Embryogenesis expression from somatic explants of olive (*Olea europaea* L.) cv. Picual

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Abstract

Somatic embryogenesis has been widely studied in olive tree (*Olea europaea* L.); however, there is no common protocol for regeneration within olive tree genotypes. In the present study, we evaluated the effect of different media, previously used to induce somatic embryos in olive tree, on the embryogenic capacity of leaf and petiole explants of Picual olive cultivar. Leaf and petiole explants taken from in vitro grown shoots of this cultivar, were cultured for 4,8 and 12 weeks on 2 different induction media. The formed calli were morphologically different; however, histological observations showed embryogenic cells in all of them. After induction in OM supplemented with 31.78 µM thidiazuron (TDZ) and 0.53 µM 1-naphthalene acetic acid (NAA) culture for 4 weeks on plant growth regulator-free MS, globular somatic embryos were observed on all the explants. The highest percentage of somatic embryos-formation (18%) was observed on petioles after 12 weeks induction. OM supplemented with 0.44µM 6-benzylaminopurine (BAP), 0.24µM indole-3-butyric acid (IBA) and 0.49µM 6-(dimethylallylamino) purine (2iP) induced the formation of multi-cotyledonary embryoids.

Key words: Embryogenic callus, histological observations, *Olea europaea* L., somatic embryogenesis.

Introduction

Olive tree (*Olea europaea* L.) is one of the major tree crops in the Mediterranean area, mainly for its fruit, oil and the trituration by-products. The socio-economic role of this species allows it to occupy a privileged place in research works. For genetic improvement-strategies in olive tree, *in vitro* techniques are not commonly used, even though they usually involve the use of a simple plant material. Embryogenic cells are, therefore, a material of choice for such manipulations. Nevertheless, the development of a reproducible multiplication and regeneration protocol through somatic embryogenesis is primordial.

Like many other fruit species, somatic embryogenesis has been studied in olive tree. However, studies have concerned a very limited number of cultivars and have mostly used zygotic embryos as explants. Thus, somatic embryos have been observed on immature (Rugini, 1988; Leva *et al.,* 1995) and mature zygotic embryos (Orinos & Mitrakos, 1991), cotyledon segments (Pritsa & Voyatzis, 1999) and on radicles taken from zygotic embryos (Rugini & Tarini, 1986; Mitrakos *et al.*,1992; Rugini *et al.* ,1995; Shibli *et al.*, 2001; Mazri *et al.,* 2012).Works using somatic

tissues such as leaves or petioles are rare (Rugini & Caricato, 1995; Capelo *et al.*, 2010; Mazri et *al.*, 2013). The first step of any somatic embryogenesis process, the induction phase, takes place in response to a stress (hormonal, thermal and/or carbon stress) and often from a juvenile material. Thereafter, when somatic embryogenesis is induced, the regeneration protocol is generally reproducible (Bhaskaran & Smith, 1992.; Germanà, 2003; Alemanno *et al.*, 1996).

In olive tree, the somatic embryogenesis process is very complex. Previous studies have shown that the success of this morphogenesis varies depending on genotypes and explants (Capelo *et al.*, 2010; Mazri *et al.*, 2011; Mazri *et al.*, 2012; Mazri *et al.*, 2013; Rugini, 1995; Shibli *et al.*, 2001; Trabelsi *et al.*, 2003).Within each genotype/explant, the formation of somatic embryos is highly affected by the culture conditions: plant growth regulators (PGR), media texture, carbon sources and basal media (Chaari Rkhiss *et al.*, 2003; Cozza *et al.*, 1997; Grigiriadou *et al.*, 2002; Rugini & Fedeli,1990; Rugini, 1984; Santos *et al.*, 2003). Moreover, the determination of an "induction window" (i.e. the appropriate time to transfer calli from the induction medium to expression) is very difficult. In fact, the formation of embryogenic cells seems to be related to the culture period on the induction medium: somatic cells may be induced after a short induction period or on the contrary after a long induction period (Capelo *et al.*, 2010; Mazri *et al.*, 2013). Therefore, the induction-"window" determination, corresponding to the induced-cells transfer on an expression medium, is also a crucial step to success the somatic embryogenesis.

The aim of this investigation was to combine some factors that allowed the somatic-embryogenesis expression in some olive cultivars in order to induce this morphogenesis on leaf and petiole explants of the Picual one.

Materials and methods

Plant material

One-year-old Picual cultivar branches were disinfected with1 g/L mercuric chloride for 2 min, and cut into bi-nodal explants (1cm long). The cuttings were grown on PGR-free Olive Medium (OM; Rugini, 1984) for 2 weeks, and transferred to OM supplemented with 2mg/L zeatin (Figure1A).The developed shoots (Figure1B) were maintained *in vitro* for 1 year with a monthly transfer to the same fresh medium. Afterwards, proximal, intermediate and distal leaf segments as well as petioles (Figure1C) were taken from shoots and used to induce somatic embryogenesis.

Figure 1. (A) Development of bi-nodal explant after 1 month of culture, **(B)** One year offshoots development, **(C)** Material explants source: distal, intermediate and proximal leaf segments and petiole.

Culture conditions

Two basal media were used in the present study: OM and Murashige and Skoog medium (MS; Murashige & Skoog, 1962). To induce

somatic embryogenesis, OM was supplemented with 31.78µM thidiazuron (TDZ) and 0.53µM 1-naphthalene acetic acid (NAA) (OM1) as suggested by Mazri *et al.* (2013), or with 0.44µM 6 benzylaminopurine (BAP), 0.24µM indole-3-butyric acid (IBA) and 0.49µM 6-(dimethylallylamino) purine (2iP)(OM2) (Capelo *et al.*, 2010). After 4, 8 or 12 weeks of culture on the induction medium, calli were transferred to PGR-free OM or MS media, in which they have been cultured for at least 4 weeks (Figure 2). All media were supplemented with 30g/L sucrose and 6g/L bactoagar (Biokar Diagnostics, Cedex, France). The pH of all media was adjusted to 5.8 before autoclaving. Cultures were kept in the dark at 25° C and transferred to a fresh medium every 4 weeks.

Histological analysis

Histological observations were carried out on all the formed calli. After each induction period (4, 8, and 12 weeks), calli were fixed for 48 h in a 0.2 M phosphate buffer (pH = 7.2) containing 2% paraformaldehyde, 1% Glutaradehyde and 1% caffeine. Dehydration was performed in steps before inclusion in paraffin or resin; then, thin sections (6µm) were stained with periodic acid Schiff (PAS) and naphthol blue black (Martoja & Martoja, 1967). The observations were performed under natural light.

Statistical analysis

In this study, a total of 2400 explants were used. For each treatment, 4 explants were placed in a Petri dish (one replicate), and 25 replicates were used (Figure 3).Statistical analyzes were performed by ANOVA, and the completely randomized design (CRD) was used at 5% significance level. Percentage data (P) were transformed by [arc sin √P] formula, and means with significant difference were separated by SNK (Student, Newman and Keul) test using SPSS software v.16.0 (IBM, Chicago, IL, USA). The graphics were made by Excel (Microsoft office 2007).

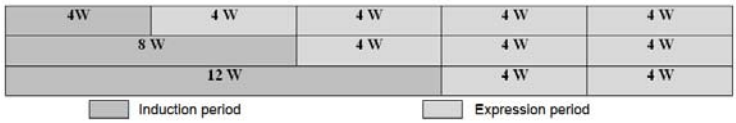

Figure 2. Induction (OM1 or OM2) and expression (MS or OM) periods; W: week.

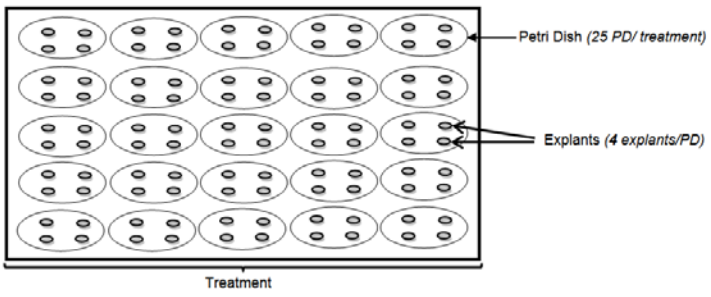

Figure 3. Treatment scheme; PD: Petri Dish.

Results and discussion

Callus induction

Petioles and leaf segments cultured on both OM1 andOM2 media started to develop calli within the first week of culture. The calli formation rate reached the maximum after 4 weeks of culture. Thereafter, only callus morphology was changing. After each induction period (i.e. 4, 8 and 12 weeks) on OM1 medium, petioles showed higher calli formation rate than

leaf segments. For instance, petioles showed 80, 77 and 82 % after 4, 8 and 12 weeks, respectively; while leaf segments showed 7, 11 and 14% after 4, 8 and 12 weeks in the same order (Figure 4). On OM2 medium, the calli formation rates were lower; the highest ones were observed again on petiole (Figure 4). In a previous study, almost similar calli formation rate (74%) was observed on leaf explants of Picual and Dahbia cultivars, cultured in the presence of the same PGR combination as OM1 medium (Mazri, 2013). However, in the wild olive *"Sylvestris"*, the calli formation rate did not exceed 57% (Capelo *et al.*, 2010). Moreover, with this same genotype, the PGR combination used in OM2 medium has induced a very low calli rate (23%), and only on petioles. Statistical analysis showed that calli formation rate was significantly influenced by the induction medium ($P< 0.05$). Whatever the induction medium used, all the studied explants started to swell slightly at the excision zones. Calli often appeared at these areas, and then grew to cover the entire explant, with a varying intensity depending on the explants type, the induction period and the induction medium. Keeping calli in the induction medium leads generally to more proliferation.

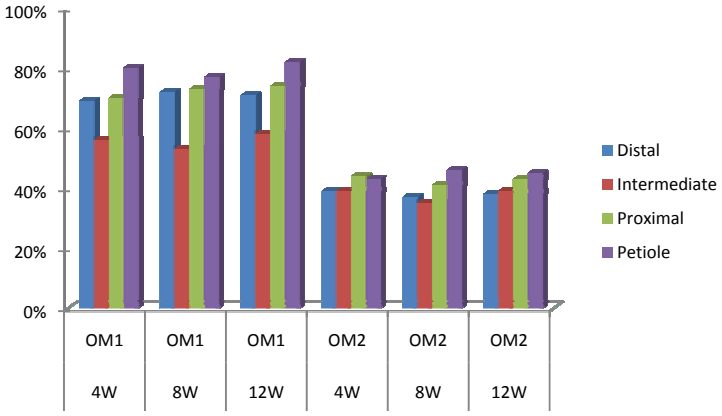

Figure 4. Callogenesis rates after the three induction periods for all explant types on OM1 and OM2; W: week.

Embryogenic nature of induced callus

Figure 5A1 shows that culture on OM1 medium containing TDZ and NAA during 4 weeks, followed by 4weeks on PGR-free MS medium, allowed the emergence of globular white and translucent structures on the entire callus periphery; calli rates with these structures were 9% in petioles, and 4%, 5% and 7% for proximal, intermediate and distal leaf segments, respectively (Table 1). Histological observations of these calli showed heart-shape clusters, not completely isolated from the original explants (Figure 5A2). However, in cultures on OM1 during 8 and 12 weeks, followed by 4 weeks on MS, it appears on the entire callus periphery a profusion of white and smooth globular structures (Figures 5B1-C1). These are heart-shaped somatic embryos with caulinary and root poles, and can be easily separated from the original explants (Figures 5B2 and 5C2). The epidermis of these embryos is highly embryogenic (Figure 5D) and could lead to a secondary embryogenesis. The callus transfer to the OM medium did not induce any embryogenic structure, even though histological observations revealed that all formed calli during the induction phase consisted of embryogenic cells: thick-walled, highly dense cytoplasm and highly developed nucleus. These characteristics disappeared and a parenchymal tissue takes place when these

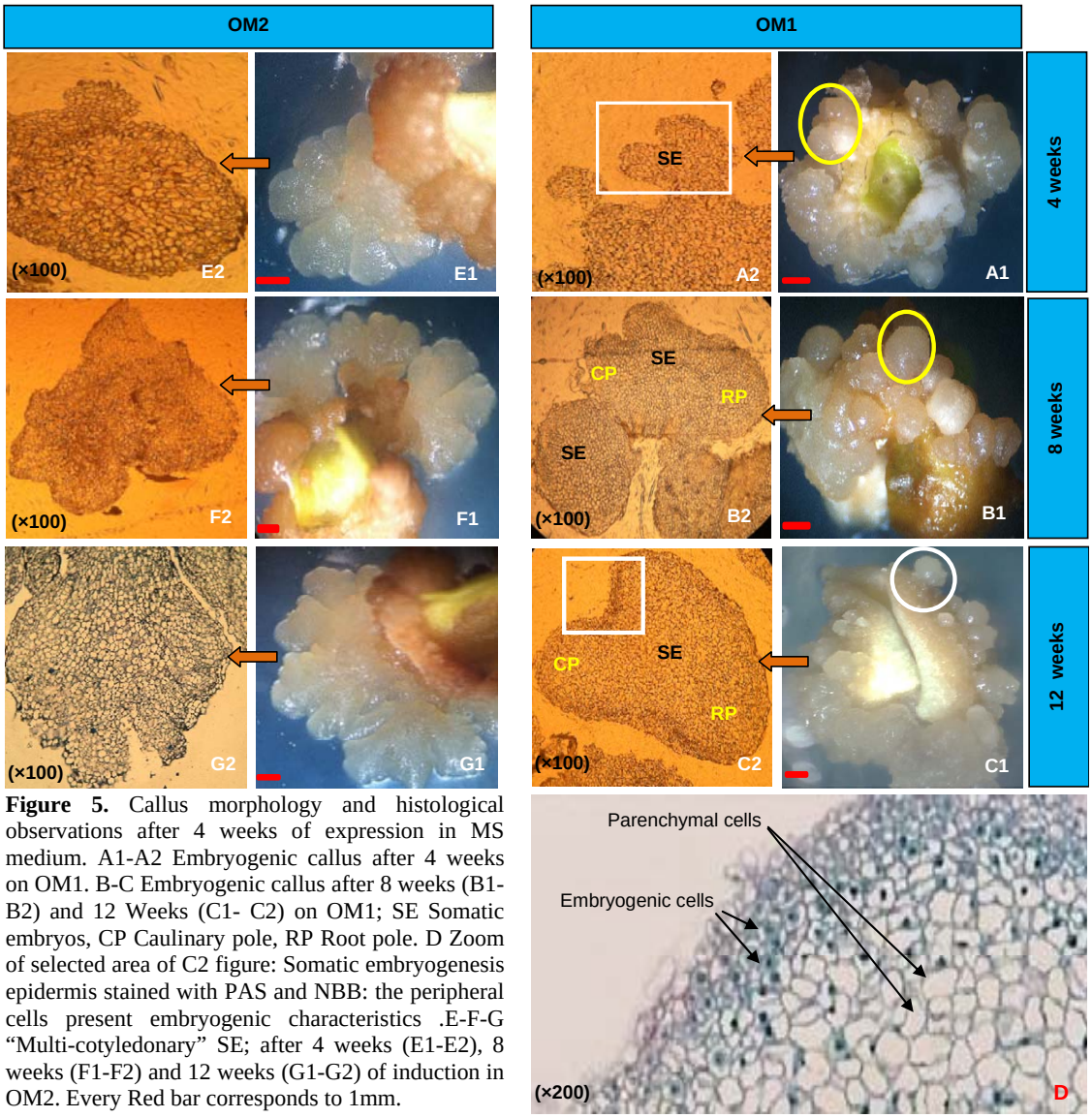

Figure 5. Callus morphology and histological observations after 4 weeks of expression in MS medium. A1-A2 Embryogenic callus after 4 weeks on OM1. B-C Embryogenic callus after 8 weeks (B1-B2) and 12 Weeks (C1- C2) on OM1; SE Somatic embryos, CP Caulinary pole, RP Root pole. D Zoom of selected area of C2 figure: Somatic embryogenesis epidermis stained with PAS and NBB: the peripheral cells present embryogenic characteristics .E-F-G "Multi-cotyledonary" SE; after 4 weeks (E1-E2), 8 weeks (F1-F2) and 12 weeks (G1-G2) of induction in OM2. Every Red bar corresponds to 1mm.

Table 1. Embryogenic calli (OM1) and «Multi-cotyledonary» calli (OM2) rates (±SD) for all induction periods after 4 weeks of expression on MS medium; w: week.

Media	**Induction periods**	**Explants**			
		Proximal	Intermediate	Distal	Petiole
OM1	4 w	4% ± 9,3	5% ± 10,2	7% ± 13,5	9% ± 14,2
	8 w	10% ± 12,5	11% ± 12,6	9% ± 12,2	13% ± 14,6
	12 w	13% ± 16 ,3	14% ± 14,5	13% ± 12,7	18% ± 18,4
OM2	4 w	15% ± 22,8	10% ± 14,4	9% ± 12,2	18% ± 23,4
	8 w	16% ± 14,2	10% ± 12,5	7% ± 11,4	21% ± 28,5
	12 w	15% ± 16,1	8% ± 11,9	7% ± 11,4	20% ± 23,9

embryogenic calli were transferred to the OM medium for expression. Table 1 shows that 12 weeks-induction gave the highest embryogenic calli rate (18% on petioles and 14% on leaf segments), followed by 8 weeks one (13% on petioles and 11% on leaf segments).

In comparison with other works on somatic explants, a long induction time in OM1 medium has not allowed the induction of embryogenic callus regardless of the explant type used in the wild olive genotype (Capelo *et al.*, 2010). In the Moroccan cultivar "Dahbia", a short induction period on MS/2 medium supplemented with hormonal combination of OM1 (TDZ and NAA), followed by a transfer first to a PGR-free medium for 2 months and then to an expression medium supplemented with PGR, have allowed the induction of embryogenic structures on leaf explants (Mazri *et al.*, 2013).

The OM2 medium, having induced a low somatic embryos rate (4%) on petioles of wild genotype (Capelo *et al.*, 2010), has allowed in our experience the formation of white translucent and multi-expansion structures (Figures 5E1, F1 and G1). These structures were easily removed from the original explants through a very low mechanical pressure, and consisted of multi-cotyledonary embryoids (Figures 5E2, F2 and G2). The percentages of calli with multi-cotyledonary embryoids remained relatively stable for all the explants after the three induction periods, with a higher percentage in petioles (21%), compared to leaf segments one (Table 1).

OM1 (TDZ+NAA) and OM2 (2iP, BAP and IBA) media have allowed the expression of embryogenic cells into globular embryos (OM1) or embryoids structures (OM2). Expression of somatic embryogenesis was performed on an expression medium (MS) devoid of any plant growth regulator. These findings differ from results reported in Dahbia cultivar, highlighting that somatic embryos were observed on media supplemented with PGR (Mazri *et al.*, 2011; 2013). This confirms that supplement requirement for somatic embryos formation differs according to genotypes. Several studies support the hypothesis that the ratio of exogenous PGR and endogenous hormones, which concentration depends on the nature of the explant, is responsible of the somatic embryogenesis expression (Gaspar *et al.*, 2003). Thus, the effect attributed to the auxins and cytokinins that are added to the medium was related to their interaction with other endogenous hormones, leading consequently to new morphogenesis development (Gaspar *et al.*, 1996; Lakshmanan & Taji, 2000). This shows (i) the importance of plant growth regulators for the induction and/or expression of somatic embryogenesis, and (ii) the difference in embryogenesis response depending on the explants type.

In olive Picual-cultivar, somatic embryogenesis has been already observed on zygotic explants (Mazri *et al.*, 2012; Cerezo et *al.*, 2011), and genetic transformation attempts based on somatic embryogenesis have been published recently for this cultivar (Pérez-Barranco *et al.*, 2009; Torreblanca *et al.*, 2009). Nevertheless, the use of zygotic material could not ensure genetic conformity. To our knowledge, this is the first report about somatic embryogenesis induction in olive Picual-cultivar, from leaf and petiole explants.

Conclusion

In this work, we have succeeded to establish a simple somatic embryogenesis induction protocol from somatic adult explants (leaves and petioles) for the first time in olive Picual-cultivar. In addition, we have demonstrated that somatic embryos formation could be achieved in PGR-free expression medium. Our protocol must be performed for use in large-scale propagation and genetic transformation of this cultivar.

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