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ORIGINAL PAPER

Enhancing frequency of regeneration of somatic embryos of avocado (*Persea americana* Mill.) using semi-permeable cellulose acetate membranes

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Abstract Regeneration of avocado via somatic embryogenesis is difficult due to poor embryo maturation, resulting in low frequencies of germination. In this study, the influence of semi-permeable cellulose acetate membranes and culture media, containing high levels of sucrose along with coconut water, on maturation and germination of somatic embryos of avocado have been evaluated. The culture of embryogenic calli on top of cellulose acetate membranes significantly increased the number of mature, white-opaque embryos that were recovered after 5 weeks of culture. These embryos showed a much more normal appearance and better quality compared with the control embryos, although the embryo size was significantly reduced. To increase the embryo size and to complete maturation, several two-step maturation treatments were tested. The culture of white-opaque somatic embryos in a modified MS medium with B5 macronutrients gelled with 10 g L^{-1} agar (B5m10A medium) over a 5-week period, followed by 5 additional weeks in B5m10A with 45 g L^{-1} sucrose and 20 % coconut water, yielded the best results, reducing the percentage of necrotic embryos and the number of calli formed. The beneficial effects of this maturation treatment were enhanced when using embryos that were pre-matured on cellulose acetate membranes. Following this two-step maturation treatment, the germination rate of the control somatic embryos, which were not cultured on cellulose membranes, was lower than 10 %, but it significantly improved when the embryos had been pre-matured on cellulose acetate membranes for 5 weeks, reaching a germination rate close to 40 %. The water availability was significantly reduced when somatic embryos were cultured on cellulose membranes, and after this pre-maturation treatment, the white-opaque embryos showed lower water potential and ABA content compared with the control embryos. These results suggest that culturing over cellulose membranes causes a controlled embryo desiccation that enhances the recovery of plants.

Keywords Cellulose semi-permeable membrane · In vitro plant regeneration · Somatic embryogenesis · Somatic embryo maturation · Water potential

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Introduction

Avocado, *Persea americana* Mill., is one of the most important fruits in the world, with an annual production estimated to be 3.5 million tons in 2008 (FAOSTAT 2010). Mexico, Chile, the United States and Indonesia are the leading producers of avocado. This species is highly heterozygous, with a long juvenile phase and a high rate of flower abscission and immature fruit drop (Litz et al. 2005). Due to these problems, breeding programs have been relatively unsuccessful, and the most important cultivars have been obtained by open-pollinated tree selection (Litz et al. 2007). Biotechnological approaches involving in vitro culture, such as genetic transformation and somaclonal variation, could be used to genetically improve this species. Furthermore, in vitro culture techniques, e.g., the micro propagation of elite selections or embryo rescue from selected crosses, could also be used in conventional breeding (Márquez-Martín et al. 2009; Pliego-Alfaro et al. 2013). However, these tools are hampered by the recalcitrance of avocado tissues to in vitro regeneration.

Journal : Large 11240 | Dispatch : 9-7-2013 | Pages : 9
Article No. : 352 | ☐ LE | ☐ TYPESET
MS Code : PCTO-D-13-00186 | ☑ CP | ☑ DISK

In avocado, somatic embryogenesis is the method that is most frequently employed for adventitious plant regeneration (Litz et al. 2005). Embryogenic cultures derived from immature zygotic embryos of different avocado genotypes have been obtained (Pliego-Alfaro and Murashige 1988; Sánchez-Romero et al. 2006; Witjaksono and Litz 1999a). However, the conversion of somatic embryos (SE) occurs at very low rates of 5–11 % (Sánchez-Romero et al. 2006; Pliego-Alfaro et al. 2013). Pliego-Alfaro and Murashige (1988) ascribed the low conversion rate of avocado SE to an abnormal development caused by the failure of the shoot apical meristem to become organized.

Somatic embryos should undergo a maturation phase during which induced biochemical and morphological changes will later facilitate plant conversion (Ammirato 1987). These changes involve the accumulation of storage proteins, the repression of precocious germination and the acquisition of desiccation tolerance (Jiménez 2005; Braybrook and Harada 2008). Generally, the accumulation of storage products turns translucent embryos into white-opaque embryos. This change has been used as a maturity indicator in several species, including avocado (Cailloux et al. 1996; Witjaksono and Litz 1999b; Perán-Quesada et al. 2004; Cerezo et al. 2011). Few attempts to study the factors that are involved in the maturation of avocado SE have been reported thus far. It has been shown that supplementing the maturation medium with filter-sterilized coconut water or increasing its sucrose concentration improves the quality of avocado SE (Witjaksono and Litz 2002; Perán-Quesada et al. 2004). Márquez-Martín et al. (2011) studied the effect of gelling agent types and concentrations, as well as the addition of osmotic agents, on SE maturation. In general, the maturation response of avocado SE was improved in culture media with high concentrations of a gelling agent. In contrast, the addition of polyethylene glycol (PEG) or sorbitol did not positively affect the SE maturation (Márquez-Martín et al. 2011). Apparently, the benefits of culture media with a high gelling agent concentration on the maturation of avocado SE are related to increased gel strength and decreased water availability. An alternative approach to obtaining mature SE by reducing water availability consists in the culture of SE on top of cellulose acetate semi-permeable membranes. This procedure has been successfully used by Niedz et al. (2002) and Cerezo et al. (2011) to normalize the development of citrus and olive SE, respectively.

The aim of this investigation was to develop an improved regeneration system for avocado SE. To achieve this goal, several factors affecting SE maturation, such as pre-maturation on cellulose acetate membranes or culture on media supplemented with coconut water and high sucrose, were analyzed. Furthermore, the effect of membrane treatments on the water status of SE was evaluated.

Materials and methods

Plant material and culture conditions

An embryogenic avocado (*P. americana* Mill.) culture line, D2.3, was established from an immature zygotic embryo, cv. 'Duke 7', according to Pliego-Alfaro and Murashige (1988) on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) that was supplemented with 0.41 μM picloram (MSP medium) and solidified with 6 g L^{-1} agar (Sigma A-1296). The D2.3 culture was incubated in darkness at 25 ± 1 °C. The embryogenic cultures were maintained in MSP medium and were subcultured at monthly intervals.

Treatments for somatic embryo maturation

For maturation, embryogenic suspensions were initiated following the protocol of Márquez-Martín et al. (2012). Approximately 0.4 g friable callus was used to inoculate 40 mL liquid MSP medium in 100-mL Erlenmeyer flasks. The suspensions were incubated in an orbital shaker (120 rpm) under a low irradiance level (5 μmol m^{-2} s^{-1}) with a 16-h photoperiod at 25 °C. After 9 days, the cultures were sequentially sieved through 2- and 1-mm pore mesh, selecting the fraction that was retained in the 1-mm pore mesh. To test the effect of the cellulose acetate semi-permeable membranes on SE maturation, 100 mg of the selected fraction was cultured on top of 4 × 4 cm dialysis tubing cellulose acetate membranes (MW cut-off 12,000, Sigma D9777) in 120-mL jars containing 40 mL B5m10A maturation medium [MS formulation with macronutrients from Gamborg et al. (1968), gelled with 10 g L^{-1} agar] (Márquez-Martín et al. 2011). The membranes were prepared following the manufacturer's instructions and were autoclaved twice in distilled water at 121 °C for 20 min. The somatic embryos were cultured on the membranes for 5 weeks, and the number of white-opaque somatic embryos (WOSE) was recorded after this culture period.

In a different experiment, the effect of culturing WOSE in several maturation media to increase their size prior to germination was evaluated. In this experiment, the WOSE that were cultured in B5m10A medium for 5 weeks, with or without the cellulose acetate membranes, were subjected to the following treatments: 1) 2 cycles of a 5-week culture in B5m10A; 2) 5 weeks of culture in B5m10A followed by 5 weeks in B5m10A-S-CW (B5m10A medium supplemented with 45 % sucrose and 20 % [v/v] filter-sterilized coconut water [Sigma, C5915]); and 3) 2 cycles of a 5-week culture in B5m10A-S-CW. The number of mature WOSE, their size and the presence of calli were recorded at the end of the maturation period. A total of 25 WOSE per treatment were used, and the experiment was conducted twice.

Journal : Large 11240 Dispatch : 9-7-2013 Pages : 9
Article No. : 352 LE TYPESET
MS Code : PCTO-D-13-00186 CP DISK

Somatic embryo germination

White-opaque SE with diameters greater than 5 mm, derived from SE pre-matured with or without the cellulose acetate membranes as described above, and later matured for 5 weeks in B5m10A followed by an additional 5-week period in B5m10A-S-CW, were germinated as described by Palomo-Ríos et al. (2012). The WOSE were transferred to liquid germination medium, MS medium supplemented with 4.44 μM 6-benzyladenine (BA) and 2.89 μM gibberellic acid (GA_3) (Witjaksono and Litz 1999a) for 3 days in a roller drum and then cultured in the same medium gelled with 6 g L^{-1} agar for 4 weeks. This treatment was repeated three times. A total of 30 WOSE per treatment were used, and the experiment was conducted twice.

Water potential measurements

The water potential (Ψw) in the control embryos and the embryos pre-matured on cellulose acetate membranes was measured using a Wescor Dew Point Microvoltmeter HR-33T in dew point mode. The isolated embryos 3 mm in length were incubated in a C-52 sample chamber, and the dew point depression was recorded after 35 min of equilibration. The Ψw was estimated in the control and membrane-treated WOSE after 5 weeks of culture in B5m10A and after an additional incubation period in Petri dishes with 100 % relative humidity for 48 h. A minimum of 19 embryos per treatment were measured.

Abscisic acid content

The abscisic acid (ABA) content was measured in the WOSE that were pre-matured with or without cellulose acetate membranes after 5 weeks of culture in B5m10A. The ABA was extracted following the procedure of Walker-Simmons et al. (2000). The somatic embryos (50 mg per treatment) were frozen in liquid nitrogen, lyophilized, and extracted with 5 mL methanol for 20 h at 4 °C. Then, the samples were centrifuged at 9,000×*g* for 10 min, and the amount of ABA in the supernatant was estimated using the ELISA Phytodetek ABA Test Kit (Agdia, USA), following the manufacturer's instructions. Two independent extractions per treatment and four measurements per extraction were performed.

Water availability of the culture medium

The effect of the cellulose membranes on the water availability was estimated by measuring the amount of water that was absorbed by 2 × 2 cm filter paper laid on the surface of the B5m10A culture medium with or without cellulose membranes. The filter papers were incubated at 25 °C for 24 h. Then, the water was removed by incubating the papers at 100 °C for 24 h. The amount of absorbed water was estimated as the difference in filter paper weight. Each treatment contained 6 replicates.

The matric potential (Ψ_m) in 0.5-cm-diameter filter papers that were incubated on the surface of the B5m10A medium with or without cellulose acetate membranes for 24 h was measured using a Wescor Dew Point Microvoltmeter HR-33T in dew point mode. The papers were incubated in a C-52 sample chamber, and the dew point depression was recorded after 5 min of equilibration. A total of 10 replicates per treatment were used.

Statistical analysis

The data were subjected to analysis of variance (ANOVA) using SPSS software. Tests for normality and homogeneity of variance were performed prior to the ANOVA, and Student's *t* test or the Mann–Whitney *U* test was used for the mean separation in the case of homogeneous or non-homogeneous variance, respectively. Frequency analyses were performed using the χ^2 test (Sokal and Rohlf 1995).

Results

Effect of cellulose acetate membranes on embryo pre-maturation

Embryogenic calli were cultured on top of cellulose acetate membranes that were placed on B5m10A medium, and the number of white-opaque SE (WOSE) was recorded after 5 weeks of culture. In the control treatment without the membrane, a mean number of 5.3 WOSE per 100 mg of calli was obtained, with 13 % of these embryos being larger than 5 mm (Table 1). A significantly higher number of WOSE was obtained when the calli were cultured over the cellulose acetate membrane (Table 1). However, all of the WOSE that were collected over the membrane were 55 % smaller than the control embryos, and none reached 5 mm in length (Table 1). Interestingly, the mature embryos that were collected over the membrane treatment were of a superior quality compared with the control embryos. The appearance of the control WOSE was rough, while the embryos that were matured over membrane were smooth with a white surface (Fig. 1a, b). Additionally, culturing on the cellulose membranes significantly reduced the amount of proliferating calli (Table 1).

Effect of maturation treatments

The optimum size for somatic embryo germination is in the range of 4–5 mm (Perán-Quesada et al. 2004;

Journal : Large 11240 Dispatch : 9-7-2013 Pages : 9
Article No. : 352 LE TYPESET
MS Code : PCTO-D-13-00186 CP DISK

Table 1 Effect of cellulose acetate membranes on the pre-maturation of avocado SE

	Control	Membrane
Number of WOSE/100 mg calli		
<5 mm	4.6 ± 0.6 b	7.1 ± 0.5 a
≥5 mm	0.7 ± 0.9 a	0 b
Total	5.3 ± 0.7 b	7.1 ± 0.5 a
Mean WOSE size (mm)	2.7 ± 0.1 a	1.2 ± 0.3 b
Amount of calli (g/100 mg)	1.1 ± 0.0 a	0.1 ± 0.0 b

A total of 100 mg calli was cultured on B5m10A medium either without (control) or with the cellulose acetate membranes. The number of mature, white-opaque SE (WOSE); their size; and the amount of calli formed were recorded after 5 weeks of culture

Data correspond to the mean ± SE. Means with different letters within rows are significantly different using the Mann–Whitney *U* test at $P = 0.05$

Márquez-Martín et al. 2011). After 5 weeks of culture in B5m10A, independent of the use of the cellulose acetate membranes, most of the WOSE were smaller than 5 mm. Thus, several 2-step maturation treatments were tested to increase the embryo size: (1) two cycles of a 5-week culture in B5m10A (treatment 1); (2) a 5-week culture in B5m10A followed by another 5-week period in B5m10A medium supplemented with 45 g L^{-1} sucrose and 20 % (v/v) coconut water (B5m10A-S-CW) (treatment 2); and (3) two cycles of a 5-week culture in B5m10A-S-CW medium (treatment 3). In the embryos that were obtained without cellulose membranes, the mean size after 10 weeks of culture was similar in the three maturation treatments (Fig. 2a). Similarly, no differences in embryo size were observed in the three maturation treatments when using embryos that were pre-matured on cellulose membranes (Fig. 2a). However, these embryos were slightly smaller than the control WOSE, although the differences were not statistically significant. The average embryo size was 5 mm and 6 mm in embryos that were pre-matured with and without the cellulose acetate membranes, respectively. Treatments 2 and 3 (maturation in a medium with high sucrose and coconut water) increased the percentage of SE that formed calli when using the embryos that were not pre-matured on cellulose membranes (Fig. 2b); however, this effect was not observed in the WOSE that were cultured on the membrane, and this percentage was even lower in the treatments using B5m10A-S-CW medium than in treatment 1 (10 weeks in B5m10A), although the differences were not statistically significant. Finally, lower percentages of explants that were showing signs of necrosis were observed in the WOSE that were cultured for 5 weeks in B5m10A followed by culturing in B5m10A-S-CW compared with those in the other treatments, although the differences with treatment 3, in the case of membrane-treated SE, were not statistically significant. The reduction of necrosis was enhanced by the use of the cellulose membranes in SE pre-maturation (Fig. 2c). Therefore, based on these observations, treatment 2 was chosen for the maturation of WOSE. The appearances of the SE that were pre-matured with or without the membranes and that were matured in B5m10A followed by B5m10A-S-CW are shown in Fig. 1c, d.

Effect of cellulose acetate membranes on embryo germination

The WOSE whose size was ≥5 mm and that were pre-matured with or without (Control) the cellulose acetate membranes and matured following the optimal media sequence described above were subjected to the germination protocol reported by Palomo-Ríos et al. (2012). The germination frequency in the control embryos was lower than 10 % (Fig. 3). The use of the membranes to pre-mature the SE significantly increased the germination rate, which reached values close to 40 %. In most cases, only shoot emergence was observed, while the shoot and the root rarely appeared simultaneously. The shoots obtained were weaker than in the plants that were derived from zygotic embryos, as is normal in this species (Sánchez-Romero et al. 2006); however, for plant recovery, these shoots were multiplied and rooted following the protocol of Barceló-Muñoz et al. (1999). Figure 1e, f shows the germinated SE at different developmental stages, and Fig. 1g shows an acclimated plant that was derived from embryos that had been previously matured on the membrane.

Water potential measurements and ABA content

To gain insight regarding the role of cellulose acetate membranes in the pre-maturation process of avocado SE, the effect of these membranes on the water availability of the B5m10A medium, as well as the water potential and abscisic acid content in the pre-matured SE, were measured after the 5-week culture period in this medium. The effect of the cellulose membranes on the water availability was determined by measuring the amount of water that was absorbed by the filter papers that were overlaid on the gel or on the membrane surface. The amount of water that was absorbed by the filter papers that were placed on the cellulose membranes was 2.8-fold lower than in those located in the surface of the medium: 0.023 ± 0.002 versus 0.064 ± 0.003 g in the membrane and control treatments, respectively. Moreover, the matric potential (Ψm) in the filter papers was significantly lower in those that were incubated over the membrane, with average values of

Journal : Large 11240 Dispatch : 9-7-2013 Pages : 9
Article No. : 352 LE TYPESET
MS Code : PCTO-D-13-00186 CP DISK

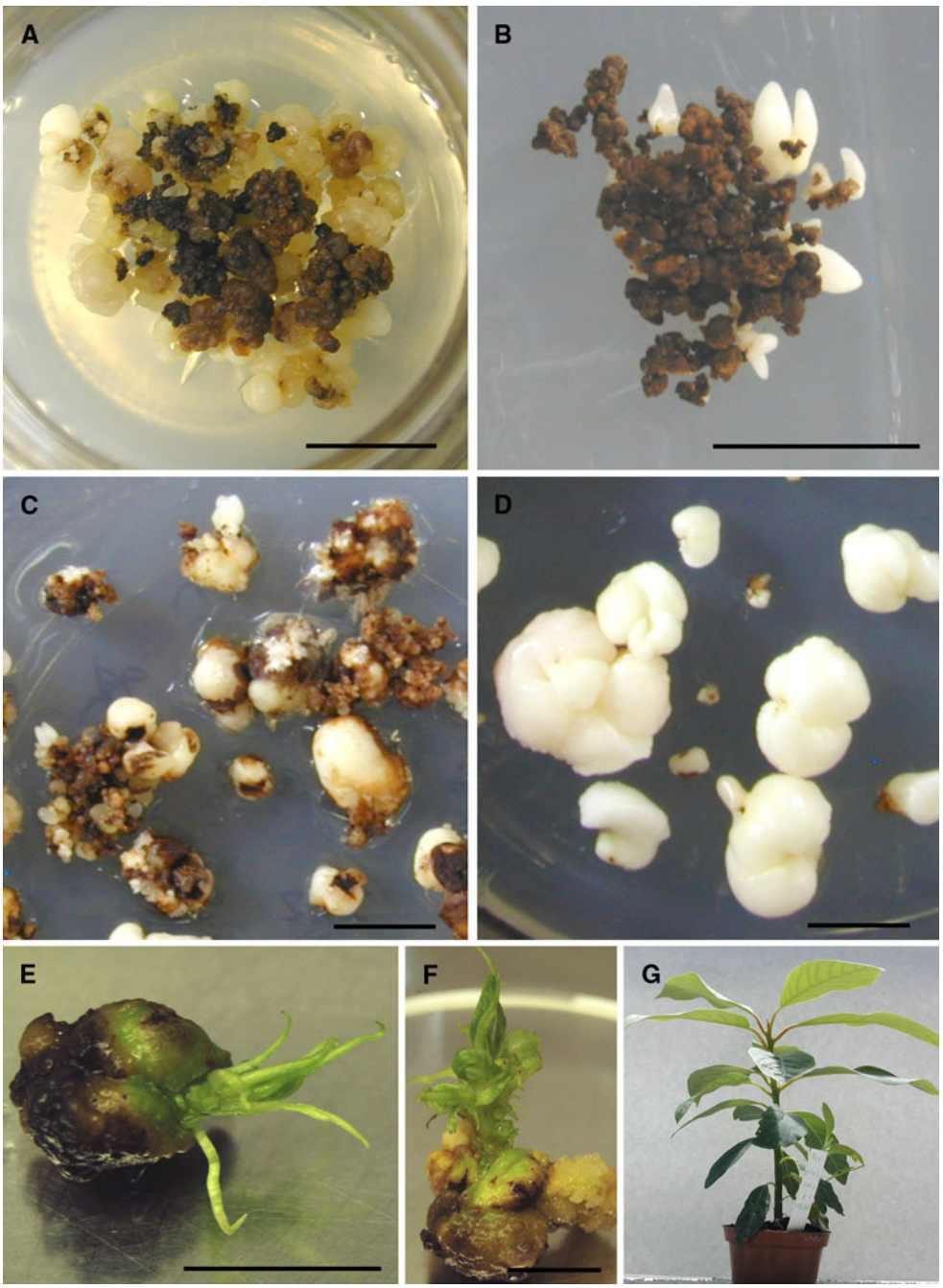

Fig. 1 The appearance of the white-opaque SE that were obtained after a 5-week culture in B5m10A without (**a**) or with the cellulose acetate membranes (**b**). The appearance of the WOSE that were pre-matured without (**c**) or with the cellulose acetate membranes (**d**) after a maturation treatment consisting of a 5-week culture in B5m10A followed by an 5 additional weeks in B5m10A-S-CW medium. The germinated embryos (**e**, **f**) and acclimated plants (**g**) that were derived from the embryos that were pre-matured over the cellulose acetate membranes and matured for 5 weeks in B5m10A, followed by 5 additional weeks in B5m10A-S-CW. In all figures, *scale bars* correspond to 1 cm

-37.7 ± 4.0 versus -21.3 ± 2.7 MPa in the filters that were directly overlaid on the medium.

The water potential (Ψw) was significantly lower in the WOSE that were cultured on the cellulose acetate membrane than in control embryos (Fig. 4a). Some of the WOSE were incubated in Petri dishes with 100 % RH for 48 h to determine their hydration capacity. As expected, the Ψw in the control embryos increased significantly, reaching a value close to -0.5 MPa (Fig. 4b). Similarly, the Ψw of the membrane-treated embryos increased, but even in this situation, the Ψw value was significantly lower than in the control embryos. Regarding ABA content, the

Journal : Large 11240 Dispatch : 9-7-2013 Pages : 9
Article No. : 352 LE TYPESET
MS Code : PCTO-D-13-00186 CP DISK

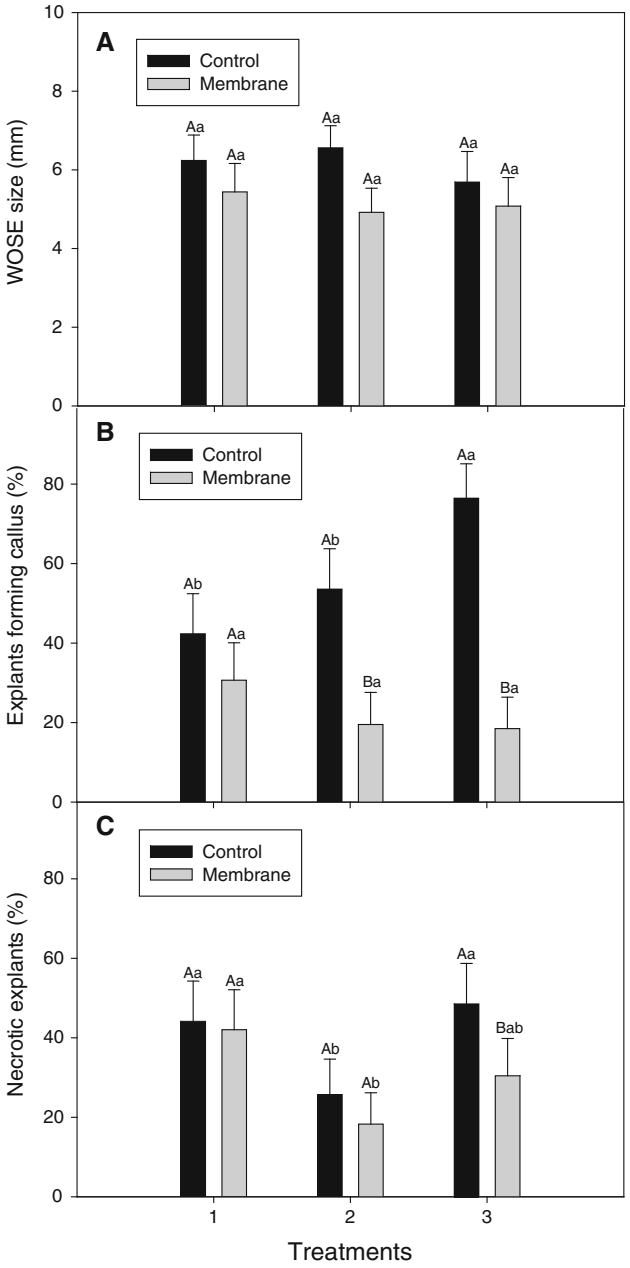

Fig. 2 The effect of different maturation treatments on the SE size (**a**), explant with calli (**b**) and percentage of necrosis (**c**) in the WOSE that were obtained without (control) or with the cellulose acetate membranes. Treatment 1: 2 cycles of a 5-week culture in B5m10A; treatment 2: 5 weeks in B5m10A followed by 5 weeks in B5m10A-S-LC; treatment 3: 2 cycles of a 5-week culture in B5m10A-S-LC. The data correspond to the mean ± SE. The data were statistically analyzed by ANOVA (SE size) and the χ^2 test (explant-forming calli and necrotic SE), at $P = 0.05$. The mean separation between the control and membrane-treated WOSE within each treatment is indicated by *capital letters*. The mean separation within the maturation treatments was performed separately for the control and membrane-treated SE and is indicated by *lower-case letters*

WOSE that were pre-matured on the cellulose acetate membranes showed statistically significant lower values compared with the control WOSE (Fig. 5).

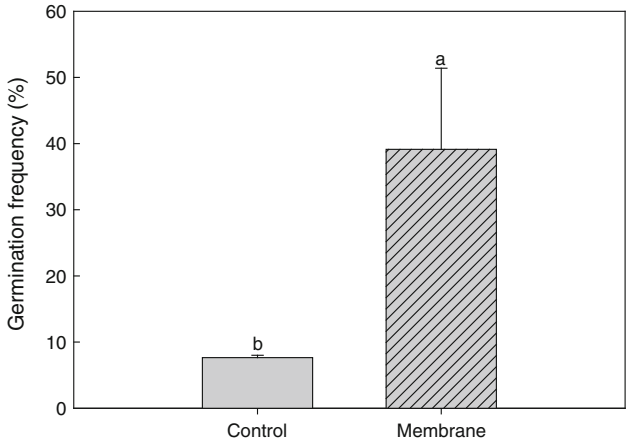

Fig. 3 The germination frequencies of the WOSE that were pre-matured without (control) or with the cellulose acetate membranes and matured by culturing for 5 weeks in B5m10A followed by another 5 weeks in B5m10A-S-CW medium. The data correspond to the mean ± SE. The mean separation was performed using the χ^2 test at $P = 0.05$

Discussion

Generally, incomplete development is the cause of the low germination rate observed in the SE of many species. Only mature embryos with a normal morphology that have accumulated enough storage materials and acquired desiccation tolerance develop into normal plants at the end of the maturation phase (von Arnold et al. 2002). In avocado, plant recovery through somatic embryogenesis is mainly limited by poor SE maturation, which leads to very low germination rates (Perán-Quesada et al. 2004; Márquez-Martín et al. 2011; Pliego-Alfaro et al. 2013). The culture of embryogenic calli in media with a decreased water potential, either from increasing the concentration of a gelling agent or from the addition of osmotic agents, is a common procedure for the production of high quality mature embryos (Krajnáková et al. 2009; Troch et al. 2009; Buendía-González et al. 2012). According to Perán-Quesada et al. (2004), avocado SE maturity is enhanced by B5 major salts. A higher number of mature, white-opaque SE were obtained when a medium with B5 macroelements was gelled with 10 g L^{-1} agar, a concentration higher than that which is considered standard; however, the addition of osmotic agents, such as PEG or sorbitol, did not improve maturation (Márquez-Martín et al. 2011). The increase of the gelling agent concentration increased the medium gel strength, although it did not affect the medium water potential (Márquez-Martín et al. 2011). Increasing the gel strength with a high gellan gum concentration also improved the maturation and germination of *Pinus strobus* SE (Klimaszewska and Smith 1997; Klimaszewska et al. 2000). The benefits of a high gelling agent concentration

Journal : Large 11240 Dispatch : 9-7-2013 Pages : 9
Article No. : 352 LE TYPESET
MS Code : PCTO-D-13-00186 CP DISK

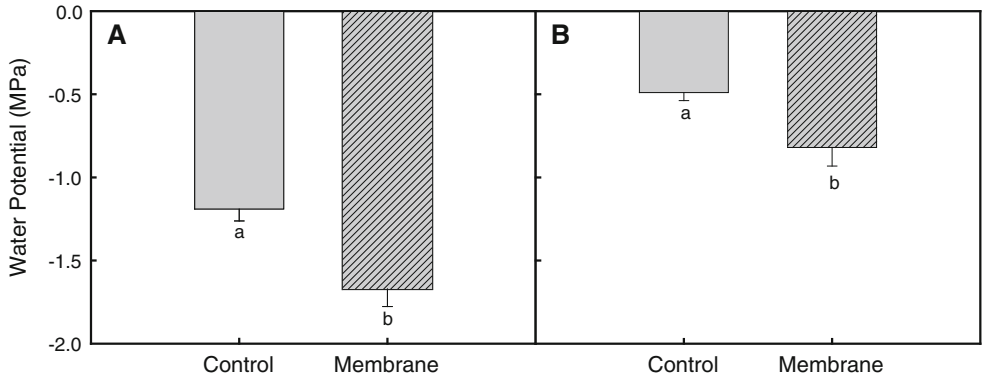

Fig. 4 The water potential (Ψw) of the white-opaque SE that were pre-matured without (control) or with the cellulose acetate membranes. **a** The water potential of the SE after 5 weeks in culture with B5m10A. **b** The water potential of the SE that were pre-matured for 5 weeks in B5m10A and incubated for 48 h at 100 % relativity humidity. The data correspond to the mean ± SE. The mean separation was performed using Student's *t* test at $P = 0.05$

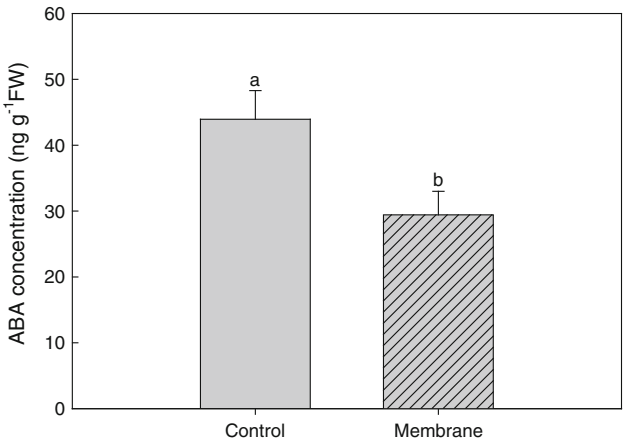

Fig. 5 The abscisic acid content in the WOSE that were pre-matured without (control) and with the cellulose acetate membranes. The data correspond to the mean ± SE. The mean separation was performed using Student's *t* test at $P = 0.05$

on SE maturation are related to reduced water availability (Owens and Wozniak 1991; Klimaszewska et al. 2000). In a few species, the culture of SE on cellulose semi-permeable membranes has been used as an alternative approach to restrict the water availability and improve maturation. The use of membranes normalized the development of citrus SE into heart-shaped-stage embryos that were capable of germination (Niedz et al. 2002; Niedz 2006). Similarly, Cerezo et al. (2011) used cellulose acetate membranes to obtain mature olive SE of superior quality and greater germination rates. In avocado, the culture of embryogenic calli on cellulose acetate membranes over B5m10A medium significantly increased the number of WOSE that were obtained. These embryos were smaller but more homogeneous in size compared with the control embryos that were pre-matured without membranes. Moreover, the appearance of the membrane-derived WOSE was different from that of the control, as they were white and smooth, compared to the brown color and irregular surface of the control embryos. Additionally, the membrane treatment reduced the proliferation of embryogenic calli. The homogeneity in the size and appearance of the avocado embryos that were matured on the cellulose acetate membranes suggests normalized development and better quality compared with control embryos.

It has been previously shown that white-opaque avocado SE should be 4–5 mm in size to germinate, and several re-cultures in maturation medium are needed to reach this size (Perán-Quesada et al. 2004; Márquez-Martín et al. 2011). Perán-Quesada et al. (2004) observed a positive effect of a high sugar concentration (175 mM) on somatic embryo maturation. Moreover, supplementing the maturation medium with filter sterilized-coconut water has also been shown to improve the quality of the recovered SE (Witjaksono and Litz 2002). In this study, the effect of a medium containing a high sucrose concentration and coconut water, B5m10A-S-CW medium, on the maturation of the control and membrane-treated SE was evaluated. Although the SE that were pre-matured on the cellulose membrane were significantly smaller than the controls, these WOSE increased in size after two additional re-cultures, reaching an average size similar to that of the control embryos independent of the maturation treatment employed. The culture in B5m10A followed by B5m10A-S-CW yielded the best results, as this treatment reduced the percentage of necrotic explants without increasing the amount of calli that developed from the WOSE. These effects were enhanced by the cellulose membrane treatment. As expected, the normalization of embryo development and the improvement in embryo quality as a result of pre-maturation on the cellulose acetate membranes significantly improved the germination rate of the WOSE, which was fourfold higher than in the control treatment. It is

Journal : Large 11240 Dispatch : 9-7-2013 Pages : 9
Article No. : 352 LE TYPESET
MS Code : PCTO-D-13-00186 CP DISK

noteworthy that the germination rate achieved with this treatment, 40 %, is much higher than the previous values that have been reported for avocado somatic embryo germination (Márquez-Martín et al. 2011; 2012). A similar effect of cellulose membranes on the germination rate was also observed in citrus and olive SE (Niedz et al. 2002; Cerezo et al. 2011).

The mechanisms underlying the benefits of semi-permeable membranes in SE maturation are not well known. The low amount of water that was absorbed by the filter papers overlaid on the cellulose membrane that was placed on the B5m10A medium demonstrates that the availability of water to the SE that were cultured on the membrane was substantially reduced. A similar result was previously observed by Niedz et al. (2002). Increasing the gel concentration in the medium also reduces the water availability (Klimaszewska et al. 2000). However, according to Niedz et al. (2002), the level of water availability reduction that is exerted by the membrane would be difficult to achieve with gels. In fact, these authors were unable to reproduce the benefits of the membranes in citrus SE with the use of different concentrations and types of gelling agents or with other compounds that reduce the osmotic potential of the medium, such as PEG (Niedz et al. 2002). At the physiological level, the culture of avocado SE on cellulose membranes significantly reduced their water potential and ABA content. The water potential is frequently used as a measure of the water status of plant tissues (Taiz and Zeiger 2010). Olive SE that were matured on cellulose membranes showed a decrease in Ψw, reportedly due to a reduction in the solute potential as result of the accumulation of storage products (Cerezo et al. 2011). The average Ψw value observed in the membrane-treated avocado SE was similar to that reported by Cerezo et al. (2011) in olive. The re-hydration of the avocado WOSE in 100 % RH increased the Ψw in both the control and membrane-treated SE. However, the membrane-derived SE showed a lower capacity to gain water, as those embryos maintained a Ψw lower than that of the control. These results suggest that membrane treatments induce the accumulation of storage products in SE. The maturation of oak and *Pinus strobus* SE in media with a high gelling agent concentration also reduced the Ψw of SE (Klimaszewska et al. 2000; Prewein et al. 2004). Interestingly, the reductions in Ψw that were reported in those papers are much lower than those observed in avocado or olive SE (Cerezo et al. 2011), which were both pre-matured over cellulose membranes. These data indicate that membranes exert a stronger effect on the SE Ψw compared with simply raising the agar concentration.

It is thought that desiccation improves the germination frequency, either by reducing the endogenous ABA level or by changing the sensitivity to ABA (Prewein et al. 2004; Jiménez 2005). In avocado, the somatic embryos that were pre-matured over the cellulose acetate membranes showed a lower ABA concentration and a significantly higher germination rate compared with the control embryos. Similarly, Prewein et al. (2004) reported a decrease in the ABA content in oak SE that were derived from media that were gelled with 1 % agar compared with those cultured that were in media with 0.8 % agar. Additionally, it has been shown that, as observed in avocado SE, the decrease in the ABA content is parallel to a decrease in the osmotic potential (Xu et al. 1990; Prewein et al. 2004). The reduction in the ABA content during SE development seems to be a prerequisite for embryo germination (Cvikrova et al. 1998; Rajasekaran et al. 1992; Prewein et al. 2004). The role of ABA in SE maturation could be related to the synthesis and deposition of storage and late embryogenesis-abundant (LEA) proteins (Dodeman et al. 1997; von Arnold et al. 2002).

In conclusion, we have improved the conversion of avocado somatic embryos through the culture of the small size fraction of embryogenic calli over cellulose acetate membranes on a B5-based pre-maturation medium that was solidified with 10 g L^{-1} agar, followed by a re-culture of the resulting WOSE in B5m10A and then another culture in the same medium supplemented with a high level of sucrose and coconut water. The membranes restricted the water availability to the SE, reducing their Ψw and ABA concentration. The WOSE that were obtained using this protocol were of high quality and showed a germination rate much higher than that of the control embryos. Our findings indicate that the use of cellulose membranes could be an excellent alternative for the maturation of SE. This approach avoids the negative effects that sometimes appear when using osmotic agents to lower the medium Ψw. Moreover, the use of cellulose acetate membranes induces stronger changes in the SE water status compared with the use of media with high gelling agent concentrations.

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References

Ammirato PV (1987) Organizational events during somatic embryogenesis. In: Green CE, Somers DA, Hackett WP, Biesboer D (eds) Plant biology, plant tissue and cell culture vol 3, Alan RLiss, New York, pp 57–81

Barceló-Muñoz A, Encina CL, Simón-Pérez E, Pliego-Alfaro F (1999) Micropropagation of adult avocado. Plant Cell Tiss Organ Cult 58:11–17

Braybrook SA, Harada JJ (2008) LECs go crazy in embryo development. Trends Plant Sci 13:624–630

Buendía-González L, Estrada-Zúñiga ME, Orozco-Villafuerte J, Cruz-Sosa F, Vernon-Carter EJ (2012) Somatic embryogenesis

Journal : Large 11240 | Dispatch : 9-7-2013 | Pages : 9
Article No. : 352 | LE | TYPESET
MS Code : PCTO-D-13-00186 | CP | DISK

of the heavy metal accumulator *Prosopis laevigata*. Plant Cell Tiss Organ Cult 108:287–296

Cailloux F, Julien-Guerrier J, Linossier L, Coudred A (1996) Long-term somatic embryogenesis and maturation of somatic embryos in *Hevea brasiliensis*. Plant Sci 120:185–196

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

Cvikrova M, Mala J, Eder J, Hrubcova M, Vagner M (1998) Abscisic acid, polyamines and phenolic acids in sessile oak somatic embryos in relation to their conversion potential. Plant Physiol Biochem 36:247–255

Dodeman VL, Ducreuw G, Kreis M (1997) Zygotic embryogenesis versus somatic embryogenesis. J Exp Bot 48:1493–1509

FAOSTAT (2010) http://faostat.fao.org/

Gamborg OL, Muller RA, Iojima K (1968) Nutrient requirements of suspension cultures of soybean roots cells. Exp Cell Res 50:151–158

Jiménez VM (2005) Involvement of plant hormones and plant growth regulators on in vitro somatic embryogenesis. Plant Growth Regul 47:91–110

Klimaszewska K, Smith DR (1997) Maturation of somatic embryos of *Pinus strobus* is promoted by a high concentration of gellan gum. Physiol Plant 100:949–957

Klimaszewska K, Bernier-Cardou M, Cyr DR, Sutton BCS (2000) Influence of gelling agents on culture medium gel strength, water availability, tissue water potential, and maturation response in embryogenic cultures of *Pinus strobus* L. In Vitro Cell Dev Biol Plant 36:279–286

Krajnáková J, Häggman H, Gömöry D (2009) Effect of sucrose concentration, polyethylene glycol and activated charcoal on maturation and regeneration of *Abies cephalonica* somatic embryos. Plant Cell Tiss Org Cult 96:251–262

Litz RE, Witjaksono, Raharjo S, Efendi D, Pliego-Alfaro F, Barcelo′-Muñoz A (2005) *Persea americana* avocado. In: Litz RE (ed) Biotechnology of fruit and nut crops. CABI Publishing, Wallingford, pp 326–347

Litz R, Raharjo S, Gómez-Lim M (2007) Avocado. In: Pua EC, Davey MR (eds) Biotechnology in agriculture and forestry. Transgenic crops V. Springer, Berlin, pp 167–187

Márquez-Martín B, Guzmán-García E, Barceló-Muñoz A, Pliego-Alfaro F, Sánchez-Romero C (2009) Effects of an in vitro maturation treatment on plant recovery from avocado zygotic embryos. Sci Hortic 122:532–539

Márquez-Martín B, Sesmero R, Quesada MA, Pliego-Alfaro F, Sánchez-Romero C (2011) Water relations in culture media influence maturation of avocado somatic embryos. J Plant Physiol 168:2028–2034

Márquez-Martín B, Barceló-Muñoz A, Pliego-Alfaro F, Sánchez-Romero C (2012) Somatic embryogenesis and plant regeneration in avocado (*Persea americana* Mill.): influence of embryogenic culture type. J Plant Biochem Biotechnol 21:180–188

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Niedz RP (2006) Regeneration of somatic embryos from sweet orange (*C. sinensis*) protoplast using semi-permeable membranes. Plant Cell Tiss Organ Cult 84:353–357

Niedz RP, Hyndman SE, Wynn ET, Bausher MG (2002) Normalizing sweet orange (*C. sinensis* (L.) Osbeck) somatic embryogenesis with semi-permeable membranes. In Vitro Cell Dev Biol-Plant 38:552–557

Owens LD, Wozniak CA (1991) Measurement and effects of gel matric potential and expressibility on production of morphogenic callus by cultured sugarbeet leaf discs. Plant Cell Tiss Org Cult 26:127–133

Palomo-Ríos E, Barceló-Muñoz A, Mercado JA, Pliego-Alfaro F (2012) Evaluation of key factors influencing *Agrobacterium*-mediated transformation of somatic embryos of avocado (*Persea americana* Mill.). Plant Cell Tiss Organ Cult 109:201–211

Perán-Quesada R, Sánchez-Romero C, Barceló-Muñoz A, Pliego-Alfaro F (2004) Factors affecting maturation of avocado somatic embryos. Sci Hortic 102:61–73

Pliego-Alfaro F, Murashige T (1988) Somatic embryogenesis in avocado (*Persea americana* Mill.) in vitro. Plant Cell Tiss Org Cult 12:61–66

Pliego-Alfaro F, Barceló-Muñoz A, López-Gómez R, Ibarra-Laclette E, Herrera-Estrella L, Palomo-Ríos E, Mercado JA, Litz RE (2013) Biotechnology. In: Schaffer B, Wolstenholme BN, Whiley AW (eds) The avocado: botany production and uses. CABI Publishing, Wallingford, pp 268–300

Prewein C, Vagner M, Wilhelm E (2004) Changes in water status and proline and abscisic acid concentrations in developing somatic embryos of pedunculate oak (*Quercus robur*) during maturation and germination. Tree Physiol 24:1251–1257

Rajasekaran K, Vine J, Mullins MG (1992) Dormancy in somatic embryos and seeds of *Vitis*: changes in endogenous abscisic acid during embryogeny and germination. Planta 154:139–144

Sánchez-Romero C, Márquez-Martín B, Pliego-Alfaro F (2006) Somatic and zygotic embryogenesis in avocado. In: Mujib A, Samaj J (eds) Somatic Embryogenesis. Springer, Berlin, pp 271–283

Sokal RR, Rohlf FJ (1995) Biometry: the principles and practice of statistics in biological research, 3rd edn. WH Freeman and Company, New York

Taiz L, Zeiger E (2010) Plant physiology, 5th edn. Sinauer Associates, USA

Troch V, Werbrouck S, Geelen D, van Labeke C (2009) Optimization of horse chestnut (*Aesculus hippocastanum* L.) somatic embryo conversion. Plant Cell Tiss Organ Cult 98:115–123

von Arnold S, Sabala I, Bozhkov P, Dyachok J, Filonova L (2002) Developmental pathways of somatic embryogenesis. Plant Cell Tiss Organ Cult 69:233–249

Walker-Simmons MF, Rose PA, Hogge LR, Abrams SR (2000) ABA immunoassay and gas chromatography/mass spectrometry verification. In: Tucker GA, Roberts JA (eds) Plant hormone protocols. Humana Press, Nottingham, pp 33–49

Witjaksono, Litz RE (1999a) Induction and growth characteristics of embryogenic avocado cultures. Plant Cell Tiss Organ Cult 58:19–29

Witjaksono, Litz RE (1999b) Maturation of avocado somatic embryos and plant recovery. Plant Cell Tiss Organ Cult 58:141–148

Witjaksono, Litz RE (2002) Somatic embryogenesis of avocado (*Persea americana* Mill.) and its application for plant improvement. Acta Hortic 575:133–138

Xu N, Coulter M, Bewley JD (1990) Abscisic acid and osmoticum prevent germination of developing alfalfa embryos, but only osmoticum maintains the synthesis of developmental proteins. Planta 182:382–390

Journal : Large 11240 | Dispatch : 9-7-2013 | Pages : 9
Article No. : 352 | ☐ LE | ☐ TYPESET
MS Code : PCTO-D-13-00186 | ☑ CP | ☑ DISK