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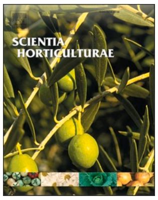

Effect of sucrose preculture and culture inoculum density on cryopreservation of olive somatic embryos

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ABSTRACT

This study reports improvement of a protocol for cryopreservation of olive somatic embryos by using the droplet-vitrification method on aluminum foil strips. Two different approaches were considered in order to optimize cryopreservation: investigating the effect of somatic embryos growth conditions, specifically, the influence of the inoculum density of suspension cultures from which cryopreservation explants were collected, and studying the impact of preculture with different sucrose concentrations in both solid and liquid medium. The results obtained showed a significant effect of inoculum density on cryopreservation evaluated as regrowth rate, although a significant influence on recovery percentage could not be inferred. Sucrose preculture significantly improved recovery rates after cryopreservation. The best results were achieved with somatic embryos previously incubated in liquid ECO medium supplemented with 0.2 M sucrose for 28 days. After this treatment, 90% of explants resumed embryogenesis 12 weeks after thawing. Preculture in liquid medium containing 0.2 M sucrose also had a significant influence on the initial response after cryopreservation, with first signs of regrowth resumption 3 days after thawing, compared to 24-25 days in control, non-precultured embryos. Surviving explants continued actively growing, exhibiting the growth pattern normally observed in olive. As revealed by the histological analysis, cell proliferation greatly increased in somatic embryos precultured on sucrose-enriched media. Cellular features of meristematic and proembryogenic cells, mostly constituting the proliferative regions, make them more prone to survive cryopreservation, thus explaining higher recovery rates found after these treatments.

1. Introduction

The olive (*Olea europaea* L.) is an evergreen tree species native to Middle East and cultivated in the Mediterranean basin from ancient times (Rallo et al., 2011). Olive constitutes the most important fruit crop in this region, with fruits being consumed as olive oil or table olives. Olive oil is a very valuable food product and an important component of the Mediterranean diet from antiquity (Baldoni and Belaj, 2009). Due to its unique chemical composition, olive oil is considered responsible for a large part of the health benefits associated to the Mediterranean diet (Jimenez-Lopez et al., 2020).

The interest in this species justifies the existence of genetic improvement programs with different objectives, such as increased fruit production, increased oil content and quality, shortening of the juvenile period, reduced alternate bearing, and resistance to disease and abiotic stresses. Especially important in the last years is breeding focused on the obtainment of cultivars adapted to high-density planting systems and new cultivation techniques (Fabbri et al., 2009; Lavee, 2013).

Biotechnology can help to accelerate the development of new cultivars. Somatic embryogenesis is essential for application of biotechnological tools in olive, as it constitutes the regeneration method normally used in this species (Sánchez-Romero, 2019). In fact, somatic embryos are the initial explants mainly used in genetic transformation investigations (Sánchez-Romero, 2019).

Embryogenic cultures can be indefinitely maintained by periodic subcultures in proliferation medium. However, long-term maintenance of actively growing embryogenic tissues under *in vitro* conditions may lead to cultures contamination, loss of embryogenic competence, decline of morphogenic potential and increased occurrence of somaclonal variation, with the consequent appearance of genetic and epigenetic alterations in the regenerated plants. Some of these detrimental effects have been reported in olive embryogenic cultures maintained through cyclic embryogenesis for different time periods (Bradaï et al., 2019, 2016a, 2016b).

Cryopreservation, i.e., storage of biological material at ultralow temperatures, can mitigate these problems. At these temperatures, all cellular divisions and metabolic processes are stopped and, therefore,

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Abbreviations

2iP	2-isopentenyladenine
BAP	6-benzylaminopurine
ECO	olive cyclic embryogenesis
IBA	indole-3-butyric acid
LS	loading solution
LSD	least significant difference
MS	Murashige and Skoog
OM	olive medium
PVS2	plant vitrification solution 2

the plant material can be stored without modifications for a theoretically unlimited time (Engelmann, 2004). However, cryopreservation may cause a series of injuries in plant water-rich cells: during freezing due to ice crystals formation, and during the desiccation treatments aimed to reduce intracellular water (Ramon et al., 2002). Moreover, during cryopreservation procedures, plant cells are exposed to different types of stress, such as dehydration, osmotic, oxidative and temperature stress, as well as to mechanical and pH changes (Kaczmarczyk et al., 2012). The combination of cell physical damage and stresses underwent during the freeze-thaw cycle can result in necrosis (Baust et al., 2009). In fact, numerous stress factors associated with cryopreservation have been linked to initiators of apoptotic cell death processes (Baust et al., 2009).

Different treatments may help to overcome damages caused during the cryopreservation procedures, such as slow drying, cold-hardening, exposure to non-lethal heat shocks or incubation with sugars, sugar alcohols, amino acids, abscisic acid or antioxidants (Hoekstra et al., 2001; Reinhoud et al., 2000). Preculture on high sucrose medium has been repeatedly used to cryopreserve embryogenic tissues of different species. During this phase, cells are subjected to mild osmotic stress, which enhances desiccation and chilling tolerance (Lambardi et al., 2008).

Nevertheless, in embryogenic cultures, explants culture conditions also play a relevant role on cell cryotolerance (Sala et al., 2006; Withers and Street, 2006; Wu et al., 2007). Thus, previous investigations revealed a significant influence of culture method, in solid or liquid medium, culture period and the interaction between both factors on cryopreservation of olive somatic embryos (Bradaï et al., 2017). Cell initial density is an important variable influencing growth of embryogenic cultures. Besides, inoculum density has an important influence on the somatic embryogenesis process, with a determinant influence on some steps of this developmental program (Brittain-Loucas et al., 1998; Márquez-Martín et al., 2012). Although some works have studied the effect of cell density in the embryogenic suspensions subjected to cryostorage (Find et al., 1998; Marum et al., 2004); to our knowledge, the effect of the inoculum density of the embryogenic suspensions from which the cryopreservation explants are collected has not been investigated.

The aim of this work was to analyze the effect of inoculum density of embryogenic suspensions cultured for different time periods on subsequent cryopreservation of olive somatic embryos. The influence on cryopreservation of a preculture with different sucrose concentrations was also investigated in both solid and liquid medium. In order to study the influence of the sucrose preculture on explants cryotolerance, somatic embryos subjected to the treatments giving rise to the best results were histologically analyzed and compared with their corresponding controls maintained in culture media with standard sucrose concentration.

2. Material and methods

2.1. Plant material

Embryogenic cultures of olive (*Olea europaea* L.) were initiated from zygotic embryos cv. `Picual', provided by the World Olive Germplasm Bank of Cordoba. As previously described by Orinos and Mitrakos (1991), radicle segments were excised from mature embryos and cultured on OMc medium (Cañas and Benbadis, 1988) supplemented with 25 μM indole-3-butyric acid (IBA), 2.50 μM 2-isopentenyladenine (2iP) and 6 g l^{-1} agar. After three weeks, explants were transferred to OMc medium without hormonal supplement. Embryogenic tissue growing on explants surface was cultured in ECO medium (Pérez-Barranco et al., 2009) solidified with 3 g l^{-1} phytagel. ECO medium consisted of macronutrients of OMe medium (Cañas and Benbadis, 1988), ¼ MS micronutrients (Murashige and Skoog, 1962), ½ OM vitamins (Rugini, 1984), 20 g l^{-1} sucrose, 50 mg l^{-1} myo-inositol, 550 mg l^{-1} glutamine and the supplements proposed by Rugini and Caricato (1995), i.e., 0.25 μM IBA, 0.44 μM 6-benzylaminopurine (BAP), 0.5 μM 2-iP, 200 mg l^{-1} cefotaxime and 1 g l^{-1} casein hydrolysate. Olive embryogenic cultures were maintained through subculturing to fresh medium at 6-7-week intervals, and incubated in the dark at 25 ± 1°C.

The pH of all culture media was adjusted to 5.74 before adding the gelling agent, consisting of agar 6 g l^{-1}, except for the ECO medium, which was gelled with 3 g l^{-1} phytagel. Media sterilization was carried out by autoclaving for 20 min at 121°C and 0.1 MPa. Cefotaxime was filter-sterilized and added to the cooled sterilized media.

2.2. Somatic embryo cryopreservation

Olive somatic embryos were cryopreserved by using the droplet-vitrification method on aluminum foil strips optimized by Bradaï et al. (2017). Somatic embryos 1–6 mm in size were harvested at the end of a maintenance cycle (6-7 weeks after the last subculture). One hundred mg, consisting of 8-12 embryos, were placed in plastic vessels containing approximately 10 ml filter sterilized loading solution (LS), consisting of basal ECO medium (i.e., without growth regulators and cefotaxime) with 2 M glycerol and 0.4 M sucrose. After 20 min in the dark at room temperature, LS was replaced by filter-sterilized and ice-cooled plant vitrification solution 2 (PVS2) (Sakai et al., 1990), consisting of 3.26 M glycerol, 2.42 M ethylene glycol, 1.9 M dimethyl sulfoxide and 0.4 M sucrose dissolved in basal ECO medium. Dehydration in PVS2 was conducted on ice for 30 min. Just before the end of dehydration, somatic embryos surrounded by a droplet of PVS2, were placed onto a sterilized aluminum foil strip and rapidly frozen in liquid nitrogen for at least 30 min. For rewarming, explants were rinsed in filter-sterilized unloading solution for 15 min at room temperature. The unloading solution consisted of basal ECO medium supplemented with 1.2 M sucrose. Subsequentlty, somatic embryos were placed onto two sterile filter paper discs on top of ECO medium containing 0.3 M sucrose and 0.001% (w/v) ascorbic acid. After one day, samples were transferred to standard proliferation medium.

The pH of all cryopreservation solutions was adjusted to 5.74. Solutions sterilization was carried out by filtration through 0.22 μm pore size membranes.

Five replicates were executed per treatment and each cryopreservation experiment was repeated at least twice.

2.2.1. Effect of suspension density and culture time

The effect of inoculum density and time in culture on cryopreservation of olive somatic embryos was investigated in a factorial design experiment.

Somatic embryos 1-6 mm in size were collected from stock cultures at the end of a subculture cycle in maintenance medium. For initiation of suspensions, 200, 400 or 800 mg of somatic embryos were inoculated into 100 ml Erlenmeyer flasks containing 40 ml of ECO medium without the gelling agent. Cultures in liquid medium were incubated on a rotary shaker at 100 rpm for 21, 28 and 35 days, incubation times at which positive cryopreservation results were obtained in previous investigations (Bradaï et al., 2017). Subsequently, 100 mg of somatic embryos derived from each treatment were subjected to cryopreservation as previously indicated. The results obtained were compared with those of somatic embryos 1-6 mm in size directly harvested from stock cultures at the end of a maintenance cycle.

To investigate the influence of cryopreservation on the parameters recorded, explants directly coming from the different culture treatments were included in the experiment.

2.2.2. *Effect of sucrose preculture in solid medium*

Somatic embryos 1-6 mm in size selected from stock cultures, 6-7 weeks after the last subculture were cultured in solidified ECO medium supplemented with 0.2 or 0.4 M sucrose for 6 weeks. After sucrose preculture, 100 mg somatic embryos were cryopreserved following the droplet-vitrification method optimized by Bradaï et al. (2017). Cryopreservation response of sucrose-precultured embryos was compared with that of embryos directly collected from embryogenic cultures maintained under standard proliferation conditions.

To examine the effect of preculture on high-sucrose media on the toxicity of the cryoprotective solutions and the freezing damage, two different controls were included in this experiment: treated controls (-LN), consisting of samples treated with LS and PVS2 but not immersed in liquid nitrogen, and untreated controls (control), consisting of samples directly collected from stock cultures, without any cryopreservation treatment.

2.2.3. *Effect of sucrose preculture in liquid medium*

Somatic embryos 1-6 mm in size collected from stock cultures at the end of a maintenance cycle were used to establish cultures in liquid medium. Four hundred mg of selected embryos were inoculated into 100 ml Erlemeyer flasks containing 40 ml of liquid ECO medium supplemented with 0.2 or 0.4 M sucrose. Embryogenic suspensions were incubated on a rotary shaker at 100 rpm for 28 days. After sucrose preculture in liquid medium, 100 mg somatic embryos were cryopreserved as indicated above. Cryopreservation response of embryos precultured in liquid high-sucrose media was compared with that of somatic embryos directly collected from stock cultures 6-7 weeks after the last subculture.

In order to explore the effect of sucrose preculture in liquid medium on the tolerance to the cryoprotective solutions and freezing, treated (-LN) and untreated controls (control) were included in this experiment.

2.3. *Histological analysis*

In order to investigate the effect of a sucrose preculture on the response of somatic embryos to cryopreservation, somatic embryos subjected to different culture conditions were histologically analyzed.

Somatic embryos directly collected from stock cultures at the end of a maintenance cycle in proliferation medium were subjected to the preculture treatments giving rise to the best cryopreservation results, i.e., culture in liquid medium supplemented with 0.2 M sucrose for 28 days and culture in solid medium supplemented with 0.2 M sucrose for 42 days. To elucidate the influence of high sucrose concentration on cryotolerance, different controls were included in the experiment: somatic embryos directly harvested from stock cultures (control), and the corresponding preculture controls consisting of somatic embryos cultured under the same preculture conditions but in culture medium containing standard sucrose concentration (20 g l^{-1}).

Plant samples representative of the structures obtained after the aforementioned treatments were collected from at least three independent cultures and immediately fixed in freshly prepared and precooled FAA [70% ethanol:37% formaldehyde:acetic acid:, 90:5:5 (v/v/v)] for approximately 72 h at 4°C. Fixed samples were subsequently dehydrated by immersion in a set of ethanol solutions of increasing concentration and embedded in paraffin. Serial tissue sections, 7 μm thick, were cut using a rotary microtome (Leica Biosystems, Nussloch, Germany, mod. RM2125) and floated on the surface of a distilled water bath at 37-40 °C to flatten them previous to be mounted on poly-lisinated glass slides. After passed through a schedule of de-waxing, sections were stained by the Gerlach procedure (Gerlach, 1969) for cellular activity and anatomical analysis. Accumulation of starch granules was monitored using periodic acid–Schiff reagent (PAS) (Arbeloa, 1986; Herrero, 1979).

Stained sections were analyzed with a Nikon microscope (mod. Eclipse E 800) and scanned with a VS120 virtual slide microscope (Olympus, Tokyo, Japan) using UPlanSApo 60× and 100× objectives.

2.4. *Data taken and statistical analysis*

Data of survival, recovery, regrowth rates and culture appearance were monitored during two successive recultures of 6 weeks each, 6 and 12 weeks after rewarming. Survival was assessed as the percentage of samples showing new tissue growth, while recovery was recorded as the percentage of explants showing embryogenesis resumption. The regrowth capacity was estimated by determining the fresh weight increase, i.e. the difference between the final and initial fresh weights, for each reculture cycle.

Percentage data were subjected to frequency analysis with an R × C test of independence or with a three-way log-linear analysis to evaluate interactions between variables. The rest of data were analyzed by one or two-way ANOVA, followed by mean comparison by the least significant difference (LSD) test. The significance level used was 0.05 in all cases (Sokal and Rohlf, 2013).

3. Results

3.1. *Somatic embryo cryopreservation*

3.1.1. *Effect of suspension density and culture time*

Survival and recovery data were identical in all cases, as in all surviving explants, new growing tissues exhibited embryogenic features.

Cryopreservation significantly affected olive somatic embryos recovery (Fig. 1). Six weeks after rewarming, only 10.53% of explants directly collected from stock cultures maintained in solid medium (control) showed signs of regrowth (Fig. 1A). Six weeks later, recovery percentage slightly increased up to 15.79% (Fig. 1B).

A culture step in liquid medium prior to cryopreservation improved recovery rates after thawing (Fig. 1). The results obtained revealed a significant influence of time in culture on cryopreservation success (Figs. 1 and 2, Table S1), with better results obtained with somatic embryos cultured in liquid medium for 28 days. Globally, lower recovery rates were achieved when somatic embryos were cultured in liquid medium for shorter or longer time periods (Fig. 1).

Inoculum density also affected cryopreservation, although a significant influence of this factor only could be inferred in regrowth data (Fig. 2, Table S1). Nevertheless, after 28 days in suspension culture, significantly different recovery percentages were attained depending on the inoculum size, with higher recovery values achieved at 0.2 g suspension density (Fig. 1). Overall, throughout the different time periods tested, slightly superior recovery results were obtained with 0.4 and 0.2 g inoculum densities (Fig. 1B). However, while recovery rates obtained with 0.2 g initial density significantly varied depending on the culture period, no significant differences were appreciated in somatic embryos derived from suspension cultures initiated with 0.4 g inoculum size (Fig. 1).

A significant time in culture x inoculum density interaction only was evident in regrowth data, 12 weeks after rewarming (Fig. 2B, Table S1), although different recovery percentages were achieved at 0.2 g inoculum density depending on time in culture (Fig. 1).

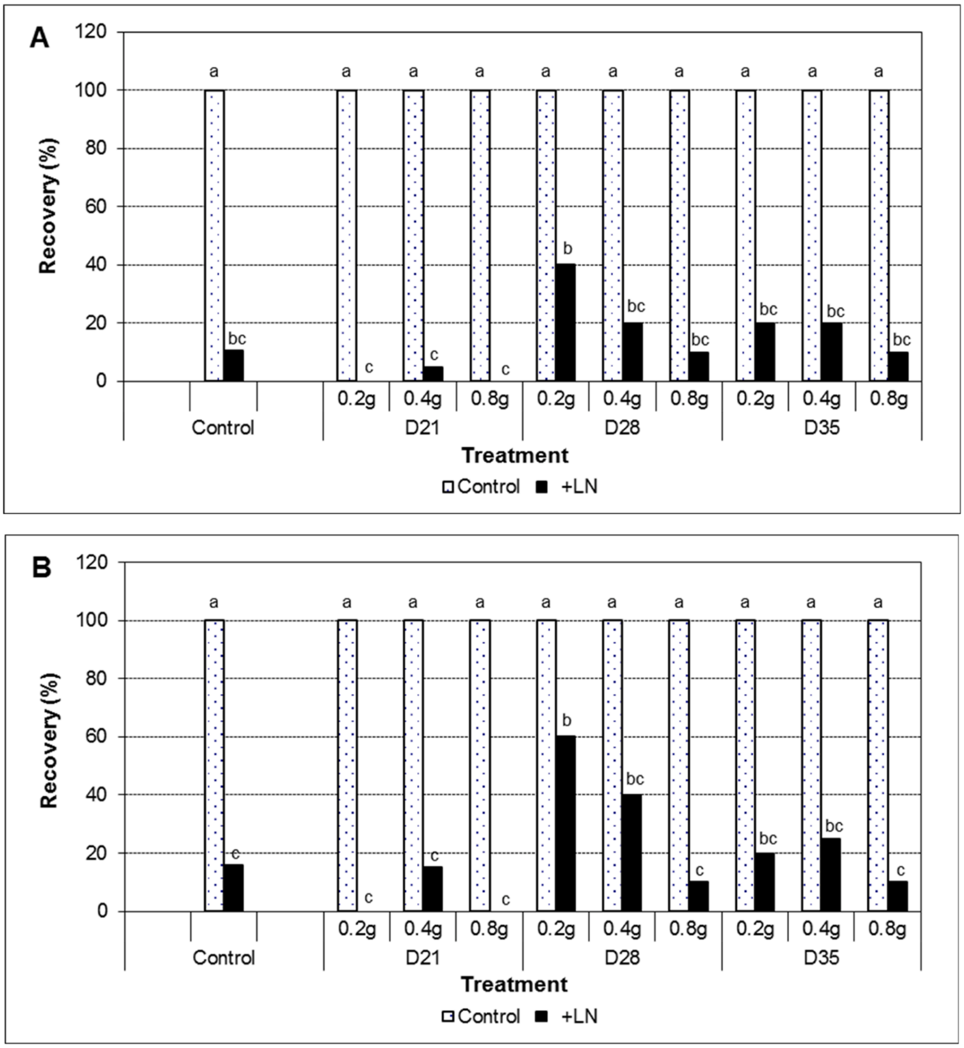

Fig. 1. Recovery rates of olive somatic embryos derived from suspension cultures established with different inoculum densities and cultured for different time periods, untreated (control) or subjected to cryopreservation (+LN). Assessments made A) 6 and B) 12 weeks after treatment. Different letters indicate significant differences by the R × C test with a significance level of 0.05.

3.1.2. Effect of a long-term sucrose preculture in solid medium

Long-term preculture on media containing high sucrose concentrations reduced regrowth of control, non-cryopreserved embryos (Fig. 3). After 6 weeks in proliferation medium, fresh weight increase of somatic embryos treated with 0.2 M sucrose was significantly lower than that of control, non-precultured embryos (Fig. 3A, Table S2). Nevertheless, 6 weeks later, no significant differences could be observed among non-treated embryos derived from the different sucrose pretreatments (Fig. 3B, Table S2). Preculture with high sucrose concentrations did not affect the embryogenic capacity of cultures, as all growing explants exhibited embryogenic features (Fig. 4).

In treated controls (-LN), sucrose preculture significantly improved explants regrowth 6 weeks after treatment with cryoprotective solutions (Fig. 3A, Table S2). However, 6 weeks later, a significant influence of sucrose preculture on tolerance to cryoprotectants was not evident, as no significant differences in the regrowth (Fig. 3B) and recovery (Fig. 4B) rates were found in embryos treated but not subjected to freezing (-LN) derived from different sucrose precultures (Table S2).

After rewarming of cryopreserved explants (+LN), a significant reduction of recovery was observed in non-precultured somatic embryos (Fig. 4A). Preculture on high-sucrose media significantly affected embryos response to cryopreservation (Fig. 4, Table S2). The best results were achieved after a 6-week pretreatment period with 0.2 M sucrose, with recovery rates significantly higher than those obtained in non-precultured explants (80% versus 20%) (Fig. 4B, Table S2). Intermediate values (33.33%), not significantly different from the control, were recorded in somatic embryos treated with 0.4 M sucrose.

Long-term preculture with high sucrose concentrations did not advance cultures response after rewarming, with first signs of growth resumption observed at 21-25 days, independently of the explant's pretreatment.

3.1.3. Effect of a long-term sucrose preculture in liquid medium

Long-term sucrose preculture in liquid medium significantly affected somatic embryos behavior in standard proliferation medium. Although incubation in 0.4 M sucrose for 28 days caused culture deterioration and a slight decline in recovery (Fig. 5), sucrose-pretreated embryos exhibited higher proliferation rates than untreated control (Fig. 6).

In relation to tolerance to cryoprotective solutions (-LN), pretreatment with 0.2 M sucrose slightly increased both culture recovery and explant regrowth in embryos that underwent treatment with cryoprotective solutions but not freezing (Figs. 5 and 6).

However, high-sucrose pretreatment in liquid medium had a significant effect on explants response to cryopreservation (+LN), with an increase in the recovery rate, from 40% in non-precultured explants to 90% in those treated with 0.2 M sucrose (Fig. 5B, Table S3). Besides,

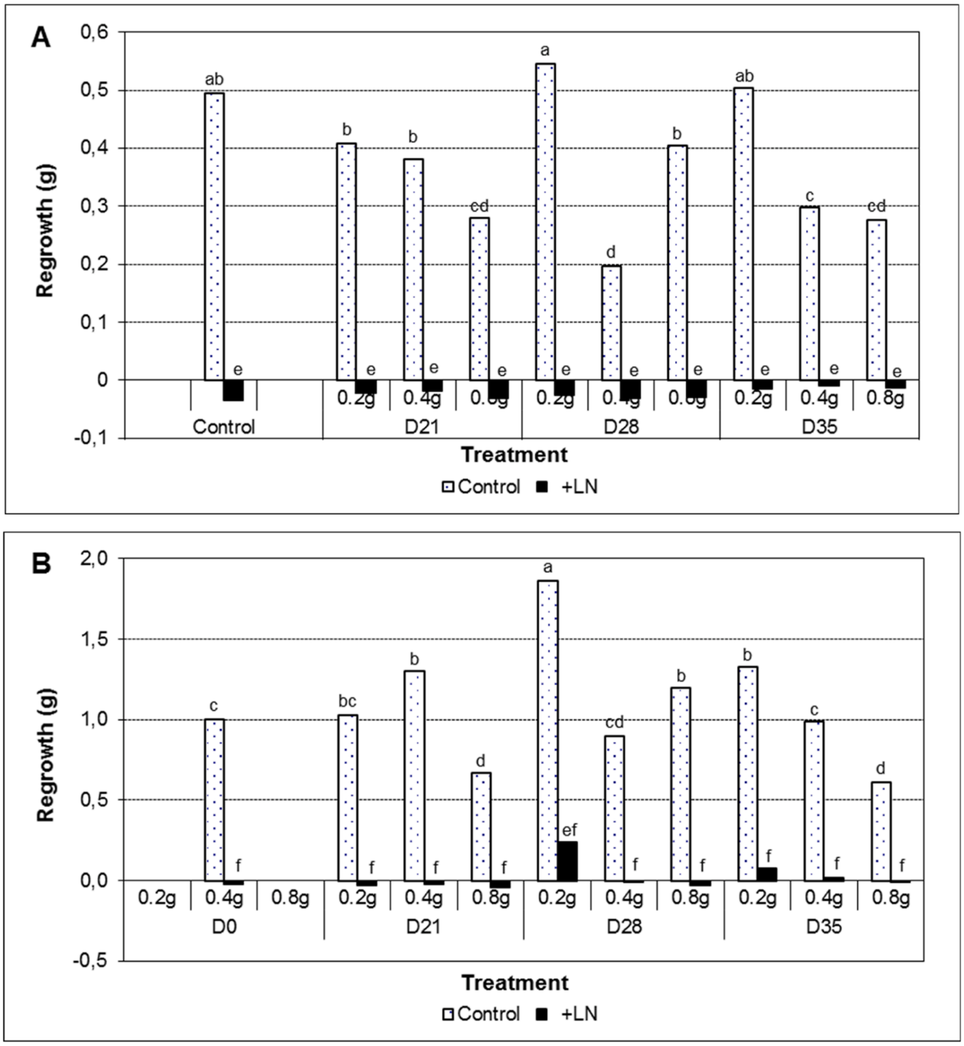

Fig. 2. Regrowth rates of olive somatic embryos derived from suspension cultures established with different inoculum densities and cultured for different time periods, untreated (control) or subjected to cryopreservation (+LN). Assessments made A) 6 and B) 12 weeks after treatment. Different letters indicate significant differences by the LSD test with a significance level of 0.05.

cultures established from somatic embryos frozen in liquid nitrogen after this treatment showed slightly higher proliferation capacity (Fig. 6B) than those grown from cryopreserved untreated explants. Incubation with 0.4 M sucrose had a negative effect, as no explant cryopreserved after this treatment survived freezing (Fig. 5).

Preculture in liquid ECO medium supplemented with 0.2 M sucrose for 28 days significantly accelerated explants response after cryopreservation, with signs of growth resumption 3 days after thawing, compared to 24-25 days in control, non-pretreated embryos. Surviving explants continued actively growing, exhibiting a normal proliferation pattern (Fig. 7).

3.2. Histological analysis

Control embryos, directly collected from stock cultures presented a smooth epidermis, formed by rectangular or isodiametric cells (Fig. 8A, B). In the absence of histodifferentiation signs, cells in the interior of the embryos were large and oval, with clear cytoplasm and rounded nucleus, which in many cells appeared displaced to the periphery, probably due to the large size of the vacuole (Fig. 8B).

Meristematic cells, characterized by being small in size and generally cubic, with a high nucleus/cytoplasm ratio, dense and little vacuolized cytoplasm, an intensely stained rounded nucleus and prominent nucleolus, were rarely observed at embryo surface and, when present, they were restricted to small specific areas. After 28 days of culture in standard liquid medium (Fig. 8C), increased proliferative activity was found in some regions, with actively dividing cells occupying several of the outermost cell layers (Fig. 8D). Aggregate formation and development of secondary somatic embryos could also be observed under these culture conditions (Fig. 8E). Embryos cultured in liquid medium with high sucrose concentration presented a similar morphogenic response although both processes, cell proliferation and aggregate formation, were clearly stimulated in sucrose-enriched medium (Fig. 8F). Somatic embryos cultured on standard solid medium for 42 days displayed few areas with meristematic activity (Fig 8G). Embryos cultured for 42 days on solid medium supplemented with 0.2 M sucrose frequently produced cellular aggregates, which were mostly composed by meristematic cells and globular somatic embryos, although more advanced developmental stages could also be reached (Fig. 8H).

Compared to control embryos (Fig. 9A, B), starch grains significantly increased in somatic embryos cultured in liquid medium for 28 days, presenting a homogenous distribution throughout the embryos surface (Fig. 9C). They appeared in both proliferative and non-proliferative areas, although important differences could be observed in their abundance and size, with larger and more abundant granules in meristematic cells (Fig. 9D). Higher starch accumulation was found in embryos

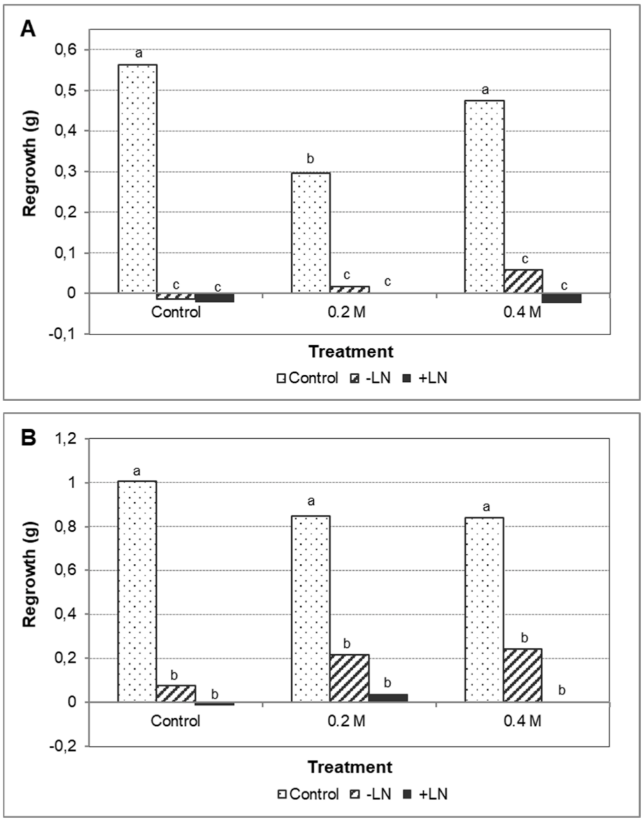

Fig. 3. Regrowth rates of olive somatic embryos after a 6-week preculture on solid medium supplemented with different sucrose concentrations, untreated (control) or subjected to cryoprotection, with (+LN) or without (-LN) subsequent immersion in liquid nitrogen. Assessments made A) 6 and B) 12 weeks after treatment. Different letters indicate significant differences by the LSD test with a significance level of 0.05.

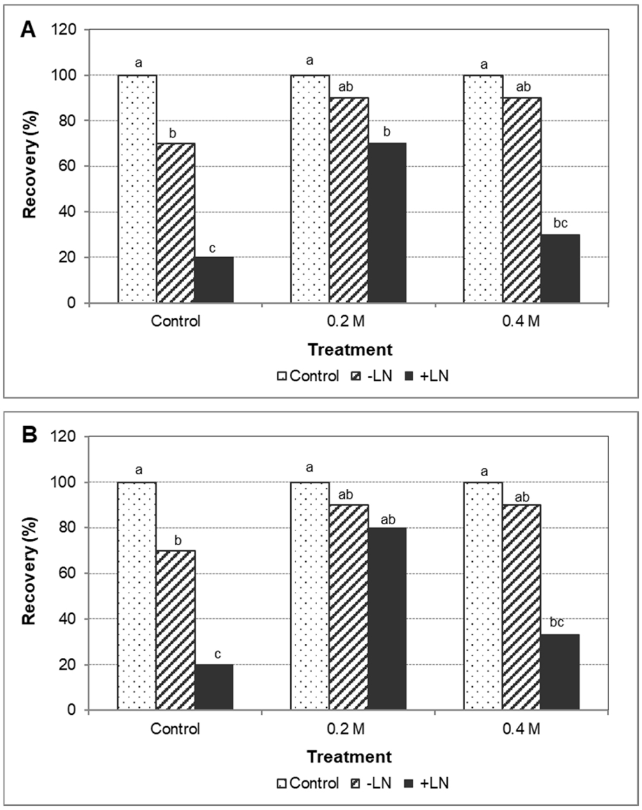

Fig. 4. Recovery rates of olive somatic embryos after a 6-week preculture on solid medium supplemented with different sucrose concentrations, untreated (control) or subjected to cryoprotection, with (+LN) or without (-LN) subsequent immersion in liquid nitrogen. Assessments made A) 6 and B) 12 weeks after treatment. Different letters indicate significant differences by the R × C test with a significance level of 0.05.

cultured in liquid medium supplemented with 0.2 M sucrose, compared to those maintained in liquid medium with standard sucrose concentration (Fig. 9E, F). As found in liquid media, a higher number of starch grains could be observed in solid medium when sucrose concentration was increased (Fig. 9G, H).

4. Discussion

Sucrose preculture significantly improved cryopreservation of olive somatic embryos. The best results were obtained after preculture in liquid ECO medium supplemented with 0.2 M sucrose for 28 days, with 90% of samples recovered six weeks after rewarming.

In the present investigation, a significant effect of time in culture, inoculum density and the interaction time in culture x inoculum density was found when cryopreservation was evaluated as regrowth rate, although a statistically significant influence on the recovery rate could only be inferred for time in culture.

The state of growth has a critical influence on cryopreservation survival (Bajaj, 1976; Sala et al., 2006; Withers and Street, 2006). Therefore, growth conditions during explants culture are decisive in their subsequent cryopreservation response (Wu et al., 2007). Different variables define growth conditions used for somatic embryos culture.

The inoculum density is a very important factor determining the growth pattern and kinetics of embryogenic suspensions. In avocado, significantly different relative fresh weight increases were obtained after a culture cycle (Márquez-Martín et al., 2012). In grapevine, it has proposed that the initial cell population density may play a modulating role in the embryogenic process by influencing the secretion of extracellular proteins with positive or negative effects on proliferation (Maës et al., 1997).

Culture period also has an important influence on cryopreservation. As time in culture progresses, batch cultures pass by different growth phases which exhibit a differential response to cryopreservation (Find et al., 1998). Variations in the morphology and composition of embryogenic suspensions have been reported with culture duration (Jiménez et al., 2013). Cell composition has a determinant influence on cryopreservation survival as only small and slightly vacuolated cells survive freezing, while large and highly vacuolated cells are more prone to cryoinjuries (Lambardi et al., 2008). Moreover, both the period of time in culture (Heine-Dobbernack et al., 2008) and inoculum density (Márquez-Martín et al., 2012) may affect the physiological state of embryogenic cultures, an important factor determining cryotolerance (Engelmann et al., 2008a). Important processes influencing cryopreservation, such as the dehydration tolerance and the capacity for producing vigorous recovery growth after freezing can vary depending on the physiological state of cells and tissues (Sakai and Engelmann, 2007).

Sucrose preculture played an important role in cryopreservation of olive somatic embryos. Incubation in culture media supplemented with 0.2 M sucrose for 28 days in liquid medium or 42 days in solid medium significantly improved cryopreservation results.

As revealed by the histological analysis, preculture in sucrose-enriched media increased the proliferation activity in olive somatic embryos, augmenting the areas with actively dividing cells, regardless of the culture method, in liquid or solid medium. Similar results were found in other species, such as iris (Jéhan et al., 1994) melon (Nakagawa et al., 2001) or *Macrotyloma uniflorum* (Mohamed et al., 2005). In fact, increasing proliferation may be one of the ways in which sucrose can improve cryotolerance.

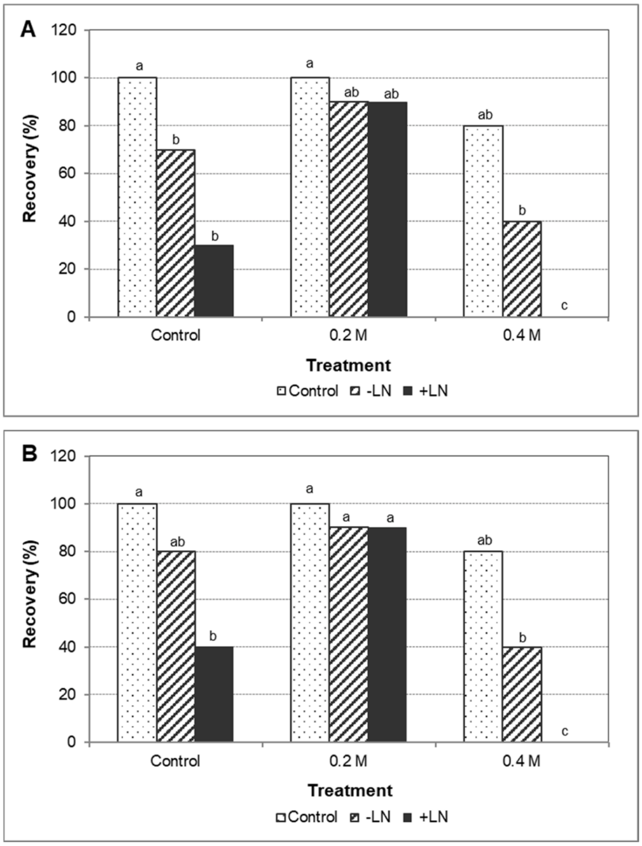

Fig. 5. Recovery rates of olive somatic embryos after a 28-day preculture in liquid medium supplemented with different sucrose concentrations, untreated (control) or subjected to cryoprotection, with (+LN) or without (-LN) subsequent immersion in liquid nitrogen. Assessments made A) 6 and B) 12 weeks after treatment. Different letters indicate significant differences by the R × C test with a significance level of 0.05.

The carbohydrate source and its concentration in the culture medium play an important role modulating *in vitro* morphogenesis, with a significant influence on the nature and intensity of the *in vitro* explant's response (El Maataoui et al., 1998). Sucrose is considered as a highly callogenic sugar, due to its great capacity to induce cell proliferation (El Maataoui et al., 1998). In this line, Farrar et al. (2000) found that sucrose can induce resumption of cell division arrested in the G1 and G2 phases. Furthermore, carbohydrates can act as carbon and energy sources, thus favoring proliferation (Nakagawa et al., 2001).

Explants with stimulated cell proliferation exhibited a higher proportion of meristematic and embryogenic cells, characterized by their small size, high nucleus/cytoplasm ratio, and little vacuolized cytoplasm; cell features that make them more prone to survive cryopreservation, compared to large, highly-vacuolized cells, mostly composing differentiated tissues (Lambardi et al., 2008). Differences in water content observed between both cell types (Pérez, 2000) can also explain their differential response, as formation of intracellular ice crystals is widely considered to be one of the most damaging events during cryopreservation (Panis, 1995). Moreover, more hydrated cells require stronger dehydration treatments, with higher risks of severe injuries such cell wall breakage and plasmalemma detachment. Important differences are also observed related to their physiological state, which, as previously indicated, has a significant influence on the final cryopreservation results (Engelmann et al., 2008b). Successful cryopreservation is not only determined by cell survival after thawing but also by the proliferation capacity of surviving cells. While meristematic cells, characterized by high metabolic activity and proliferation rate, are programmed to continue undergoing mitotic cycles, differentiated cells are involved in a development program in which cell divisions are not frequent.

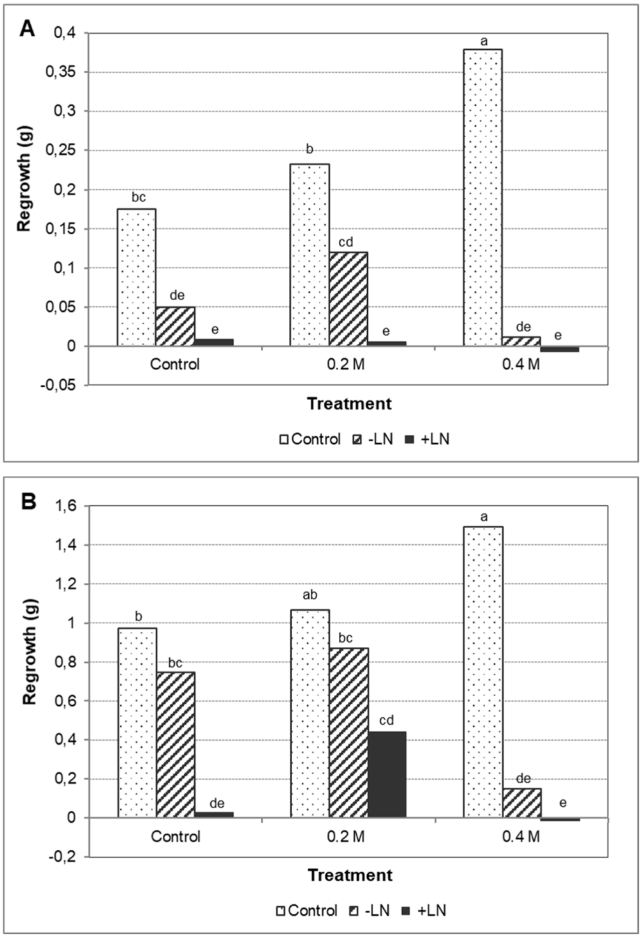

Fig. 6. Regrowth rates of olive somatic embryos after a 28-day preculture in liquid medium supplemented with different sucrose concentrations, untreated (control) or subjected to cryoprotection, with (+LN) or without (-LN) subsequent immersion in liquid nitrogen. Assessments made A) 6 and B) 12 weeks after treatment. Different letters indicate significant differences by the LSD test with a significance level of 0.05.

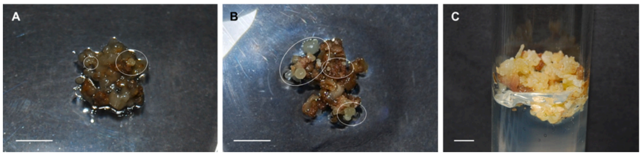

Fig. 7. Embryogenesis resumption after cryopreservation of olive somatic embryos A) directly derived from stocks cultures and B) after a 28-day preculture in liquid medium supplemented with 0.2 M sucrose. C) General aspect of embryogenic cultures derived from cryopreserved somatic embryos. Bars = 5 mm.

Nevertheless, although increased proliferation can help to explain improved recovery rates observed in sucrose-precultured explants, other mechanisms could be involved in sucrose contribution to acquisition of cryopreservation tolerance. Thus, preculture in high sucrose concentration influences the water status of cryopreservation explants. The reduced osmotic potential of sucrose-enriched media has a direct effect decreasing tissue water content and, consequently, the amount of intracellular freezable water (Panis and Thinh, 2001), thus avoiding detrimental ice crystal formation during freezing. Moreover, concentration of the intracellular solution depresses the freezing point

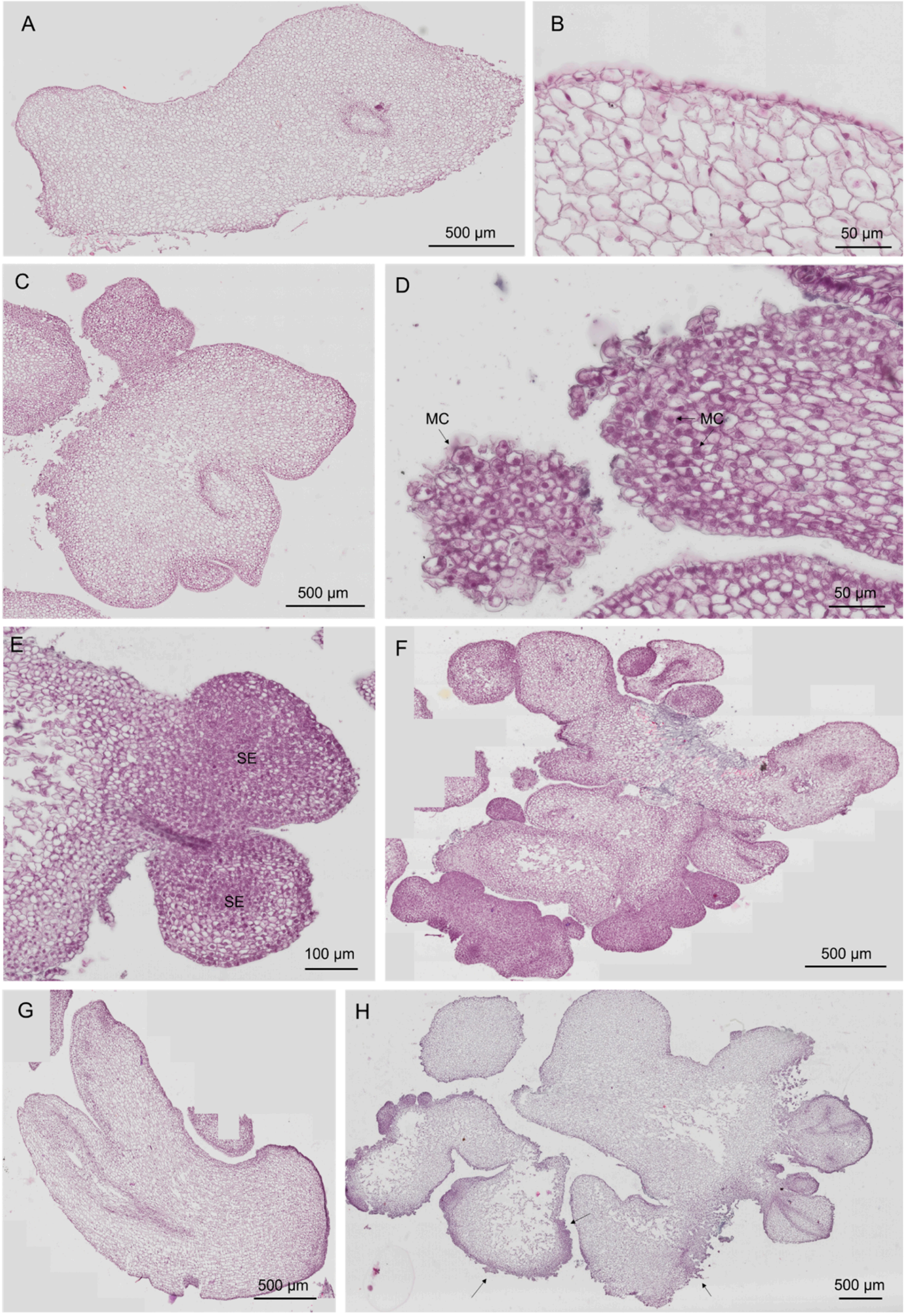

Fig. 8. Light micrographs of olive somatic embryos stained with Gerlach. A) General view and B) detail of control embryos directly collected from stock cultures at the end of a maintenance cycle. C) General view of embryos cultured in liquid ECO medium for 28 days, and details of D) a proliferative region with abundant meristematic cells (MC), and E) development of secondary embryos (SE). General views of F) embryos cultured in liquid ECO medium supplemented with 0.2 M sucrose for 28 days, G) embryos cultured on solidified ECO medium for 42 days and H) embryos cultured on solidified ECO medium supplemented with 0.2 M sucrose for 42 days. Meristematic activity indicated by arrows.

(Benson, 2008). These effects, which have been described in somatic embryos of different species, such as cocoa (Fang et al., 2009) and oil palm (Palanyandy et al., 2020), could prepare tissues for cryopreservation (Folgado et al., 2015).

Increased levels of endogenous sugars have also been observed after sucrose treatment in different types of explants, including somatic embryos (Fang et al., 2009; Lynch et al., 2011). Accumulated sugars play a fundamental role protecting cell membranes and proteins during dehydration and freezing (Hoekstra et al., 2001; Oliver et al., 2002). Sugars replace water present in the hydrate state when the hydration shell is gradually lost, thus helping to tolerate dehydration (Crowe et al., 1998). Furthermore, the interaction of endogenous sucrose accumulated following sucrose treatments with intracellular water through hydrogen and multimolecular linkages could increase the non-freezable water content (Hitmi et al., 2000).

In olive somatic embryos, the histological analysis showed higher starch accumulation after incubation in culture media with high sucrose concentration. Similar results have been described in *Albizzia julibrissin* (El Maataoui et al., 1998) and *Phoenix dactylifera* (Bagniol et al., 1992). As found in olive, in these cases, starch grains were also mainly located in superficial cells (Bagniol et al., 1992), belonging to calli and neoformed meristems (El Maataoui et al., 1998).

Moreover, sucrose has been identified as an important signaling molecule regulating metabolism and gene expression (Carpentier et al., 2010; Gupta and Kaur, 2005; Koch, 1996; Wang et al., 2020). Thus, sucrose treatments can increase tolerance to dehydration and cooling by

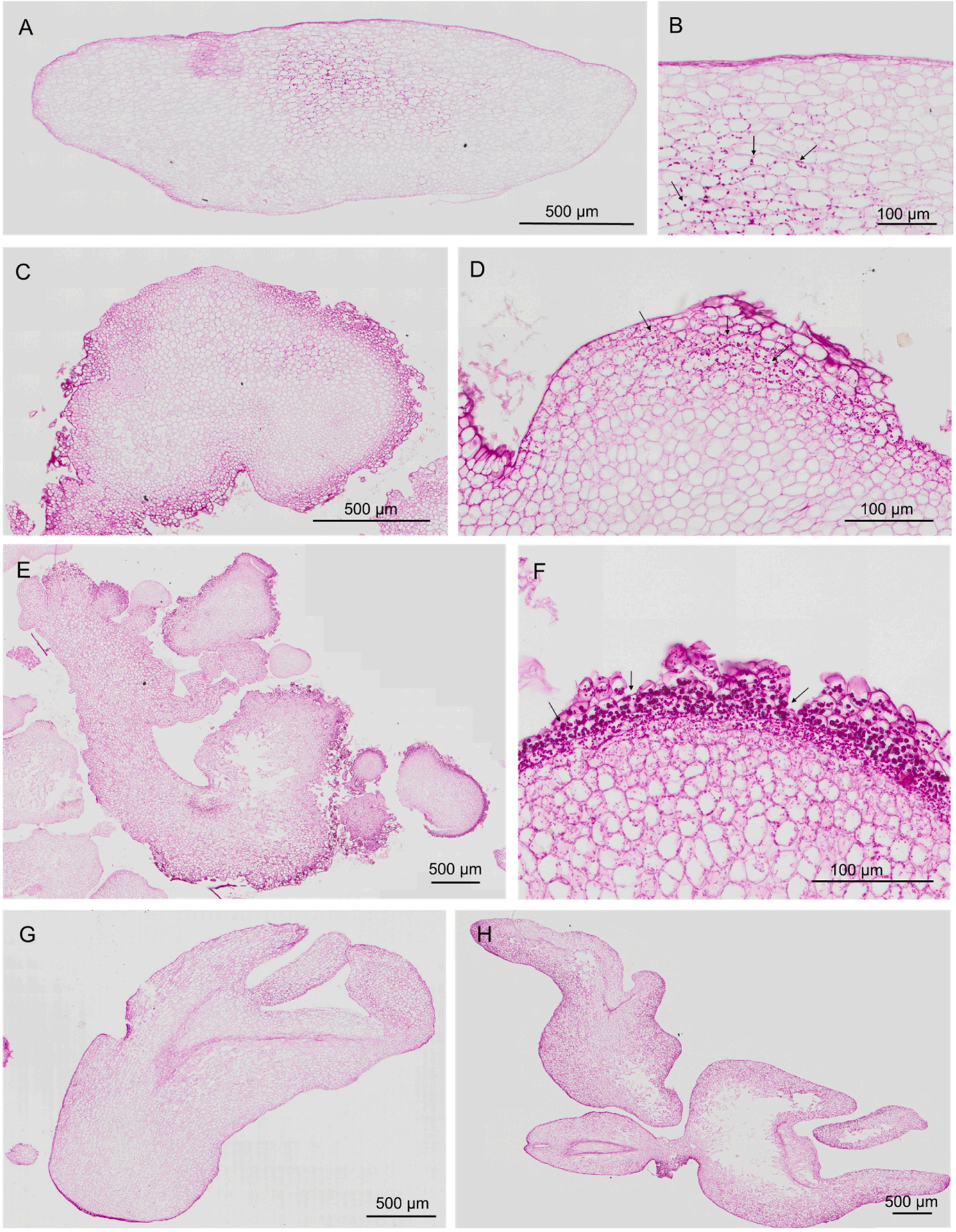

Fig. 9. Light micrographs of olive somatic embryos stained with PAS. A) General view and B) detail of control embryos directly collected from stock cultures at the end of a maintenance cycle. C) General view and D) detail of embryos cultured in liquid ECO medium for 28 days. E) General view and F) detail of embryos cultured in liquid ECO medium supplemented with 0.2 M sucrose for 28 days. General view of embryos cultured for 42 days on G) solidified ECO medium with standard sucrose concentration, and H) solidified ECO medium supplemented with 0.2 M sucrose. Starch granules indicated by arrows.

inducing changes at multiple levels, such as: 1) Provoking modifications in the proteome, with variations in the abundance of specific proteins (Carpentier et al., 2010; Folgado et al., 2015). 2) Inducing alterations in fatty acids metabolism, causing changes in the membrane components (Ramon et al., 2002; Zhu et al., 2006). 3) Increasing polyamines levels, which may act as radical scavengers and bind to important cellular components, such as DNA, RNA, phospholipids, acidic protein residues and cell wall constituents (Ramon et al., 2002). 4) Inducing the accumulation of compatible solutes, as proline (Pociecha et al., 2009; Suzuki et al., 2006). 5) Causing a transient increase of abscisic acid levels (Suzuki et al., 2006).

Nevertheless, sucrose treatments can become toxic when relatively high concentrations are used or they are applied for prolonged periods. In fact, high sucrose sensitivity has been previously reported in somatic embryos of different species (Danso and Ford-Lloyd, 2004; Vasanth and Vivier, 2011) In olive somatic embryos, preculture in liquid medium supplemented with 0.4 M sucrose had a dramatic effect on cryostorage. Although this treatment only slightly reduced survival of control non-cryoprotected explants, it significantly decreased embryogenesis resumption after cryoprotection, and no survival was accomplished after freezing. Nonetheless, this prejudicial effect was not evident in somatic embryos cultured in solid medium supplemented with 0.4 M sucrose. Culture in liquid medium favors the close contact of the tissues with the culture medium (Soomro and Memon, 2007), thus enhancing the availability of medium components (Moscatiello et al., 2013) and improving their absorption (Ibrahim and Tresniawati, 2020). These characteristics could explain the differential response observed depending on culture method.

In conclusion, preculture with 0.2 M sucrose for 28 days in liquid ECO medium or 42 days in solid ECO medium significantly improved cryopreservation of olive somatic embryos following the droplet-vitrification method. Higher recovery rates, faster response after cryopreservation and increased regrowth capacity obtained allows the practical application of cryopreservation to olive breeding programs using non-conventional techniques.

CRediT authorship contribution statement

Fatiha Bradaï: Investigation, Formal analysis, Visualization. **Javier Almagro-Bastante:** Investigation, Formal analysis. **Carolina Sánchez-Romero:** Conceptualization, Formal analysis, Methodology, Supervision, Visualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors are unable or have chosen not to specify which data has been used.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scienta.2023.112385.

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