49.

Khabou W, Trigui A, Drira N (1999) Etude de la rhizogenèse des boutures semi ligneuses de l'olivier (*Olea europaea* L.). In: Proceedings of 5èmes Journées Nationales des Acquis de la Recherche Agricole , Hammamet, Tunisia, pp. 146-150.

Lambardi M, Benelli C, Ozden-Tokatli Y, Ozudogru EA, Gumusel F (2006) A novel approach to olive micropropagation: the temporary immersion system. Proceedings of 2nd Int. Seminar Olivebioteq 2006, Vol. 1, Marsala-Mazara del Vallo, Italy, pp. 319-326.

Leva AR, Petruccelli R, Bartolini G (1994) Mannitol "in vitro" culture of *Olea europaea* L. (cv. Maurino). Acta Horticulturae 356: 43-46.

Maalej M, Chaari-Rkhis A, Drira N (2006) Contribution to the improvement of olive tree somatic embryogenesis by mineral and organic analysis of zygotic embryos. Euphytica 151: 31-37.

Maalej M, Chaari-Rkhis A, Trigui A, Drira N (2002) Preliminary results of somatic embryogenesis from young zygotic embryos of the olive tree (*Olea europaea* L.). Acta Horticulturae 586: 899-902.

Mencuccini M (1995) Micropropagazione e miglioramento genetico in vitro dell'olivo: stato dell'arte e prospettive future. Rivista di Frutticoltura 12: 73-82

Micheli M, El Behi AW, Zakhour D, Yasin M, Standardi A (2009) In vitro proliferation of olive ('Dolce Agogia' and 'Moraiolo'): effect of different cytokinins. In: Abstract of XIth Intern. Symposium on Plant Bioregulators in Fruit Production, Bologna, Italy.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiology 15: 473-479.

Nas MN (2004) Inclusion of polyamines in the medium improves shoot elongation in hazelnut (*Corylus avellana* L.) micropropagation. Turkish Journal of Agriculture and Forestry 28: 189-194.

Peixe A, Raposo A, Lourenco R, Cardoso H, Macedo E (2007) Coconut water and BAP successfully replaced zeatin in olive (*Olea europaea* L.) micropropagation. Scientia Horticulturae 113: 1-7.

Pospisilova J, Ticha I, Kadelcek P, Haisel D, Plzakova S (1999) Acclimatization of micropropagated plants to *ex vitro* conditions. Biologia Plantarum 42: 481-497.

Pospisilova J, Wilhelmova N, Synkova H, Castsky J, Krebs D, Ticha I, Hannackova B, Snopel J (1998) Acclimation of tobacco plantlets to *ex vitro* conditions as affected by application of abscisic acid. Journal of Experimental Botany 49: 863-869.

Rama P, Pontikis CA (1990) In vitro propagation of olive (*Olea europaea* L.) "Kalamon." Journal of Horticulture Science 65: 347-353.

Revilla MA, Pacheco J, Casares A, Rodriguez R (1996) In vitro reinvigoration of mature olive trees (*Olea europaea* L.) through micrografting. In Vitro Cellular & Developmental Biology - Plant 32: 257-261.

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

Rugini E (1990) In vitro culture of the olive: an overview of the present scientific status. Acta Horticulturae 286: 93-96.

Rugini E, Politi V, Bignami C, De Agazio M, Greco S (1990) Effect of polyamine treatments on rooting cutting of three olive cultivars. Acta Horticulturae 286: 97-100.

Serret MD, Trillas MI, Araus JL (2001) The effect of in vitro culture conditions on the pattern of photoinhibition during acclimation of gardenia plantlets to ex vitro conditions. Photosynthetica 39: 67-73.

Sghir S, Chatelet PH, Ouazzani N, Dosba F, Belkoura I (2005) Micropropagation of eight Moroccan and French olive cultivars. Hort Science 40: 193-196.

Şaş Sertkaya G, Çınar A (1999) Detection of a new medium for budwood culture in vitro of citrus. Turkish Journal of Agriculture and Forestry 23: 333-340.

Trigui A, Msallem M (2002) Oliviers de Tunisie: catalogue des variétés autochtones et types locaux, Vol 1. Ministère de l'Agriculture, Sfax, Tunisia.

Zryd JP (1998) Cultures de cellules, tissues et organes végétaux. In: Presses Polytechniques Romandes, Lavoisier TEC & DOC, Paris, France, p. 308.

Zuccherelli G, Zuccherelli S (2002) In vitro propagation of fifty olive cultivars. Acta Horticulturae 586: 931-934.