doi:10.14720/aas.2022.118.4.2581 Original research article / izvirni znanstveni članek

Somatic embryogenesis and plant regeneration from radicles of olive (*Olea europaea* 'Chemlal') zygotic embryos

Khayreddine TITOUH [1, 2, 3], Khadidja HADJ-MOUSSA [1, 3], Nazim BOUFIS [4], Lakhdar KHELIFI [3]

Received February 28, 2022; accepted November 14, 2022.
Delo je prispelo 28. februarja 2022, sprejeto 14. novembra 2022

Somatic embryogenesis and plant regeneration from radicles of olive (*Olea europaea* 'Chemlal') zygotic embryos

Abstract: Olive improvement by biotechnological tools such as genetic transformation requires an efficient *in vitro* regeneration system. Somatic embryogenesis seems the most suitable process. Our work describes for the first time the regeneration of whole plants in the main olive cultivar in Algeria 'Chemlal' via somatic embryogenesis induced from radicles of mature zygotic embryos. The obtained results showed that the establishment of a competent embryogenic culture is highly influenced by the chemical composition of the calli induction and maintenance media as well as addition of growth regulators. More than 10 and 13 % of nodular calli were obtained after callogenesis respectively on MS and OMc solid media containing IBA and zeatin followed by transfer to the same media without zeatin and a reduced concentration of auxin, while embryogenesis rates of 3.3 and 6.7 % were obtained respectively with IAA on MS medium and NAA on both tested media. However, no embryogenesis was observed with 2, 4-D or control which induced less callogenesis. Subsequently, an ECO medium with IBA, zeatin and BA particularly in liquid culture, allows better calli proliferation and embryogenic expression compared to OM and MS media. Finally, matured somatic embryos germinate quickly on a solid OM basal medium and generate normal well-developed plantlets easily acclimatized to natural conditions.

Key words: embryogenic callus; growth regulators; olive; proliferation rate; somatic embryo

Somatska embriogeneza in vzgoja rastlin iz radikule zigotskih embrijev oljke (*Olea europaea* 'Chemlal')

Izvleček: Izboljšanje oljke z biotehnološkimi orodji kot jer genetska transformacija zahteva učinkovit *in vitro* sistem vzgoje rastlin. V tem pogledu se zdi somatska embriogeneza najprimernejši način vzgoje. Raziskava opisuje prvič vzgojo rastlin najvažnejše sorte oljke Chemlal v Alžiriji s somatsko embriogenezo iz radikule zrelih zigotskih embrijev. Rezultati so pokazali, da je vzpostavitev primerne embriogene kulture močno odvisna od kemične sestave gojišča, v katerem poteka indukcija kalusa in gojišča za njegovo vzdrževanje kot tudi od dodatka rastnih regulatorjev. Več kot 10 in 13 % nodularnih kalusov je bilo pridobljenih na trdih MS in OMc gojiščih, ki sta vsebovali IBA in zeatin, čemur je sledil prenos v isto gojišče brez zeatina in zmanjšano koncentracijo auksina, 3,3 in 6,7 % embriogeneze pa je bilo dosežene z IAA na MS gojišču in dodatkom NAA na obeh preiskušenih gojiščih. Nasprotno pa ni bilo embriogeneze pri dodatku 2,4-D ali pri kontroli, kjer je bila indukcija kalogeneze slabša. Naknadno je ECO gojišče z IBA, zeatinom in BA, še posebej v tekoči kulturi, omogočilo boljšo proliferacijo in pojav embriogeneze v primerjavi z OM in MS gojiščem. Na koncu so zreli somatski embriji hitro vzkalili na trdnem OM osnovnem gojišču in dali normalne, dobro razvite rastlinice, ki so se zlahka prilagodile naravnim razmeram.

Ključne besede: embriogeni kalus; rastni regulatorji; oljka; hitrost proliferacije; somatski embrio

1 National Institute of Agronomic Research of Algeria (INRAA), Research Center of Oued Ghir, Béjaïa, Algeria
2 Corresponding author, e-mail: khayreddine.titouh@inraa.dz
3 National Higher School of Agronomy (ENSA), Laboratory of Genetic Resources and Biotechnologies (LRG-B), Algiers, Algeria
4 National Institute of Agronomic Research of Algeria (INRAA), Research Center of Baraki, Algiers, Algeria

1 INTRODUCTION

The olive tree (*Olea europaea* L.) is one of the most important crops in the Mediterranean region. In Algeria, several varieties of olive tree exist, of which the most cultivated are 'Chemlal' for oil extraction and 'Sigoise' as a table olive (Haddad et al., 2020) although other more interesting local cultivars also exist (Boukhari et al., 2020). Recently, the emergence of verticillium wilt caused by the fungus *Verticillium dahliae* Kleb. in newly established olive orchards has raised concerns about the development of the culture worldwide (Montes-Osuna and Mercado-Blanco, 2020) including in Algeria (Boutkhil et al., 2016). This soil-borne pathogen penetrates the plant via the roots and causes the necrosis of the organs as consequence of the obstruction of the vessels following the development of the fungal mycelium (Montes-Osuna and Mercado-Blanco, 2020). Indeed, the use of resistant genotypes remains the best solution to control this disease (Boutkhil et al., 2016; Narváez et al., 2019). However, the classical methods of breeding and multiplication achieved limited results and became unable to produce alone significant gains due to the long juvenile period of the species (10 to 15 years) specially in the current context of climate change (Rugini et al., 2020). Therefore, the use of biotechnological methods such as genetic transformation could be of great benefit for varietal creation in olive tree. However, the application of these techniques requires an effective *in vitro* regeneration system mainly for recalcitrant genotypes. Somatic embryogenesis seems to be the most appropriate and powerful *in vitro* method in several woody species including olive tree (Sánchez-Romero, 2019 and 2021).

Somatic embryogenesis has been induced from different explants of several olive cultivars. However, juvenile tissue is generally more successful for recalcitrant species (Von Arnold, 2008). Thus, the best results of olive embryogenic calli formation and regeneration were often obtained with juvenile tissues particularly radicles of mature zygotic embryos (Pires et al., 2020) because they include undifferentiated cells with a very high embryogenic activity (Sánchez-Romero 2019) while the petioles and the external parts of the leaf blade remain the most promising adult tissues for olive regeneration (Mazri et al., 2013) and even in wild olive (Narváez et al., 2019).

Nutritional and hormonal requirement for somatic embryogenesis depends directly on the type of explant and its degree of development and more on the genotype (Von Arnold, 2008). Induction and maintenance of olive embryogenic calli have often been achieved under dark conditions on solid medium based on the chemical composition of Olive Medium 'OM' (Rugini, 1984) or MS (Murashige and Skoog, 1962) supplemented with growth regulators. Later, the germination of somatic embryos can be successfully achieved under photoperiod on a simple medium even without hormones (Mazri et al., 2020) or a preliminary rigorous phase of maturation (Sánchez-Romero, 2019). Plants with a stable phenotype are regenerated despite some phenotypic variations observed after long periods of *in vitro* maintenance (Bradaï et al., 2016) and easily acclimatized to *ex vitro* conditions. However, the high genotype dependence limits the applicability and standardization of the pre-established protocols (Sánchez-Romero, 2021).

Thereby, our work describes for the first time an efficient protocol for *in vitro* regeneration of whole olive plants by somatic embryogenesis induced from radicles of zygotic embryos of the main cultivar in Algeria 'Chemlal', in order to apply some biotechnological techniques of genetic improvement particularly genetic transformation and induction of somaclonal variation. Thus, the effects of chemical composition of the culture medium and addition of growth regulators on the induction, proliferation of embryogenic calli as well as maturation and germination of somatic embryos were studied. In addition, acclimatization of the obtained plants to natural conditions was tested.

2 MATERIALS AND METHODS

2.1 PLANT MATERIAL AND DISINFECTION

Seeds extracted from the stones of olive cultivar 'Chemlal' mature fruits were sterilized with ethanol 70 % (v/v) for 1 min followed by soaking in sodium hypochlorite (NaClO 12°) at a concentration of 10 % (v/v) during 10 to 15 min. The disinfected seeds were rinsed three times with sterile distilled water for 5 min each time and kept immersed in water for 48 h at 25 ± 2 °C. After that, seeds were sterilized again as previous and conserved under sterile conditions, to extract the zygotic embryos from which radicles were carefully isolated to be used as explants for callus induction.

2.2 INDUCTION OF EMBRYOGENIC CALLI

Callogenesis was induced by culturing the previously isolated radicles during three weeks on two different solid media OMc based on Olive Medium 'OM' (Rugini, 1984) as adapted for callogenesis by Cañas and Benbadis (1988) or MS (Murashige and Skoog, 1962). Both media contain 0.5 mg l^{-1} of zeatin (Zea) and 5 mg l^{-1} of one of various auxins: indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), naphtyl-acetic acid (NAA)

or 2,4-dichloro-phenoxy-acetic acid (2,4-D). Then, explants were transferred during four weeks on the same media but without zeatin and with a reduced concentration of the auxin to 1/10th. The obtained calli were maintained for eight weeks on the cyclic embryogenesis olive medium (ECO) (Cerezo et al., 2011) with 0.1 mg l^{-1} of zeatin, 0.1 mg l^{-1} of benzylaminopurine (BA) and 0.05 mg l^{-1} of the previous auxin used during calli induction in addition to 0.55 g l^{-1} of glutamine and 1 g l^{-1} of casein hydrolyzate. All the used media during the establishment step of embryogenic calli contain 20 g l^{-1} of sucrose and 50 mg l^{-1} of myo-inositol. The pH was adjusted to 5.74 with NaOH or HCl (1 N) before adding 6 g l^{-1} of agar. Six radicles per Petri dish containing 25 ml of the culture medium and five dishes per treatment were incubated in the dark at 25 ± 2 °C. The changes of the radicle shape, appearance of callus as well as its texture and structure have been observed.

2.3 PROLIFERATION OF EMBRYOGENIC CULTURES

Three cell lines of calli induced on OMc with IBA and zeatin and considered as embryogenic for their good friability, nodular structure, low browning and well embryogenic expression were selected to estimate the embryogenic potential based on their proliferation rate (mass increase) in addition to embryo production and degree of necrosis on the maintenance medium. Thus, an embryogenic mass of 0.1 g was cultured on solid medium or in liquid culture with 100 rpm of stirring. Three chemical compositions (ECO, OM and MS) were tested. All the tested media contain 0.1 mg l^{-1} zeatin, 0.1 mg l^{-1} BA and 0.05 mg l^{-1} IBA in addition to 0.55 g l^{-1} glutamine and 1 g l^{-1} casein hydrolyzate. Furthermore, five combinations of growth regulators (including the control) composed of 0.1 mg l^{-1} of BA and 0.05 mg l^{-1} of IBA or NAA combined with 0.1 mg l^{-1} of zeatin or Thidiazuron (TDZ) were added to the ECO basal medium. At least three Petri dishes or three Erlenmeyer flasks of 100 ml containing 25 ml of medium were incubated in the dark at 25 ± 2 °C. After four weeks, the whole produced callus was recovered, weighed and the increase of the fresh mass in addition to the callus morphological traits such as texture, appearance of embryogenic structures, levels of friability and necrosis were determined, and a new inoculum of 0.1 g has been taken for the next subculture. The follow-up was carried out at least with three successive subcultures. Elsewhere, at the end of the liquid culture, the callus recovered by filtration was weighed and all the somatic embryos produced at different stages of development were determined.

2.4 MATURATION AND GERMINATION OF SOMATIC EMBRYOS

The pro-embryos used for maturation were produced by culturing a mass of 1 g of callus from each embryogenic line under total obscurity in 50 ml of liquid ECO medium containing growth regulators with stirring of 100 rpm. After four weeks, all the embryos at the globular, cordiform or torpedo stages exceeding 2 mm in size were transferred to a basal ECO solid medium supplemented with 1 g l^{-1} of activated charcoal for maturation during eight weeks while the cotyledonary embryos were directly germinated. More than three Petri dishes, containing at least nine pro-embryos each one, were incubated in total darkness at 25 ± 2 °C. Necrosis, cell proliferation and differentiation of mature embryos were recorded.

Germination of the matured and cotyledonary embryos has been achieved on OM basal medium with 20 g l^{-1} of sucrose, 100 mg l^{-1} of myo-inositol and solidified with 6 g l^{-1} of agar. More than sixteen embryos were cultivated individually in test tubes for eight weeks under a 16 h light photoperiod at a temperature of 25 ± 2 °C. Germination, root emergence, shoot length and number of developed leaves were observed. The obtained plantlets exhibiting an acceptable length with several well-developed leaves were acclimatized within laboratory for about two months on a humidified mixture of sand/potting soil/perlite at a rate of 2/2/1 (v/v/v). The reacting plants were transferred under natural conditions in a greenhouse on a substrate rich in organic matter and frequently irrigated before being permanently planted in the field.

2.5 STATISTICAL ANALYSIS

All statistical analyzes of the data (Analysis of variance and tests) were performed using the "XLSTAT" program version 2016.02.27444. In the case of a significant difference, the separation of the means was carried out by Fisher's LSD (Least Significant Difference) test. The percentages were analyzed by the chi-square test. The letters in the tables indicate homogeneous groups at significance level of 5 %.

3 RESULTS AND DISCUSSION

3.1 INDUCTION OF EMBRYOGENIC CALLI

Callogenesis from radicles of olive zygotic embryos was significantly influenced by the chemical composition of the induction medium as well as the used growth regu-

lators particularly the type of auxin. After three weeks, more calli were observed on OMc medium than on MS independently of the added growth regulators' balance except for 2,4-D with zeatin. The 'OMc' medium adapted for callogenesis by Cañas and Benbadis (1988) has been commonly used with olive juvenile tissues such as radicles and cotyledonary segments while the MS chemical composition was more beneficial for olive petioles and leaves segments (Trabelsi et al., 2011; Mazri et al., 2013) and even wild olive (Narváez et al., 2019). In fact, the best rate of callogenesis (100 %) was obtained on OMc with zeatin and IBA while less than 13 % of calli was obtained on controls and 26.7 % on OMc with 2,4-D (Table 1, Figure 1A and B). Mazri et al. (2012) noted that olive radicles on OMc medium containing IBA and 2iP (6-dimethylallylamino-purine) increase in size from the first week with formation of white calli on the injured part after three weeks in most of explants. However, Rugini (1995) indicated that callus can be formed even in the absence of auxins although obtaining embryogenic calli imperatively requires the combination of growth regulators inducing the conversion of somatic cells to a meristematic state (Maalej et al., 2002). Furthermore, Rugini (1988) indicated that 2, 4-D with BA induces less calli, inhibits cell differentiation and causes rapid and marked browning. These observations corroborate perfectly with our results where a weak amorphous callogenesis was observed on the controls media in addition to a rapid browning of the few calli obtained with 2, 4-D.

Transferring the explants to a medium without zeatin and with a reduced concentration of auxin induced their browning. However, 53.3 and 56.7 % of calli induced on OMc containing respectively IAA or IBA continue to proliferate better with production of nodules (Figure 1C) while 76.7 and 86.7 % of calli obtained respectively on OMc and MS containing 2,4-D showed a high browning with a low callogenesis (6 to 10 %) close to the control allowing 6.7 % of proliferation (Table 1). Mazri et al. (2012) noted that olive calli in the absence of cytokinin and reduced concentrations of auxins turn brown quickly due to a high accumulation of phenolic compounds reducing cell growth (Trabelsi et al., 2011). Nevertheless, calli produced in the presence of NAA were slightly browned (16 to 30 %) but with a significant loss of their proliferation capacity. Our results agree with the observation of Rugini (1988) indicating that NAA, despite the poor induced callogenesis, promotes embryogenic expression with less necrosis of cell masses.

Later, the passage of the cultures to the ECO solid medium including the appropriate combination of growth regulators indicated that obtaining and establishment of embryogenic calli were more determined by the auxin used during callogenesis than by the chemical composition of the induction medium. Pires et al. (2020) noted that a second transfer to an ECO medium rich in cytokinins allows a slow restart of cell proliferation with a significant embryogenic expression by formation of an easily separated nodules (Cerezo et al., 2011) considered as the initial marker of somatic embryogenesis (Mazri et al., 2013). However, low embryogenesis rates were often

Table 1: Effect of the chemical composition (OMc or MS) of the induction medium and growth regulators combination (different auxins + zeatin) on callogenesis, browning and somatic embryogenesis rates from radicles of olive zygotic embryos, 'Chemlal'*

		Callogenesis (%)		Browning (%)		Embryogenesis (%)
Induction medium	Auxins	3 weeks (+ Growth regulators)	4 weeks (- zeatin)	4 weeks (- zeatin)	+ 8 weeks (ECO)	8 weeks (ECO)
OMc	Control	13.3 d	6.7 d'	10.0 ef"	23.3 **de**	0.0 **c'**
	IAA	83.3 a	53.3 a'	73.3 ab"	90.0 **ab**	0.0 **c'**
	IBA	100.0 a	56.7 a'	70.0 ab"	83.3 **ab**	13.3 **a'**
	NAA	50.0 b	30.0 b'	30.0 de"	60.0 **c**	6.7 **abc'**
	2,4-D	26.7 cd	6.7 d'	86.7 a"	100.0 **a**	0.0 **c'**
MS	Control	10.0 d	6.7 d'	6.7 f"	20.0 **e**	0.0 **c'**
	IAA	60.0 b	20.0 bcd'	56.7 bc"	73.3 **bc**	3.3 **bc'**
	IBA	93.3 a	26.7 bc'	43.3 cd"	63.3 **c**	10.0 **ab'**
	NAA	46.7 bc	13.3 cd'	16.7 ef"	40.0 **d**	6.7 **abc'**
	2,4-D	43.3 bc	10.0 d'	76.7 ab"	100.0 **a**	0.0 **c'**

*Results are presented as the percentage to the total introduced explants. The different small letters of the same format within columns indicate the homogeneous groups of a significant difference at level of 5 %

obtained and vary between 7 and 13 % in the cultivars 'Picholine Marocaine', 'Dahbia' and 'Arbequina' (Mazri et al., 2012) while Cerezo et al. (2011) and Pires et al. (2020) reported 25 and 17 % of embryogenesis respectively in 'Picual' and 'Galega Vulgar'. Our results agree these observations since IBA combined with zeatin allowed the best rates of embryogenesis with 13.3 and 10 % respectively on OMc and MS (Figure 1D) while nearly 7 % of the calli obtained in the presence of NAA on both media and 3.3 % with IAA on MS were embryogenic (Table 1). In addition, the obtained embryogenic calli present a nodular and friable texture with white-yellowish globules of different sizes easy to separate allowing good proliferation during subculturing, whereas, some calli lines with strongly joined nodules often show marked browning and very slow proliferation capacity as well as few embryogenic structures. However, calli induced with 2,4-D were necrotic without any cell proliferation or subsequent embryogenic expression while apical browning followed by a total necrosis was observed in the radicles of the control media.

3.2 PROLIFERATION OF THE EMBRYOGENIC CULTURES

The obtained results indicated that proliferation capacity of olive embryogenic callus varied significantly among the genotype (cell lines) and according to the culture conditions, particularly the chemical composition of the maintenance medium and the followed method of culture, but much more by the added growth regulators.

3.2.1 Effect of the type and chemical composition of the proliferation medium

Regardless to the callus line and chemical composition of the medium; suspension culture allowed a significantly greater mass increase (Figure 1F) than solid medium (Figure 1E). Cell proliferation of the lines C1 and C3 was better on ECO liquid medium with 0.3 and 0.26 g of mass increase respectively while the OM chemical composition was more beneficial for the line C2 with nearly 0.2 to 0.3 g of mass increase respectively on solid and liquid media (Table 2). The low increases in mass of C1 and C3 calli with 0.2 and 0.08 g respectively were recorded on solid MS medium while proliferation of C2 seems to be less influenced by the chemical composition of the solid medium allowing between 0.16 and 0.18 g of mass increase (Table 2). Moreover, the best cell proliferation obtained on ECO medium was accompanied by good embryogenic expression and low necrosis of the cell masses (Figure 1E and F) whereas the other media particularly MS in addition to the low mass increase induced a marked browning and formation of compact calli weakly friable with large nodules.

The continued proliferation of embryogenic cultures is determined by several factors related to the nutrient and hormonal composition of the medium but highly by the genotype in culture (Merkle et al., 1995). As in our case, Cerezo et al. (2011) reported a pronounced browning in calli of olive cultivar 'Picual' on OMc with growth regulators whereas ECO liquid medium allowed a better production of less necrotic tissues, friable and nodular masses which contain several globular embryos despite the absence of a significant difference in the mass increase. Furthermore, ECO medium was more beneficial than MS for proliferation of calli induced from leaves and petioles of cultivar 'Dahbia' (Mazri et al., 2013). This beneficial effect of ECO medium may be due to its low content of macroelements, mainly nitrogen, which induces situation of stress favoring the proliferation of embryogenic structures (Cerezo et al., 2011). In addition, the improved result with suspension culture is the result of good cellular organization and synchronization

Table 2: Effect of the type (solid or liquid) and chemical composition (ECO, OM or MS) of the proliferation medium on the mass increase (in gram) of three lines (C1, C2 and C3) of embryogenic olive callus,'Chemlal' after four weeks of culture*

Type of culture	Solid medium			Liquid medium		
Chemical composition	C1	C2	C3	C1	C2	C3
ECO	0.29 ± 0.03 a	0.16 ± 0.02 b	0.14 ± 0.03 bc	0.29 ± 0.02 **ab**	0.20 ± 0.03 **d**	0.26 ± 0.01 **abc**
OM	0.28 ± 0.04 a	0.18 ± 0.01 b	0.09 ± 0.01 cd	0.22 ± 0.02 **cd**	0.30 ± 0.02 **a**	0.13 ± 0.02 **e**
MS	0.20 ± 0.03 b	0.18 ± 0.00 b	0.08 ± 0.01 d	0.20 ± 0.01 **d**	0.25 ± 0.03 **bcd**	0.11 ± 0.01 **e**

*Results are presented as mean ± standard deviation. The different small letters of the same format within columns indicate the homogeneous groups of a significant difference at level of 5 %. Liquid culture kept under 100 rpm of stirring

(Von Arnold, 2008) as well as a better oxygenation and availability of nutrients provided by continuous agitation (Neumann et al., 2009).

3.2.2 Effect of the growth regulators' combination

The calli of the three lines remained in proliferation even on the control media, although the presence of growth regulators and their combinations were essential for the proliferation of olive embryogenic calli, especially in suspension culture which allowed better proliferation compared to solid medium. According to Sánchez-Romero (2019) the embryogenic olive callus can proliferate even in the absence of growth regulators although these are important for the development of somatic embryos (Trabelsi et al., 2011). Indeed, our best results of mass increase were obtained in the presence of IBA and zeatin with formation of embryogenic masses less browned regardless to the followed type of culture for the line C1 producing more than 0.29 g of mass and line C3 on liquid medium with 0.26 g (Table 3). In concordance with previous studies; the combination of IBA, BA and 2iP instead of zeatin has been widely used for callus proliferation of several olive cultivars such as 'Picual' (Cerezo et al., 2011), 'Picholine Marocaine', 'Dahbia' and 'Arbequina' (Mazri et al., 2012) and 'Galega Vulgar' (Pires et al., 2020). However, Hegazi et al. (2017) observed that TDZ was more efficient when combined with IBA than with NAA to maintain calli obtained from radicles and cotyledons of the cultivar 'Coratina'. These authors suggested the existence of a relationship between the used auxin and cytokinin, which may be related to the endogenous hormonal balance of the explants directly varying with genotype (Mazri et al., 2012). Our results agree with this suggestion since that TDZ was more efficient when combined with IBA for the proliferation of lines C1 and C2 independently the culture method except for the C3 line for which TDZ combined with NAA allowed a better mass increase particularly in liquid culture with 0.23 g (Table 3). Furthermore, the cell proliferation of C2 and C3 was significantly reduced in presence of NAA combined with zeatin especially on solid medium with 0.04 and 0.06 g of mass increase respectively (Table 3) in addition of a marked browning preventing the appearance of embryogenic structures.

3.3 PRODUCTION AND MATURATION OF SOMATIC EMBRYOS

After one month in ECO liquid medium, the average number of recovered embryos and their degree of differentiation varied from one line of callus to another. Indeed, despite the low proliferation recorded in the lines C2 and C3 (Table 2 and 3); these lines produced more embryos (72.3 and 56 embryos/gfm) sufficiently differentiated (torpedo and cotyledonary) compared to C1 producing 33.3 embryos/gfm generally in the primary stages of differentiation (globular or cordiform) (Table 4). Subsequently, maturation of embryos varied significantly between the studied lines. About 15 % of the C1 pro-embryos turned necrotic more than the two other lines presenting 7.4 % of browning although no signifi-

Table 3: Effect of the growth regulators' combination (IBA or NAA combined with Zea or TDZ in addition to BA) added to the solid and liquid ECO proliferation medium on the mass increase (in gram) of three lines (C1, C2 and C3) of embryogenic olive callus, 'Chemlal' after four weeks of culture*

Type of culture	Solid medium			Liquid medium		
Growth regulators' combination	C1	C2	C3	C1	C2	C3
Control	0.04 ± 0.00 g	0.02 ± 0.00 g	0.02 ± 0.00 g	0.03 ± 0.00 **jk**	0.04 ± 0.01 **ijk**	0.02 ± 0.00 **k**
IBA-Zea	0.29 ± 0.03 a	0.16 ± 0.02 bc	0.14 ± 0.03 bcde	0.29 ± 0.02 **a**	0.20 ± 0.03 **cde**	0.26 ± 0.01 **ab**
IBA-TDZ	0.15 ± 0.03 bcd	0.11 ± 0.02 cdef	0.07 ± 0.01 efg	0.17 ± 0.03 **def**	0.21 ± 0.03 **bcd**	0.15 ± 0.01 **efg**
NAA-Zea	0.21 ± 0.04 b	0.04 ± 0.01 fg	0.06 ± 0.00 fg	0.1 ± 0.01 **ghi**	0.14 ± 0.02 **fg**	0.13 ± 0.01 **fgh**
NAA-TDZ	0.14 ± 0.02 bcde	0.08 ± 0.01 defg	0.17 ± 0.0 bc	0.16 ± 0.02 **def**	0.06 ± 0.02 **hij**	0.23 ± 0.02 **bc**

*Results are presented as mean ± standard deviation. The different small letters of the same format within columns indicate the homogeneous groups of a significant difference at level of 5 %. Liquid culture kept under 100 rpm of stirring

Table 4: Average number of immature somatic embryos (SE) produced per gram of fresh material (gfm) from three lines (C1, C2 and C3) of olive embryogenic callus, 'Chemlal' after four weeks in liquid culture with stirring and maturation after eight weeks on ECO basal solid medium with activated charcoal*

		Maturation			
Embryogenic callus lines	Average number of SE produced /gfm	Necrosis (%)	Proliferation (%)	Differentiation (%)	Average number of recovered matures SE
C1	33.3 ± 5.8 c	14.8 a'	74.1 a"	11.1 **b**	1.0 ± 0.0 **c'**
C2	72.3 ± 6.0 a	7.4 a'	44.4 b"	48.1 **a**	1.3 ± 0.2 **b'**
C3	56.0 ± 4.6 b	7.4 a'	48.1 b"	44.4 **a**	2.6 ± 0.4 **a'**

*Results are presented as mean ± standard deviation. The different small letters of the same format within columns indicate the homogeneous groups of a significant difference at level of 5 %

cant difference has been revealed statistically. Consequently, more than 74.1 % of the C1 embryos proliferated but only 11.1 % of explants differentiated few mature embryos while C2 and C3 explants presenting more differentiation with 48.1 and 44.4 % respectively produced 1.3 and 2.6 mature embryos recovered after eight weeks of incubation (Figure 1G; Table 4).

According to Rugini et al. (2005) olive pro-embryos on the maturation medium may show marked necrosis, amorphous cell proliferation or differentiate to advanced stages of development. Our results are close to those of Cerezo et al. (2011) and Narváez et al. (2019) indicating maturation rates varying from 49 to 59 % in pro-embryos of cultivar 'Picual' and wild olive respectively with an average of 1.7 to 2.2 mature produced embryos whereas about 6 % of embryos turned necrotic. Similarly, Bradaï et al. (2016) obtained about 5 mature embryos from young lines of 'Picual' callus.

3.4 GERMINATION OF SOMATIC EMBRYOS AND PLANTS REGENERATION

Germination of olive somatic embryos as well as the development of the resulted plantlets depend directly on the genotype (callus line). Under photoperiod, white-opaque embryos turn greenish with swelling and spreading of the two cotyledons in addition to the elongation of their roots, which turned yellow just before the emergence of a small shoot (Figure 1H). The same observations have been reported by Toufik et al. (2017) on embryos of the cultivar 'Dahbia' resulting in roots emergence and germination after two weeks under photoperiod conditions. In our study, embryos of C2 germinated with root emergence from the second week of introduction whereas no germination or rooting had been observed before two weeks with embryos of C3 (Data not shown). Rugini (1988) indicated that germination of olive somatic embryos is faster on OM medium. However, low germination rates have been often obtained with numerous cultivars where less than 13 % of germination obtained for 'Picual' (Cerezo et al., 2011) while Mazri et al. (2020) indicated that germination of 'Dahbia' somatic embryos was only achieved in the presence of growth regulators. This low conversion is mainly due to deficiencies in embryogenic maturation and development (Merkle et al., 1995). In our case, using cotyledonary somatic embryos after a maturation step, germination rates of 50 and 31.3 % respectively for C2 and C3 were obtained on OM basal medium. Consequently, C2 embryos resulted in well-developed plants (1.6 cm) with multiple leaves while small plants were obtained from C3 embryos (Data not shown). Finally, well-developed plantlets have been easily acclimatized and exhibited normal growth and phenotype *in vivo* even after transfer to field conditions (Figure 1I).

4 CONCLUSIONS

To our knowledge, our study describes for the first time an efficient regeneration of whole plants without morphological abnormalities in the main olive cultivar in Algeria 'Chemlal' via somatic embryogenesis induced from juvenile material, radicles of zygotic embryos. More embryogenic calli were induced on OMc medium containing IBA and zeatin, while the maintenance of cell lines on ECO medium particularly in liquid culture, allows better cell proliferation and production of embryos easily convertible into whole plantlets. The optimized approach will allow the application of biotechnological improvement methods particularly genetic transformation

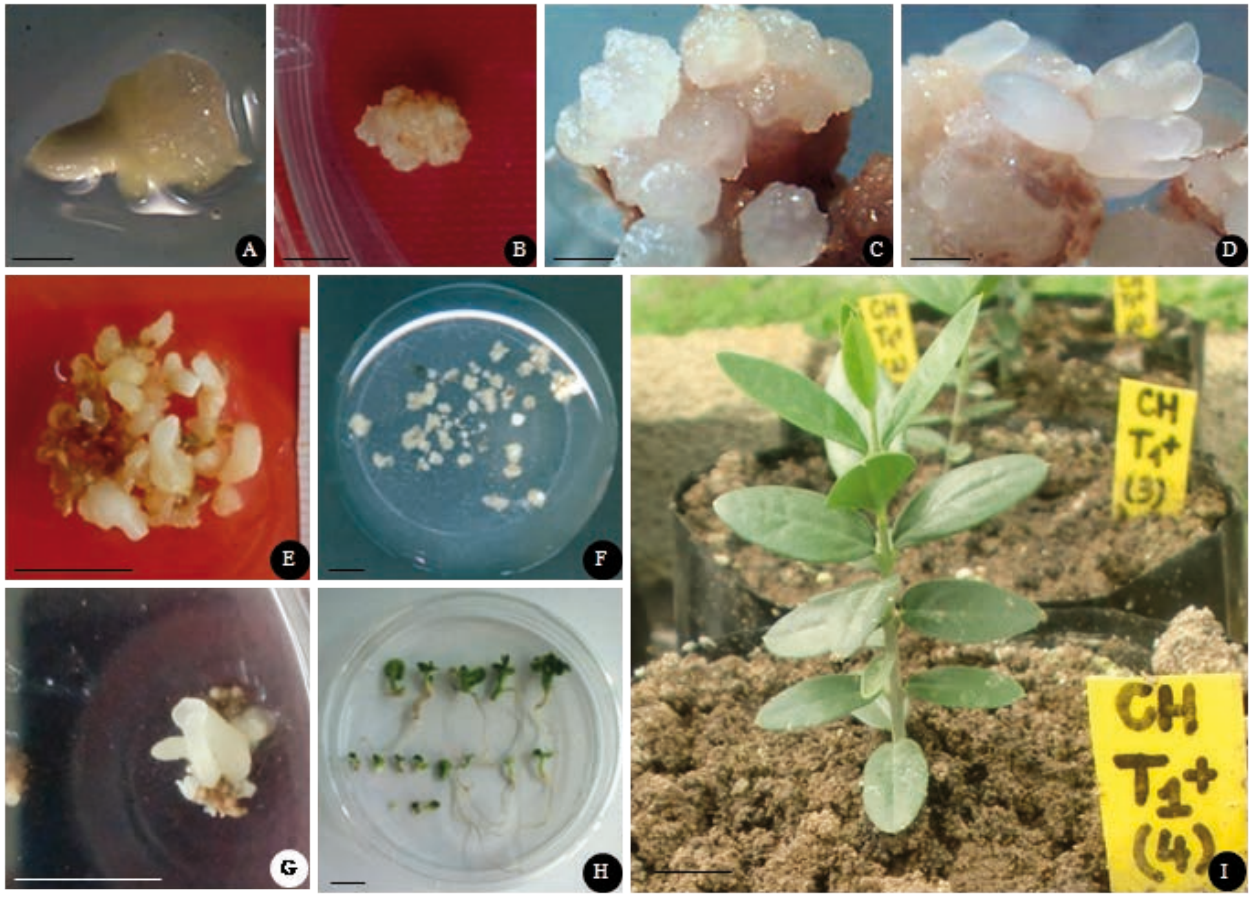

Figure 1: Plant regeneration via somatic embryogenesis induced from radicles of olive zygotic embryos, cv. 'Chemlal'. A and B. Aspect of radicles after 1 and 3 weeks on OMc solid medium containing IBA and zeatin. C. Appearance of nodules after 4 weeks on OMc without zeatin and reduced concentration of IBA. D, E and F. Somatic embryos (SE) appearance and callus proliferation after 4 weeks on solid or liquid ECO medium with IBA, zeatin and BA. G. Maturation of SE after 8 weeks on ECO solid basal medium with activated charcoal. H and I. Germination of SE on OM solid basal medium and acclimatized plantlets. (Bars correspond to 1 cm except for A, C and D the bar corresponds to 0.1 cm)

and induction of somaclonal variation as alternatives for varietal creation and improvement in olive tree against various stresses.

5 ACKNOWLEDGMENTS

The authors gratefully acknowledge funding support from the National Higher School of Agronomy (ENSA) and the Algerian Ministry of High Education and Scientific Research (MESRS).

6 REFERENCES

Boukhari, R., Ameur Ameur, A., Innal, H., Gaouar, S.B.S. (2020). First morphological characterization of autochthonous olive (*Olea europaea* L.) denominations from central and eastern of Algeria. *Acta Agriculturae Slovenica*, *116*(2), 311-325. https://doi:10.14720/aas.2020.116.2.1923

Boutkhil, S., Benine, M.L., Benzaoui, M.Y., Diaf, M., Kaldi, A., Chama, Z., Tifrit, A., Abbouni, B. (2016). Isolation and characterization of *Verticillium* wilt of olive trees in Algeria. *International Journal of Microbiological Research*, *7*(3), 93-101. https://doi:10.5829/idosi.ijmr.2016.93.101

Bradaï, F., Pliego-Alfaro, F., Sánchez-Romero, C. (2016). Long-term somatic embryogenesis in olive (*Olea europaea* L.): Influence on regeneration capability and quality of regenerated plants. *Scientia Horticulturae*, *199*, 23-31. http://dx.doi.org/10.1016/j.scienta.2015.12.010

Cañas, L.A., Benbadis, A. (1988). *In vitro* plant regeneration from cotyledon fragments of the olive tree (*Olea europaea* L.). *Plant Science*, *54*, 65-74. https://doi.org/10.1016/0168-9452(88)90056-8

Cerezo, S., Mercado, J.A., Pliego-Alfaro, F. (2011). An efficient regeneration system via somatic embryogenesis in olive.

Plant Cell, Tissue and Organ Culture, *106*, 337-344. https://doi.org/10.1007/s11240-011-9926-6

Haddad, B., Gristina, A.S., Mercati, F., Saadi, A., Aiter, N., Martorana, A., Sharaf, A., Carimi, F. (2020). Molecular analysis of the official Algerian olive collection highlighted a hotspot of biodiversity in the central Mediterranean basin. *Genes*, *11*(3), n°303, 17p. https://doi.org/10.3390/genes11030303

Hegazi, E.S., Abd Allatif, A.M., Abd Elraof, O.A. (2017). Effect of growth regulators on callus induction and somatic embryogenesis in olive. *Research Journal of Pharmaceutical, Biological and Chemical Sciences*. *8*(4), 274-280. https://www.rjpbcs.com/pdf/2017_8(4)/[35].pdf

Maalej, M., Drira, N., Chaari-Rkhis, A., Trigui, A. (2002). Preliminary results of somatic embryogenesis from young zygotic embryos of olive tree. *Acta Horticulturae*, *586*, 899-902. https://doi.org/10.17660/ActaHortic.2002.586.196

Mazri, M.A., Naciri, R., Belkoura, I. (2020). Maturation and conversion of somatic embryos derived from seeds of olive (*Olea europaea* L.) cv. Dahbia: Occurrence of secondary embryogenesis and adventitious bud formation. *Plants*, *9*(11), n°1489, 14p. https://doi.org/10.3390/plants9111489

Mazri, M.A., Belkoura, I., Pliego-Alfaro, F., Belkoura, M. (2013). Somatic embryogenesis from leaf and petiole explants of the Moroccan olive cultivar Dahbia. *Scientia Horticulturae*, *159*, 88-95. https://doi.org/10.1016/j.scienta.2013.05.002

Mazri, M.A., Belkoura, I., Pliego-Alfaro, F., Belkoura, M. (2012). Embryogenic capacity of embryo-derived explants from different olive cultivars. *Acta Horticulturae*, *929*, 397-403. https://doi.org/10.17660/ActaHortic.2012.929.57

Merkle, S.A., Parrot, WA., Flinn, BS. (1995). Morphogenic aspects of somatic embryogenesis, In: Thorpe, T.A. (editor). *In vitro embryogenesis in plants: Current plant science and biotechnology in agriculture.* Vol. 20, Dordrecht, Holland, Springer, pp. 155-204. https://doi.org/10.1007/978-94-011-0485-2_5

Montes-Osuna, N., Mercado-Blanco, J. (2020). *Verticillium* wilt of olive and its control: What did we learn during the last decade? *Plants*, *9*(6), n°735, 31p. https://doi.org/10.3390/plants9060735

Murashige, T., Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum*, *15*, 473-497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Narváez, I., Martín, C., Jiménez-Díaz, R.M., Mercado, J.A., Pliego-Alfaro, F. (2019). Plant regeneration via somatic embryogenesis in mature wild olive genotypes resistant to the defoliating pathotype of *Verticillium dahliae*. *Frontiers in Plant Sciences*, *10*(1471), 11p. https://doi.org/10.3389/fpls.2019.01471

Neumann, K.H., Kumar, A., Imani, J. (2009). *Plant cell and tissue culture - a tool in biotechnology basics and application.* Berlin, Germany, Springer, 333 p. https://doi.org/10.1007/978-3-540-93883-5

Pires, R., Cardoso, H., Ribeiro, A., Peixe, A., Cordeiro, A. (2020). Somatic embryogenesis from mature embryos of *Olea europaea* L. cv. 'Galega Vulgar' and long-term management of calli morphogenic capacity. *Plants*, *9*(6), n°758, 15p. https://doi.org/10.3390/plants9060758

Rugini, E. (1995). Somatic embryogenesis in olive (*Olea europaea* L.), In: Jain, S.M., Gupta, P.K., Newton, R.J. (editors). *Somatic embryogenesis in woody plants-Angiosperms.Vol. 2*, Dordrecht, Holland, Springer, pp. 171-189. https://doi.org/10.1007/978-94-011-0491-3_11

Rugini, E. (1988). Somatic embryogenesis and plant regeneration in olive (*Olea europaea* L.). *Plant Cell, Tissue and Organ Culture*, *14*, 207-214. https://doi.org/10.1007/BF00043411

Rugini, E. (1984). *In vitro* propagation of some olive cultivars with different root-ability, and medium development using analytical data from developing shoots and embryos. *Scientia Horticulturae*, *24*, 123-134. https://doi.org/10.1016/0304-4238(84)90143-2

Rugini, E., Mencuccini, M., Biasi, R., Altamura, M.M. (2005). Olive (*Olea europaea* L.), In: Jain, S.M., Gupta, P.K. (editors). *Protocol for somatic embryogenesis in woody plants*. Dordrecht, Holland, Springer, pp. 345-360. https://doi.org/10.1007/1-4020-2985-3_27

Rugini, E., Baldoni, L., Silvestri, C., Mariotti, R., Narváez, I., Cultrera, N., Cristofori, V., Bashir, M.A., Mousavi, S., Palomo-Ríos, E., Mercado, J.A., Pliego-Alfaro, F. (2020). *Olea europaea* Olive, In: Litz, R.E., Pliego-Alfaro, F., Hormaza, J.I. (editors). *Biotechnology of fruit and nut crops*, 2nd Ed, London, UK, CABI International, pp. 343-376. https://www.cabi.org/bookshop/book/9781780648279/

Sánchez-Romero, C. (2021). Somatic embryogenesis in olive. *Plants*, *10*(3), 433: 6p. https://doi.org/10.3390/plants10030433

Sánchez-Romero, C. (2019). Somatic embryogenesis in *Olea* spp. *Plant Cell, Tissue and Organ Culture*, *138*, 403-426. https://doi.org/10.1007/s11240-019-01652-0

Toufik, I., Belkoura, I., Guenoun, F. (2017). Improving germination conditions of somatic embryos derived from radicle explants of Moroccan olive cultivar Dahbia. *European Journal of Biotechnology and Bioscience*, *5*(3), 51-56. http://www.biosciencejournals.com/archives/2017/vol5/issue3/5-3-15

Trabelsi, E.B., Naija, S., Elloumi, N., Belfeleh, Z., Msllem, M., Ghezel, R., Bouzid, S. (2011). Somatic embryogenesis in cell suspension cultures of olive (*Olea europaea* L.) 'Chetoui'. *Acta Physiologia Plantarum*, *33*, 319-324. https://doi.org/10.1007/s11738-010-0550-6

Von Arnold, S. (2008). Somatic embryogenesis, In: George, E.F., Hall, M.A., De Klerk, G.J. (editors). *Plant propagation by tissue culture*. Dordrecht, Holland, Springer, pp. 335-354. https://doi.org/10.1007/978-1-4020-5005-3_9