Journal of Plant Biology, September 2003, 46(3) : 173-180

In-Vitro Regeneration of Olive Tree by Somatic Embryogenesis

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We induced somatic embryogenesis from the cotyledon segments of *Olea europaea* (L.) cvs. 'Chetoui', 'Chemleli', and 'Arbequina'. Calli were established from all three cultivars on OMc media supplemented with IBA and 2i-P. The greatest success was obtained with media that contained zero or low concentrations of growth regulators. High levels of hormones (i.e.,>0.5 mgL^{-1} IBA and 2i-P) inhibited embryogenesis. Embryos at different maturation stages were observed with continuously proliferating secondary embryogenesis. Abnormally shaped embryos and teratoma were also noted. Four weeks was the optimal incubation period for inducing embryogenesis on the auxin-containing medium. In addition, 30 to 40 gL^{-1} sucrose was more effective than glucose in stimulating the growth and maturation of somatic embryos. Embryogenic efficiency was also higher when multivariate combinations of nitrogen sources (inorganic and organic nitrogen forms) were used. The plantlets that were derived from our germinating somatic embryos were similar to those obtained from axillary buds.

Keywords: *Olea europaea* L., regeneration, somatic embryogenesis

Fruit trees normally are asexually propagated because they are generally heterozygous, have a large genome, and are outbreed (Ochatt et al., 1990; Han, 2001). The olive tree, (*Olea europaea* L.), is a Mediterranean woody fruit crop that is still propagated by conventional methods, e.g., cuttings and, less often seedling-grafts (Rugini and Fedeli, 1990). In fact, micropropagation protocols have been established for only a few of its cultivars (Rugini and Fedeli, 1990; Mencuccini and Rugini, 1993). In contrast, tissue culture and regeneration of whole plantlets have been widely adopted for the propagation and genetic improvement of other woody species, including citrus (Bouzid, 1983; Tisserat et al., 1990; Saito et al., 1994; Guo et al., 2002) and grape (Compton and Gray, 1996; Regner et al., 1996).

These new production methods are of great interest to researchers who wish to rapidly clone olive trees rather than rely entirely classical techniques and breeding programs (Canas et al., 1987; Canas and Benbadis, 1988; Rugini and Lavee, 1992; Rugini et al., 1996). Somatic embryogenesis of this species has been conducted with juvenile tissues, immature zygotic embryos (Rugini, 1988; Leva et al., 1993, 1995; Shibli et al., 2001), and mature zygotic embryo calli (Orinos and Mitrakos, 1991; Mitrakos et al., 1992; Leitao et al., 1997). Starting with the mature tissues of two cultivars, Rugini and Caricato (1995) have now reported the first successful somatic embryogenesis and plant recovery. Therefore, the objective of our study was to improve the regeneration protocol for olive from somatic embryos. Here, we describe a technique using cotyledonary segments.

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MATERIALS AND METHODS

Plant Materials and Culture Conditions

Mature fruits of three *O. europaea* L. cultivars 'Chetoui', 'Chemleli', and 'Arbequina' were collected in late September. After the mesocarps were removed, the seeds were washed and air-dried at room temperature (RT). Before beginning the experiments, the endocarp were broken mechanically and the seeds were surface-sterilized in 0.5 gL^{-1} $HgCl_2$ for 20 min. They were then rinsed thoroughly in sterile distilled water, and placed in Petri dishes for 24 h to imbibe at RT. Afterward, the cotyledons were cut aseptically into two segments and placed in a Petri dish (1.5 × 90 mm) containing 20 mL of an OMc medium as described by Canas and

Abbreviations: Arb, cultivar 'Arbequina'; Ct, cultivar 'Chetoui'; Cm, cultivar 'Chemleli'; IBA, Indole-3-butyric acid; 2i-P, isopentenyl adenine; OM, Olive medium (Rugini, 1984); OMc, Olive modified medium (Canas and Benbadis, 1988).

Benbadis (1988). This medium had been supplemented with 5 mgL^{-1} IBA, 0.5 mgL^{-1} 2i-P, 30 gL^{-1} sucrose, and 6 gL^{-1} Difco agar (pH adjusted to 5.6 before autoclaving). The cultures were maintained for four weeks in the dark at 28°C to initiate embryogenic calli.

Four sets of experiments were conducted to test how manipulating the culture environment might affect somatic embryogenesis efficiency. These varying factors included: 1) combinations of plant growth regulators (PGRs); 2) the length of time spent on high-auxin induction media; 3) carbon source; and 4) form of nitrogen used in the media. The first experiment evaluated the effectiveness of PGRs by subculturing our newly induced calli on OMc media that contained various levels of IBA and 2i-P. Culturing took place in the dark at 26°C to monitor the differentiation of somatic embryos.

In the second experiment, we checked the effect of induction period. Explants were incubated for 3, 4, 5, or 6 weeks on OMc media that were supplemented with 5 mgL^{-1} IBA, 0.5 mgL^{-1} 2i-P, and 30 gL^{-1} sucrose, and solidified with 6 gL^{-1} agar (pH 5.6). These cultures were incubated in the dark at 26°C, then transferred to a hormone-free OMc medium for subculturing under the same conditions. For the third experiment, the role of carbon source on embryo differentiation and maturation was evaluated by incubating our induced calli on hormone-free OMc media that contained glucose or sucrose at concentrations of 20, 30, 40, 60, or 80 gL^{-1}. These calli were then held for four weeks in the dark at 26°C for subculturing and maturation.

The final experiment assessed the role of nitrogen during the differentiation phase of somatic embryogenesis. Previously induced calli were transferred to hormone-free OMc media supplemented with inorganic reduced and oxidized forms of nitrogen, i.e., $(NH_4)_2SO_4$ and KNO_3, or an organic form, casein hydrolysate (CH). These media also contained 30 gL^{-1} sucrose and 6 gL^{-1} agar (pH 5.6).

For all experiments, the resulting mature somatic embryos were separated from the calli and placed on a germinating medium (OM medium) supplemented with 20 gL^{-1} sucrose and 1 mgL^{-1} zeatin (pH 5.6). Culturing was done in 500-mL glass vessels that were kept under a 16-h photoperiod at 26 ± 2°C. After four weeks, the plantlets were removed and washed gently under running tap water. They were then transferred to plastic pots filled with a 1:1 (v:v) pre-sterilized mix of peat moss and perlite. Plant growth continued in a plastic chamber under controlled environmental conditions.

Unless otherwise stated, the growth regulators were added before the plates were autoclaved at 121°C for 20 min; the filter-sterilized (0.22-µm pore size), thermolabile substances were added to the cooled (45 to 50°C) autoclaved media. The cultures were incubated in a growth chamber at 26 ± 2°C, either in continuous darkness or under 16-h photoperiod (80 to 100 µmol m^2 s^{-1} supplied by white fluorescent tubes; OSRAM L40W). All growth regulators and vitamins were obtained from Sigma Chemical Company, USA; other chemicals were of analytical grade.

Histological Observations

The live cultures were continuously observed throughout their development. In addition, some calli morphogenetic masses were examined by light-microscopy after being fixed in FAA (formalin-acetic acid). These were dehydrated in an ethanol series and embedded in paraffin. Afterwards, 6 to 10-µm sections were made with a gyratory microtome and the paraffin was removed in a xylol series. The cut sections were stained first with hematoxylin, then with saffranin and aniline blue, before being mounted in Canada balsam for viewing.

Experimental Design and Statistical Analysis

The treatments for all experiences were repeated three times. Each replicate comprised 10 explants per Petri dish. The significance of treatment effects was determined by analysis of variance (ANOVA), employing a completely randomized design. Variations among treatment means were analyzed by Duncans multiple range test, at $p < 0.05$.

RESULTS

After four weeks of culturing in the dark on an OMc medium (5 mgL^{-1} IBA and 0.5 mgL^{-1} 2i-P), >80% of our cotyledonary explants had produced calli in all three cultivars (Fig. 1). These calli were white-translucent and friable and proliferated from the cut ends of the segments. Nodules and morphogenetic-like structures were visible at the surfaces. By the fourth week of culture, rhizogenesis was manifested with one to eight roots emerging from each callus. However, no somatic embryogenesis occurred during period. After spending four weeks auxin-containing media, cultures were transferred for development in the dark on OMc media that contained various levels of IBA and 2i-P (Fig. 2).

On the fresh hormone-free medium, callus growth was slow in the dark. No rhizogenesis was observed but

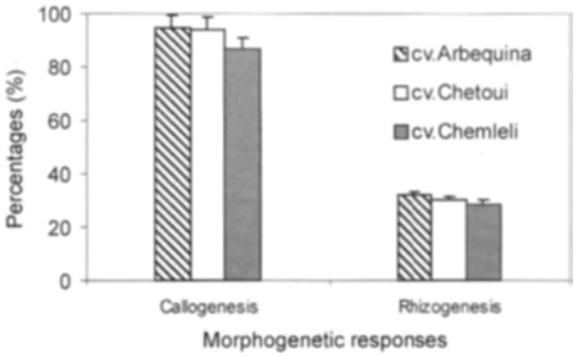

Figure 1. Callogenesis and rhizogenesis of *O. europaea* (L.) 'Chetoui', 'Chemleli', and 'Arbequina' at 28°C in the dark on OMc media supplemented with 5 mgL^{-1} IBA and 0.5 mgL^{-1} 2i-P.

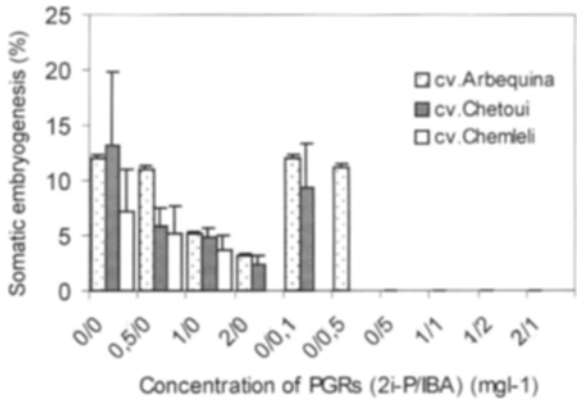

Figure 2. Effect of different hormonal types and concentrations during the realization phase of somatic embryogenesis in 60-d-old olive cotyledon cultures of *O. europaea* (L.) 'Chetoui', 'Chemleli', and 'Arbequina', at 26°C in the dark.

proembryo-like structures and soft white nodules developed at the calli surfaces. After four weeks of subculturing, histological sectioning and microscopy revealed that 1 to 12 somatic embryos per callus were being expressed at different stages (Figs. 2 and 3), for an overall frequency of 4 to 13%.

Media that contained cytokinin (2i-P) as the sole PGR promoted somatic embryogenesis only at low concentrations i.e., <0.5 mgL^{-1}; at higher amounts, embryogenesis was inhibited, and the calli turned

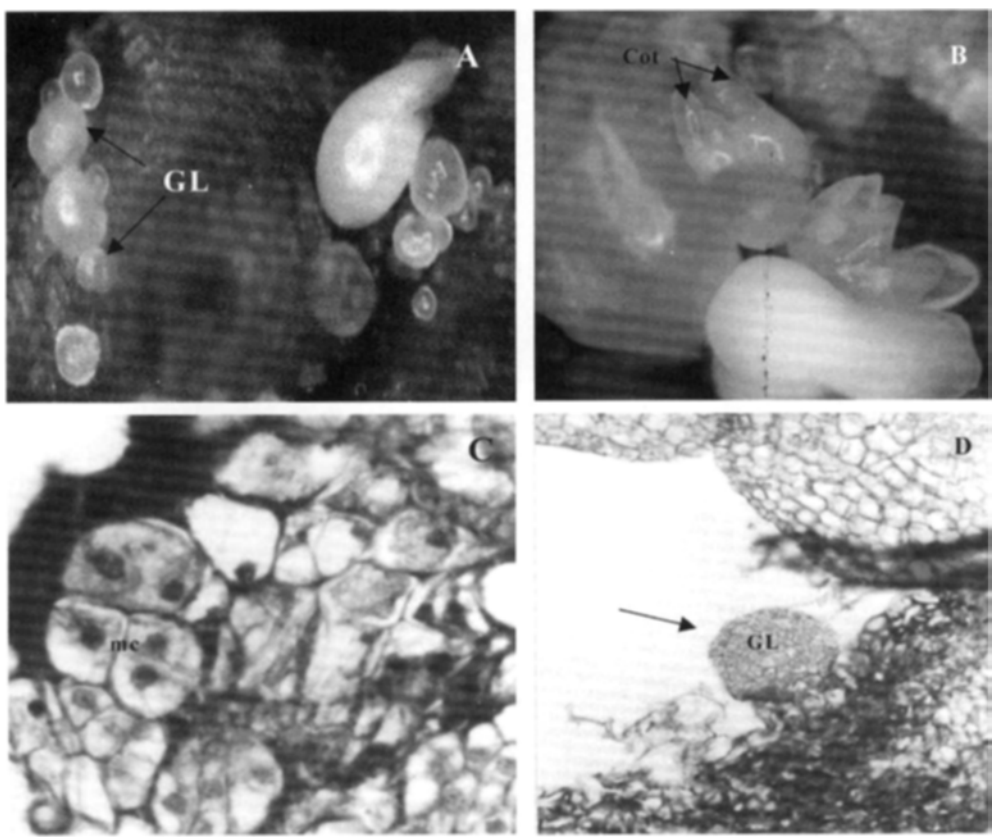

Figure 3. Embryogenic callus tissues. **A and B**, Somatic embryos at different maturation stages (x 50). **C**, Meristematic cell division in callus tissue (x 400). **D**, Globular somatic embryos (x100). GL, Globular somatic embryos; Cot, Cotyledons; mc, meristematic cells.

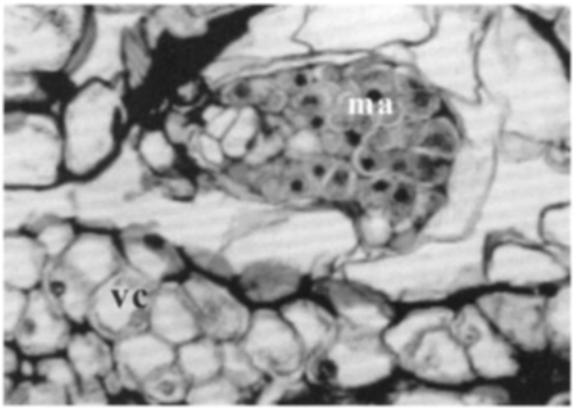

Figure 4. Cytological characteristics of embryogenic areas in callus cultures of cotyledonary explants. Cells in the meristematic regions were highly cytoplasmic with large nuclei and small vacuoles. ma, meristematic area; vc, vacuolated cells (x200).

dark brown and died within two weeks. When IBA was suplemented alone somatic embryos developed at lower concentrations (<0.5 mgL^{-1}). Higher auxin levels hampered embryogenesis and callus so that the tissue lost its competency and usually died. Treatments with high auxin and cytokinin concentrations also failed to give rise to advanced stages of embryogenesis; the calli hardened and developed a dark-brown color. Under these conditions, only parenchymatous cell masses proliferated (Fig. 4). Moreover, when the calli where grown under continuous light, they turned green and hard, and embryogenesis was less effective compared with their performance in the dark (data not shown).

Germination of somatic embryos was observed on both hormone-free OM media and that which was supplemented with 1 mgL^{-1} zeatin under a 16-h photoperiod. Plantlets derived from these germinated embryos were similar to those obtained from axillary buds, and growth was normal on the zeatin medium.

Effect of Induction Period

Four to five weeks was the optimal duration for exposure to 5 mgL^{-1} IBA and 0.5 mgL^{-1} 2i-P (Table 1), based on criteria such as percentage of embryogenic calli produced and embryo growth. Embryonic induction began around the third week of culture, but the maximum percentage was obtained at week 4. The highest number of somatic embryos was regenerated when the explants were exposed to high levels of auxin for four weeks. However, keeping the calli on such a medium for a longer period did not increase of embryo number but rather, this action had an inhibitory effect on

Table 1. Effect of time spent in media on the somatic embryogenesis of olive cotyledon-callus cultures. Explants were cultured in the dark during 3, 4, 5, 5, or 6 weeks on an OMc medium supplemented with 5 mgL^{-1} IBA and 0,5 mgL^{-1} 2i-P. They were then transferred to a hormone-free OMc medium supplemented with 30 gL^{-1} sucrose. Data are for 45 d old-cultures.

Induction period (Weeks)	Embryogenic calli (%)	Mean no. of embryos per explant (± SE)[1]
3	23	1.2 ± 0.9 c
4	82	15.4 ± 5.9 a
5	66	15.1 ± 7.3 a
6	48	8.3 ± 4.8 b

[1]Means not followed by the same letter are significantly different at $p < 0.05$, using Duncan's multiple range test.

embryogenesis. Transferred the calli to a fresh, hormone-free medium promoted rhizogenesis, a phase that was followed by callus senescence and necrosis.

Effect of Carbon Sources

The responses of our explants to various carbon sources and range of concentrations differed significantly (Table 2). Glucose did not support embryogenesis efficiently; percentages of embryogenic calli were low, the mean number of somatic embryos also considerably less. The few calli that did develop were vitreous, and did not survive very long in culture. In contrast, the presence of sucrose in the media promoted the highest degree of embryo formation (21.5 ± 3.5). The optimum sucrose concentration for growth and maturation was 30 to 40 gL^{-1}. Those amounts also increased the efficiency of secondary somatic embryogenesis, with one or more somatic embryos being derived directly from the already growing embryos. However, elevated levels of sucrose tended to decrease embryo formation and maturation; both the embryogenic calli and embryos died after developing necrosis and turning brown.

Effect of Different Nitrogen Sources

The results of testing for various nitrogen sources on somatic embryogenesis are shown in Table 3. When the culture medium was not supplied with any source of nitrogen, no calli growth or development was observed (Table 3). Calli that were white at the beginning of the subculture period became brown and generally necrotic, with death occurring within 15 d. The use of only $(NH_4)_2SO_4$ (a reduced form of N) caused a decrease in callus growth, probably resulting from a change in

Table 2. Effect of carbon source and concentration on growth and maturation of somatic embryos. Cotyledon calli were cultured. on an OMc medium without growth regulators. Explants were evaluated for embryos formation after 45 d in the dark.

Carbon source (gL^{-1})	Embryogenic calli (%)	Mean no. of embryos per explant (± SE)[1]	Callus formation (%)	Secondary embryos (%)[2]
Sucrose				
20	72	10.2 ± 4.0 c	0	0
30	88	16.3 ± 4.2 b	2	5
40	91	21.4 ± 5.2 a	5	8
60	55	7.3 ± 4.0 d	12	0
80	52	3.6 ± 3.7 e	23	0
Glucose				
20	4	2.4 ± 2.7 a	0	0
30	11	2.4 ± 2.5 a	0	0
40	8	0.8 ± 1.0 b	0	0
60	2	0.6 ± 0.9 b	0	0
80	0	0.4 ± 0.7 b	0	0

[1]Means not followed by the same letter for each sugar treatment are significantly different at $p < 0.05$, using Duncan's multiple range test.
[2]Percentage of calli developing second somatic embryos.

Table 3. Effect of different nitrogen forms on growth and maturation of somatic embryos. Cotyledon calli culture. Calli were cultured on an OMc medium without growth regulators that was supplemented with 30 gL^{-1} sucrose and solidified by 6 gL^{-1} agar (pH adjusted to 5.6 before autoclaving). Explants were evaluated for embryo formation after 45 d in the dark.

Nitrogen form (mgL^{-1})	Embryogenic calli (%)	Mean no. of somatic embryos per explant (± SE) [1]	Germinated somatic embryos (%)	Secondary somatic embryos (%)[2]	Hyper-hydricity[3]	Callus Color
Control [4]	92.2	20.3 ± 2.7a	9.6	3.7	–	White
0	0.0	0.0 ± 0.0d	0.0	0.0	–	Brown
$(NH_4)_2SO_4$	3.3	0.0 ± 0.0d	0.0	0.0	+	Brown
CH	80.1	16.6 ± 4.5b	15.2	11.6	–	White
KNO_3	88.4	11.4 ± 3.6c	12.1	5.2	–	White

[1]Means not followed by the same letter are significantly different at $p < 0.05$, using Duncans multiple range test.
[2]Percentage of calli developing second somatic embryos
[3]+, Hyperhydric calli; –, non-hyperhydric calli
[4]Control contained: 950 mgL^{-1} KNO_3, 720 mgL^{-1} NH_4NO_3 and 1000 mgL^{-1} caseine hydrolysate.

pH that was induced by the application of ammonium. In contrast, the addition of casein hydrolysate (CH) promoted the growth and maturation of somatic embryos. This response was evident at different phases of the embryogenic process, predominantly during the torpedo and the cotyledonary stages. Finally, supplementing the medium with KNO_3 as the sole nitrogen form led to enhanced growth and somatic embryogenesis, with more efficient maturation and germination, compared with the other nitrogen treatments.

DISCUSSION

The response of cotyledonary explants from *O. europaea* to attempts at somatic embryogenesis was affected by the concentrations of PGRs in the culture media. Callus production was similar for all cultivars tested as explants in the dark. As previously reported by Orinos and Mitrakos (1991) and Mitrakos et al. (1992), we also noted that rhizogenesis was induced by high auxin levels (5 mgL^{-1} IBA). This growth hormone the most important factor in regulating the induction and development of embryogenesis, having various effects at different phases. Nevertheless, a very high auxin concentration was usually inhibitory to advanced development of the embryos. Somatic embryogenesis was also expressed on hormone-free medium or at low 2i-P and IBA levels (less than 0.5 mgL^{-1}). However, higher concentrations of cytokinin caused necrosis of the calli, a response also described in other species (Gomez and Segura, 1996). Our cultivars also differed in their embryogenic capacity, with 'Arbequina' exhibiting seemingly higher competency under our experimental conditions.

Embryogenesis was successful in the dark, but ham-

pered in the light. Rugini (1988) and Rugini and Caricato (1995) have also reported that embryos, which appear after exposure to light, are first induced in the dark. Cyclic embryogenesis was observed when the embryogenic calli were subcultured either on hormone-free OMc medium in the dark or on OMc medium that was supplemented with 0.1 mgL^{-1} IBA. In the later instance, a few calli were observed on some embryos, as were some that were abnormally shaped, fused, or clustered. Embryos with more than two cotyledons were also found in some cultures. Other studies have also shown that some PGRs (e.g., ABA) seem to promote the maturation and growth of normally shaped somatic embryos (Homes, 1980; Dunstan et al., 1988; Latkowska et al., 2001).

Carbon source can significantly affect many aspect of embryogenesis (Kochba et al., 1977), including the control of precocious germination, induction of competence for non-embryogenic cells, and promotion of secondary embryogenesis (Han and Park, 1999). In our experiments, efficiency and germination were higher among the calli subcultured with sucrose than on glucose-containing medium. Compton and Gray (1996) have also demonstrated that sucrose concentrations up to 120 gL^{-1} improve the growth of embryogenic cultures. In contrast, Bensaad et al. (1996) have reported that the efficiency of *Vitis vinifera* L. anther culture is higher with glucose, in contrast to the lack of embryo formation seen from media containing either sucrose or mannitol.

The effect of nitrogen on development during the embryogenic process may be related to variations in pH of the culture medium. In our study, ammonia reduced the pH, nitrate increased it, and CH had a buffering effect. When an oxidized form of nitrogen, KNO_3, was added, the calli underwent all the embryogenic stages and successful conversion to plantlets. Pritsa and Voyiatzis (1999) have reported that exogenously applied L-glutamine also enhances embryonic-like structures in olive cotyledon explants. In other species, CH and L-glutamine similarly promote somatic embryogenesis, especially when the assimilative mechanisms of nitrate are not operative (Kirby et al., 1987; Szczygiel and Kowalczyk, 2001).

Histological examination of the cotyledon-callus transactions showed that the vacuolized cells transformed into actively dividing meristematic cells that were rich in cytoplasm and (Fig. 4). Although mitosis was found throughout the explant, activity was concentrated in the epidermis and sub-epidermal region, which were in close contact with or adjacent to the nutrient media. The use of hematoxylin as the dyeing

Figure 5. Section of somatic embryos showing different forms, closed vascular system cotyledons at shoot end, and the root meristems at the opposite end. rm, root meristem; sm, shoot meristem; cot, cotyledon (x100).

agent gave us the opportunity to localize morphological variations during the induction phase. Proembryo-like structures were visible at the bicellular, four-cells, or globular stages. Furthermore, the mature embryos had root-like structures and closed vascular systems, with cotyledons at shoot-end and root meristems at the opposite end (Fig. 5). We also observed epidermal cells dividing anticlinally, an indication of the first step in embryogenesis (Rugini and Caricato, 1995).

Densely stained meristematic cells in the epidermal layer of the primary somatic embryos evolved into globular embryos. This activity supports the hypothesis of a multicellular origin for secondary embryos, and agrees with observations on mature tissues by Lambardi et al. (1999). Keller and Espagnac (1989) have also reported that the somatic embryos of *Quercus ilex* (L.) increase their structural regulation when kept in the dark, although germination is not possible under those conditions.

Our somatic embryos germinated easily on the OMc medium containing zeatin (Fig. 6). Moreover, the use of hormone-free OMc medium (with 40 gL^{-1} sucrose

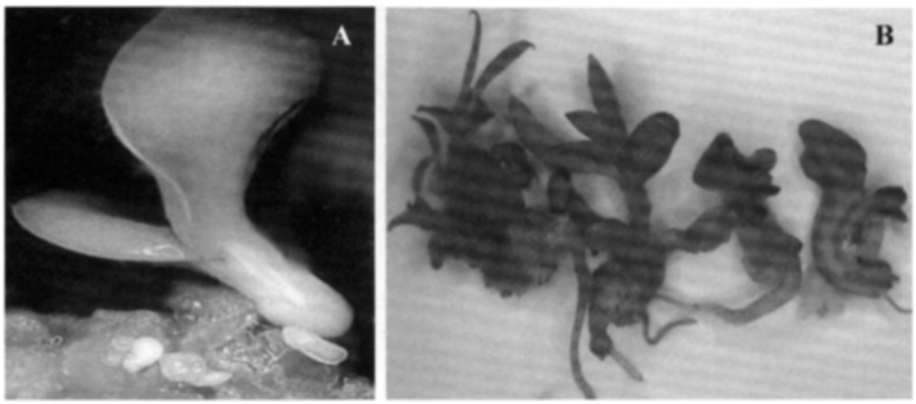

Figure 6. (A): Germinating somatic embryos on OM medium containing 0.5 mgL^{-1} zeatin (x25), (B): Olive plantlets derived from germinating somatic embryos (x10).

and 6 gL^{-1} Difco agar) enabled us to maintain continuous proliferation of cyclic somatic embryogenesis in the dark, without loss of morphogenetic capacity, for more than three years.

In conclusion, somatic embryogenesis has several advantages over organogenesis as a practical means of propagation because it is normally difficult to root olive cultivars that have been micropropagated. However, several problems must be overcome before somatic embryogenesis is applied routinely in an improvement program because most trees have marked phase changes that decrease their potential for somatic embryogenesis or micropropagation (Zimmerman, 1988; Aderkas and Bonga, 2000). The initiation of embryogenic cultures from non-embryogenic tissues has not yet accomplished for many economically important trees (Han and Park, 1999). In those species, including the olive, most of the somatic embryos have been derived from immature and/or mature zygotic embryos or juvenile tissues, which are often genetically unproven (Rugini and Caricato, 1995; Lambardi et al., 1999).

Based on our results with *O. europaea*, we have been able to show that cultures with a high potential for somatic embryogenesis can be obtained from the calli that are initiated from mature zygotic embryos (cotyledonary segments). The Plantlets that are then derived grow normally when acclimatized in a glasshouse, and are morphologically similar to plantlets that arise from axillary buds (data not shown). This is in contrast to the variability that can occur among olive plants regenerated from cotyledons via adventitious organogenesis. Moreover, success with stored seeds significantly extends the period of time during which plant material is available throughout the year, thereby facilitating future studies of somatic embryogenesis from juvenile tissues. Further modifications to the mineral, organic, and PGR components of the growth medium should also, increase efficiency. Even though we have now demonstrated an ability to establish embryogenic cultures from olive cultivars, additional researche is required to increase the number of regenerated plantlets derived from the germination of embryos. By doing so, somatic embryogenesis can become an efficient and reliable tool in olive improvement program, by providing a reliable means for propagation, germplasm storage, and genetic transformation.

ACKNOWLEDGEMENT

We are grateful for the helpful advice from the technical staff of the Laboratory of Plant Biotechnology in the National Institute of Agronomical Research of Tunisia and the National Institute of Olive of Tunisia. Thanks to Dr. Nahdi H., Laboratory of Viticulture, INRAT.

Received July 9, 2003; accepted August 2, 2003.

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