RESEARCH ARTICLE

Proliferation conditions of olive (*Olea europaea* L.) embryogenic calli

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ABSTRACT
Maintenance of the embryogenic calli in continuous proliferation for a long-time with good expression of their potential remains critical to the utility of the somatic embryogenesis process. The objective of this study is to evaluate the effect of some culture conditions on the cell proliferation of embryogenic calli induced from radicles of mature olive zygotic embryos. Our results showed that cell proliferation and embryogenic expression of olive callus were markedly enhanced by the availability of sucrose at concentrations of 20 to 40 g l^{-1}, especially in a liquid culture medium. Likewise, the addition of activated charcoal improved significantly the increase in callus weight but also the texture of the callus by promoting the maturation of pro-embryos as well as a clear reduction in the browning of cell masses. In addition, the culture duration promoted the growth of calli, although cultures exceeding six to eight weeks on solid medium or three to four weeks in suspension with stirring negatively affected the rate of proliferation due to browning.

Key words: Activated charcoal, embryogenic calli, olive, proliferation, subculture, sucrose

Introduction

The olive tree (*Olea europaea* L.) includes an important genetic diversity of which a large part is kept in living collections used in breeding programs (Rallo et al., 2018). However, classical breeding methods became unable to achieve alone significant gains (Rugini et al., 2016) due to the long juvenile period of the species (10 to 15 years), especially in the current context of climate change (Rugini et al., 2020). Therefore, biotechnological methods such as genetic transformation and cryopreservation could be an alternative for the varietal creation and preservation of the species. However, these techniques require an efficient method of in vitro regeneration. Somatic embryogenesis provides the plant material suitable for the application of these techniques (Sánchez-Romero, 2019).

Somatic embryogenesis has been achieved in several olive cultivars using different types of explants. The best results have often been obtained from juvenile tissues, particularly radicles of zygotic embryos formed by undifferentiated cells with a very high morphogenetic potential (Sánchez- Romero, 2019). Indeed, embryogenic cultures are a set of cellular masses characterized by continuous mitotic activity allowing secondary somatic embryogenesis that can be maintained by monthly subcultures on a new medium (Merkle et al., 1995; Von Arnold, 2008; Rugini et al., 2020). The maintenance of calli in proliferation is determined by several factors related to the genotype and the initial explant but also to the culture conditions; mainly the chemical and hormonal formulation of the culture medium, as well as duration and frequency of subcultures which can induce marked repercussions on the practical applicability of the whole process (Sánchez-Romero, 2019). Among the various media used during this phase; the ECO medium of cyclic olive embryogenesis developed by Pérez-Barranco et al. (2007) significantly improves the formation of mature embryos, and currently, it is widely used for the maintenance of olive embryogenic cultures (Bashir et al., 2022).

Therefore, our work aims to evaluate the effect of the mode and duration of cultures as well as the addition of sucrose and activated charcoal (AC) on the cell proliferation of embryogenic calli induced from radicles of olive zygotic embryos, cv. Chemlal; in order to maintain for a long-term period the embryogenic calli, providing the plant material necessary to the application of some biotechnological techniques of genetic improvement, particularly genetic transformation and induction of somaclonal variation.

Materials and Methods

Establishment of embryogenic cultures

Embryogenic cultures were obtained from radicles of olive zygotic embryos of the cultivar 'Chemlal' according to a modified method of Cerezo et al. (2011). After disinfection of the seed extracted from stones of mature olives; the radicles were cultured in a solid OMc medium (Cañas & Benbadis, 1988) in the presence of 0.5 mg l^{-1} of zeatin and 5 mg l^{-1} of indole-3-butyric acid (IBA) for three weeks under obscurity. Subsequently, the explants were transferred to the same medium without zeatin and with a concentration of IBA reduced to 1/10th (0.5 mg l^{-1}) during the period of four weeks. After that, the calli were transferred to a basal solid ECO medium (Pérez-Barranco et al., 2007) supplemented with 0.1 mg l^{-1} of zeatin, 0.1 mg l^{-1} of Benzylaminopurine (BAP), and 0.05 mg l^{-1} of IBA in addition to 0.55 g l^{-1} of glutamine and 1 g l^{-1} of casein hydrolyzate. All the media contained 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. The cultures were incubated in total darkness at a temperature of 25±2°C.

Proliferation of embryogenic cultures

The capacity of proliferation was evaluated by the weight increase of three lines of embryogenic calli selected for their good friable and nodular structure suitable for this type of study. An embryogenic mass of 0.1 g was cultured on 25 ml of solid or liquid basal ECO medium. The effect of some factors (concentration of sucrose, the addition of activated charcoal, and culture duration) was studied. Therefore, the sucrose was added at a concentration of 0, 5, 10, 20, 30, or 40 g l^{-1} to the ECO basal medium while the activated charcoal was added, in addition to the control (without AC), at a concentration of 0.5 or 1 g l^{-1} to the medium containing 20 g l^{-1} of sucrose. In these cases, the cultures were maintained for four weeks while the effect of culture duration was evaluated by culturing the calli for two to sixteen weeks on a solid medium or one to six weeks in a liquid medium with 100 rpm of stirring.

At least, three Petri dishes or three Erlenmeyer flasks of 100 ml were incubated in total darkness at 25±2°C. At the end of each incubation, the whole callus was collected, weighed and the increase in fresh weight, as well as the rate of proliferation (weight of cellular mass produced per week) in addition to morphological traits such as texture, the appearance of embryogenic structures of different development stages, friability, and necrosis of the callus, were determined, and a new inoculum was taken for the next subculture. The follow-up was carried out for at least three successive subcultures.

Statistical analysis

The experiments were carried out according to the total randomization design. 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 effect, the separation of the means was carried out by Fisher's LSD (Least Significant Difference) test. In all analyzes, a significance level of 5% was considered. The obtained results were represented graphically using the Excel program of Microsoft Office 2007. The letters on the graphs indicate homogeneous groups.

Results

Effect of the medium additives

Our results indicated that the presence of sucrose as a carbon source in the medium is essential for the continued proliferation of the embryogenic olive callus. Indeed, the weight of the different lines increased proportionally with the concentration of sucrose, particularly in liquid culture (Figures 1 and 3). However, doses greater than 20 g l^{-1} did not considerably improve proliferation, especially in solidified medium, despite the significant reduction of the cell necrosis and the accelerated differentiation of embryogenic structures (Figure 3). Consequently, the best results on solid medium were recorded in the presence of 40 g l^{-1} of sucrose for C1 and C3 with 0.29 and 0.15 g respectively while 30 g l^{-1} allowed more than 0.16 g of increase in the C2 line (Figure 1 A). Likewise, in liquid culture, the best weight increases of 0.33, 0.21, and 0.32 g respectively for the three lines were obtained in the presence of 40 g l^{-1} of sucrose (Figure 1 B).

In addition, the inclusion of activated charcoal in the proliferation medium, especially in liquid culture, improved significantly the weight increase of the three calli lines but also their degree of friability and embryogenic differentiation with a significant reduction in the browning of the cell masses (Figure 3). In a solid medium, the best proliferation results were obtained in the presence of 0.5 g of AC for C1 and C2 with 0.34 and 0.25 g of increase respectively, while the addition of 1 g of CA was more beneficial for C3 with 0.19 g (Figure 2 A). Furthermore, a positive correlation was revealed between the weight increase and the concentration of AC added to the liquid medium allowing 0.35, 0.25, and 0.32 g of increase respectively for the three lines in the presence of 1 g of AC (Figure 2 B).

Effect of the culture duration

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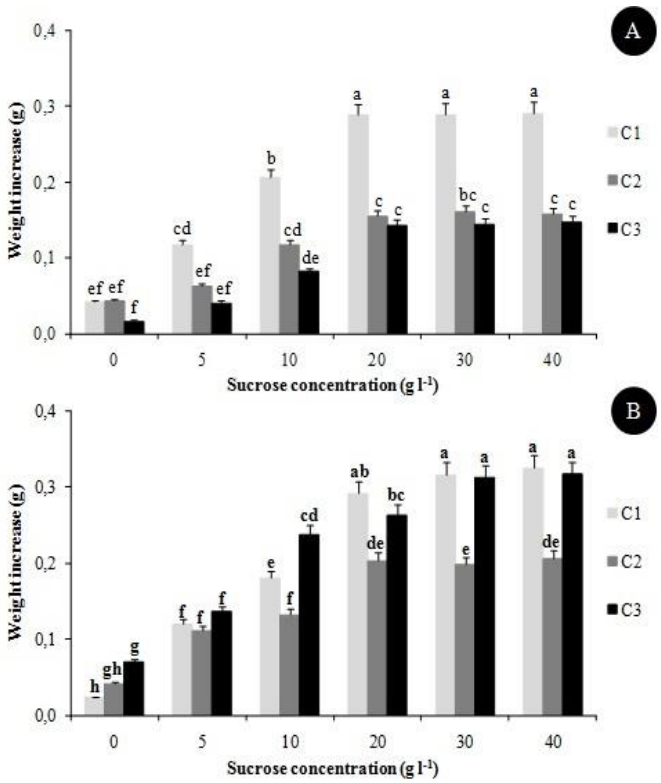

Figure 1. *Effect of sucrose concentration in solid (A) and liquid (B) ECO proliferation medium on the weight increase of three lines of embryogenic olive calli, cv. Chemlal.*

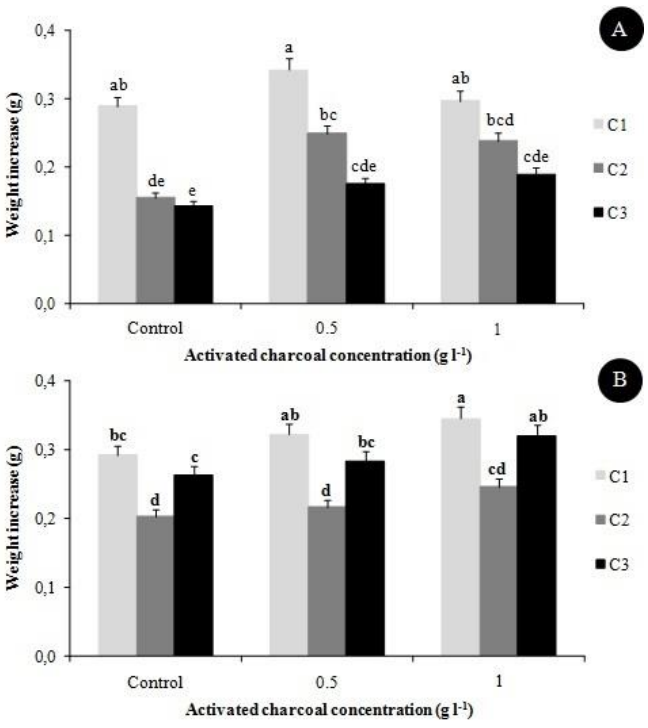

Figure 2. *Effect of the activated charcoal in solid (A) and liquid (B) ECO proliferation medium on the weight increase of three lines of embryogenic olive calli, cv. Chemlal.*

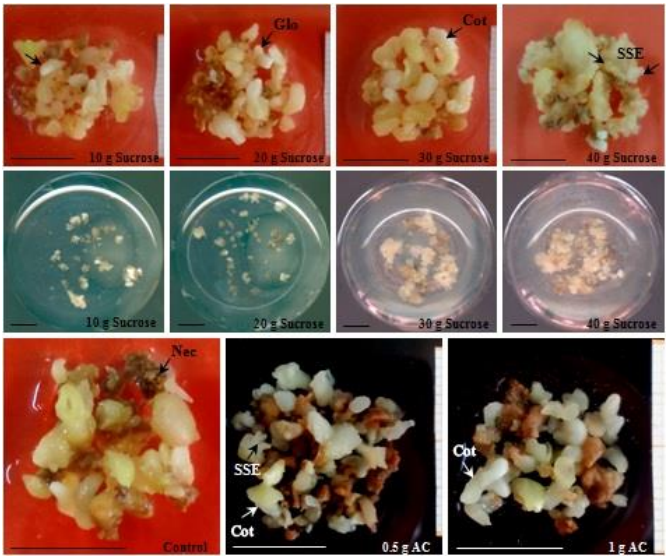

Figure 3. *Effect of sucrose and activated charcoal added to solid and liquid ECO medium on the proliferation of embryogenic olive callus, cv. Chemlal. (→: arrows indicate embryogenic structures, Bars correspond to 1 cm). (Abbreviations: Cot: Somatic embryo at the cotyledonary stage, SSE: Secondary somatic embryogenesis, Glo: Globule, Nec: Necrosis).*

The maintenance duration of callus in a culture strongly influences its proliferation. The weight increase was positively correlated with the incubation duration of cultures (Figure 4 A and B) although a period exceeding eight to ten weeks on solid medium and three to four weeks in suspension did not improve significantly the calli proliferation (Figure 4 A and B) but caused the browning of cell masses accompanied by a marked reduction in the rate of proliferation especially on liquid medium (Figure 4 C and D) in addition to the appearance of embryogenic structures more differentiated (cotyledonary stage) (Figure 5).

Conversely, the short culture incubations allowed the formation of several globules on less necrotic cell masses (Figure 5). Indeed, the best results of weight increase on solid medium were recorded after fourteen weeks of culture with 1.3, 1.1, and 0.47 g, respectively for the three lines (Figure 4 A) while 0.3, 0.2, and 0.29 g of increase were obtained from the fourth week in suspension (Figure 4 B). Nevertheless, the best proliferation rates were recorded with durations of six to eight weeks of culture on a solid medium with 0.11, 0.10, and 0.04 g/week, respectively for the three lines (Figure 4 C) and between the third and fourth week in liquid culture with 0.08, 0.05 and 0.07 g/week respectively for the lines (Figure 4 D).

Discussion

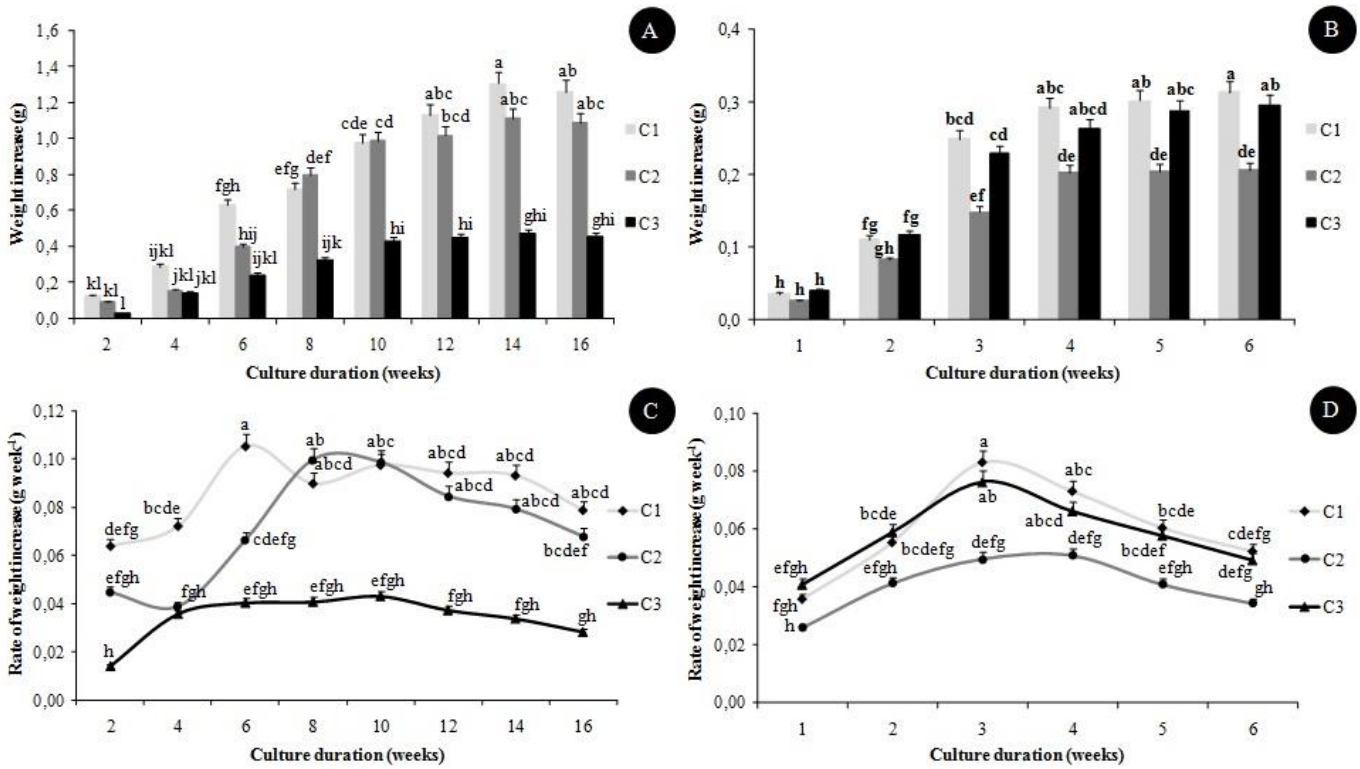

Figure 4. *Effect of the incubation time of cultures in solid (A and C) and liquid (B and D) ECO proliferation medium on the increase in weight and proliferation rate of the three lines of olive embryogenic calli, cv. Chemlal.*

Sucrose was often used during the whole process of somatic embryogenesis in olive trees (Brhadda et al., 2008). According to Shibli et al. (2001), doubling the concentration of sucrose from 30 to 60 g l^{-1} in the culture medium markedly improves the number of embryos produced by the calli of the cultivar 'Nabali', while a too high dose reduces the embryogenic expression. Furthermore, 30 to 40 g of sucrose allowed good proliferation and production of embryos from calli of the cultivar 'Chetoui' (Trabelsi et al., 2003). These authors suggested that sucrose indirectly influences secondary embryogenesis through the induction of non-embryogenic cells. However, Brhadda et al. (2008) observed that sucrose concentration above 30 g l^{-1} improves callogenesis but not embryogenic expression. Therefore, the optimal concentration of sucrose is directly related to the genotype and explant although the majority of studies indicated the addition of 20 to 30 g l^{-1} of sucrose for the proliferation of calli induced from embryo radicles of several olive cultivars (Cerezo et al., 2011; Mazri et al., 2011 and 2012; Hegazi et al., 2017; Toufik et al., 2017; Pires et al., 2020). Our results confirm these observations since the concentration of sucrose allowed a proportional increase of the calli weight, although a dose exceeding 20 or 30 g l^{-1} did not improve significantly the cell proliferation but favored the appearance of more differentiated embryos with a reduction of the necrosis degree particularly in suspension culture.

The addition of activated charcoal to the tissue culture medium improves the cell growth and development of several species (Thomas, 2008) mainly via the reduction of the explants browning by the adsorption of inhibitory substances

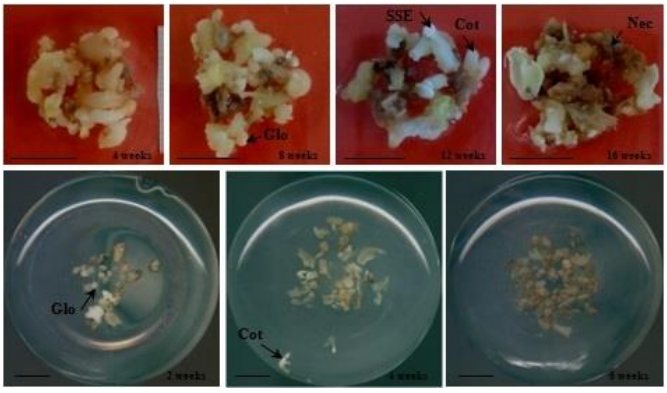

Figure 5. *Effect of the culture duration in solid and liquid ECO medium on the proliferation of embryogenic olive callus, cv. Chemlal. (→: arrows indicate embryogenic structures, Bars correspond to 1 cm). (Abbreviations: Cot: Somatic embryo at the cotyledonary stage, SSE: Secondary somatic embryogenesis, Glo: Globule, Nec: Necrosis).*

such as the released phenolic compounds while improving their morphogenetic capacity (Merkle et al., 1995). In olive trees, AC has often been used during embryo maturation to reduce the effects of residual auxins (Pérez-Barranco et al., 2007), although its addition during callogenesis significantly improves the production of embryogenic calli (Maalej et al., 2002) with a beneficial effect on the maintenance of their embryogenic capacity (Benelli et al., 2001). Indeed, Pérez-Barranco et al. (2007) indicated that maintenance of calli induced from radicles of the cultivar 'Picual' on an ECO medium containing 1 g l^{-1} of AC causes a radical change in its mode of multiplication from cyclic cell proliferation with the formation of primary embryos (globules) to the production of more structured white-opaque embryos that may even germinate on the medium as a sign of strong secondary somatic embryogenesis (Rugini and Silvestri, 2016). Similarly, Benelli et al. (2001) found that embryogenic lines of the cultivar 'Canino' on OMc medium supplemented with 0.1% of AC were less browned and able to differentiate embryos at advanced stages of development (cotyledonary). Therefore, the effect of AC on the process seems to be qualitative rather than quantitative since Benzekri and Sánchez-Romero (2012) indicated that the spreading of embryogenic masses of the cultivar 'Picual' on ECO medium supplemented with AC did not affect the total number of embryos produced but significantly improved their germination thereafter. These observations corroborate our case where the incorporation of AC clearly improves the proliferation of the three lines, particularly in liquid culture, in addition to a marked reduction in the browning of cell masses as well as the appearance of white-opaque embryos at very advanced developmental stages (cotyledonary).

The establishment of a competent olive embryogenic culture requires a considerable amount of time, particularly its maintenance in continuous proliferation (Bradaï et al., 2016) which duration and frequency of subcultures were determined by the tissue characteristics and the culture conditions mainly the depletion of the medium and the accumulation of substances harmful to growth as ethylene (Bashir et al., 2022). Shibli et al. (2001) recommended subculturing every three weeks for calli of the cultivar 'Nabali' on a solid medium supplemented with hormones to induce a proportional increase in weight followed by stability after nine weeks. Similarly, Pérez-Barranco et al. (2009), Cerezo et al. (2011) and Bradaï et al. (2016) suggested four to six weeks of incubation for calli of the cultivar 'Picual' on a solid ECO medium to reduce the browning of cell masses and stabilize their proliferation and embryogenic expression. Recently, Pires et al. (2020) observed a significant increase in the number of embryos produced by the calli of the cultivar 'Galega Vulgar' with the duration of culture on a solid ECO medium although a strong reduction in the embryogenic expression was noted after sixteen weeks. Our results agree perfectly with these observations since the calli of the three lines tested showed a proportional increase of their weight according to the duration of culture with stability from ten weeks of incubation despite the decrease in the rate of proliferation due to the marked browning.

Suspension cultures have several biotechnological applications. In olive trees, embryogenic cultures in a liquid medium were often used as a pre-treatment for the maturation of somatic embryos only for a few weeks (Sánchez-Romero, 2019) because they are subjected since the first days of incubation to phenolic oxidations inducing their rapid browning. However, cell growth restarts weakly from the second week, especially in the presence of hormones (Trabelsi et al., 2011). Pérez-Barranco et al. (2007 and 2009) and Cerezo et al. (2011) indicated that embryogenic calli obtained from radicles of the cultivar 'Picual' tolerate well the culture in a liquid medium under stirring for three to four weeks permitting a higher increase (0.9 g) compared to culture on solid medium (0.5 to 0.6 g) with the formation of many globular embryos. This beneficial effect of the liquid medium is the result of better cellular organization and synchronization (Von Arnold, 2008; Sánchez-Romero, 2019) in addition to good oxygenation and availability of nutrients assured by continuous agitation (Neumann et al., 2009). Our results confirm these observations since all the embryogenic lines tested proliferated better in a liquid culture although culture duration exceeding four weeks did not really improve the weight increase but caused a marked tissue browning with a significant decrease in the rate of proliferation.

In conclusion, our study showed that texture and proliferation of the embryogenic olive callus vary significantly with the culture conditions, particularly the presence of some additives within the medium as well as the incubation period between the successive subcultures. Consequently, frequent monthly subcultures on a solid medium rich in sucrose alternated with passages in suspension in the presence of activated charcoal allow better maintenance of the calli embryogenic potential.

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References

Bashir MA, Silvestri C, Salimonti A, Rugini E, Cristofori V, Zelasco S. 2022. Can ethylene inhibitors enhance the success of olive somatic embryogenesis? Plants, 11(168): 10.

Benelli C, Fabri A, Grassi S, Lambardi M, Rugini E. 2001. Histology of somatic embryogenesis in mature tissue of olive (*Olea europaea* L.). J. Hort. Sci. Biotech., 76: 112-119.

Benzekri H, Sánchez-Romero C. 2012. Maturation of olive somatic embryos. Acta Hort., 961: 441-447.

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. Sci. Horti., 199: 23-31.

Brhadda N, Walali LDM, Abousalim A. 2008. Effet du sucre sur l'embryogenèse somatique de l'olivier (*Olea europaea* L.) cv. 'Picholine Marocaine'. Biotechnol. Agron. Soc. Environ., 12(3): 245-250.

Cañas LA, Benbadis A. 1988. *In vitro* plant regeneration from cotyledon fragments of the olive tree (*Olea europaea* L.). Plant Science, 54: 65-74.

Cerezo S, Mercado JA, Pliego-Alfaro F. 2011. An efficient regeneration system via somatic embryogenesis in olive. Plant Cell Tiss. Organ Cult., 106: 337-344.

Hegazi ES, Abd Allatif AM, Abd Elraof OA. 2017. Effect of growth regulators on callus induction and somatic embryogenesis in olive. Res. J Pharm. Biol. Chem. Sci., 8(4): 274–280.

Maalej M, Drira N, Chaari-Rkhis A, Trigui A. 2002. Preliminary results of somatic embryogenesis from young zygotic embryos of olive tree. Acta. Hort., 586: 899-902.

Mazri MA, Belkoura I, Pliego-Alfaro F, Belkoura M. 2012. Embryogenic capacity of embryo-derived explants from different olive cultivars. Acta Hort., 929: 397-403.

Mazri MA, Elbakkali A, Belkoura M, Belkoura I. 2011. Embryogenic competence of calli and embryos regeneration from various explants of Dahbia cv, a Moroccan olive tree (*Olea europaea* L.). Afri. J. Biotechnol., 10(82): 19089-19095.

Merkle SA, Parrot WA, Flinn BS. 1995. Morphogenic aspects of somatic embryogenesis. – In: Thorpe TA. (eds), In vitro Embryogenesis in Plants. Kluwer Academic Publishers, Dordrecht, p. 155–204.

Neumann KH, Kumar A, Imani J. 2009. Plant cell and tissue culture – a tool in biotechnology Basics and application. Springer, USA, 333 p.

Pérez-Barranco G, Torreblanca R, Padilla IMG, Sánchez-Romero C, Pliego-Alfaro F, Mercado JA. 2009. Studies on genetic transformation of olive (*Olea europaea* L.) somatic embryos: I. Evaluation of different aminoglycoside antibiotics for *nptII* selection. II. Transient transformation via particle bombardment. Plant Cell Tiss. Organ Cult., 97: 243–251.

Pérez-Barranco G, Mercado JA, Pliego-Alfaro F, Sánchez-Romero C. 2007. Genetic transformation of olive somatic embryos through biolistic. Acta Hort., 738: 473–477.

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(758): 15p.

Rallo L, Barranco D, Díez CM, Rallo P, Suárez MP, Trapero C, Pliego-Alfaro F. 2018. Strategies for Olive (*Olea europaea* L.) breeding: cultivated genetic resources and crossbreeding. – In: Al-Khayri JM, Jain SM. & Johnson DV. (eds), Advances in plant breeding strategies: Fruits. Springer, Switzerland, p. 535-600.

Rugini E, Silvestri C. 2016. Somatic embryogenesis in olive (*Olea europaea* L. subsp. *europaea* var. *sativa* and var. *sylvestris*). – In: Germanà MA. & Lambardi M. (eds), *In vitro* embryogenesis in higher plants, Methods in molecular biology. Vol. 1359, Springer, p. 341-348.

Rugini E, Cristofori V, Silvestri C. 2016. Genetic improvement of olive (*Olea europaea* L.) by conventional and *in vitro* biotechnology methods. Biotechnol. Adv., 34: 687-696.

Rugini E, Baldoni L, Silvestri C, Mariotti R, Narváez I, Cultrera N, Cristofori V, Bashir MA, Mousavi S, Palomo-Ríos E, Mercado JA, Pliego-Alfaro F. 2020. *Olea europaea* Olive. – In: Litz RE, Pliego-Alfaro F. & Hormaza JI. (eds), Biotechnology of fruit and nut crops, 2nd ed., CABI International, p. 343-376.

Sánchez-Romero C. 2019. Somatic embryogenesis in *Olea* spp. Plant Cell Tiss. Organ Cult., 138: 403-426.

Shibli RA, Shatnawi M, Abu-Ein Al-Juboory KH. 2001. Somatic embryogenesis and plant recovery from callus of 'Nabali' Olive (*Olea europaea* L.). Sci. Hort., 88: 243-256.

Thomas TD. 2008. The role of activated charcoal in plant tissue culture. Biotechnol. Adv., 26: 618-631.

Toufik I, Belkoura I, Guenoun F. 2017. Improving germination conditions of somatic embryos derived from radicle explants of Moroccan olive cultivar Dahbia. European J. Biotechnol. Biosc., 5(3): 51-56.

Trabelsi EB, 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 Physiol. Plant., 33: 319-324.

Trabelsi EB, Bouzid S, Bouzid M, Elloumi N, Belfeleh Z, Benabdallah A, Ghezel R. 2003. *In vitro* Regeneration of olive tree by somatic embryogenesis. J. Plant Biol., 46(3): 173-180.

Von Arnold S. 2008. Somatic embryogenesis. – In: George EF, Hall MA. & De Klerk GJ. (eds), Plant propagation by tissue culture. Vol. 1: The background. 3rd ed., Springer, Netherlands, p. 335-354.