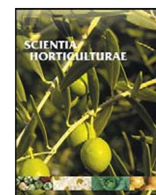
Scientia Horticulturae 122 (2009) 532–539

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti



Effects of an *in vitro* maturation treatment on plant recovery from avocado zygotic embryos

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ARTICLE INFO

Article history:
Received 28 November 2007
Received in revised form 9 June 2009
Accepted 10 June 2009

Keywords:
Avocado
Embryo rescue
In vitro maturation
Persea americana Mill.
Protein bodies
Starch grains

ABSTRACT

An efficient protocol for *in vitro* maturation of very immature, <10 mm, avocado embryos has been developed. The efficiency of plant recovery as well as the quality of the resulting plants was greatly improved by including a maturation phase prior to induction of germination. The influence of different factors, such as the gelling agent, organic supplements or abscisic acid, on embryo maturation and subsequent germination was tested. Optimum conditions were met when maturation was carried out in B5m medium supplemented with the Jensen's amino acids, an extra 88 mM sucrose and 6 g l^{-1} agar as gelling agent. At these conditions, embryos which had been collected 68 days after pollination germinated at a 65% rate in solid medium, giving rise to healthy and vigorous plantlets. Anatomical differentiation and storage product accumulation occurring during the *in vitro* maturation phase were studied by means of histological techniques. Results obtained revealed that, at the end of the *in vitro* maturation period, embryos resembled the pattern previously established for avocado embryos matured under *in vivo* conditions: histodifferentiation had been accomplished and starch granules and protein bodies were abundant.

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1. Introduction

Embryo rescue techniques have been revealed as important tools to increase the efficiency of breeding programs based on hybridization of selected genotypes. This technology is especially useful in subtropical woody crops, as avocado, that exhibit a high rate of fruit abscission (Gómez-Lim and Litz, 2004).

In vitro culture of immature embryos has been used as a rescue technique as it facilitates conversion of these embryos into plants (Raghavan, 2003). Generally, development of *in vitro* rescue protocols, including those for avocado, has normally focused on the optimisation of the germination process (Skene and Barlass, 1983; Mohapatra and Rout, 2005; Viloria et al., 2005; Sánchez-Romero et al., 2007); however, in most cases, when immature embryos are induced to germinate, the process occurs at a low rate and the resulting plantlets are weak and small or hyperhydric. This problem is especially important in species, such as avocado, showing seed recalcitrance.

Avocado embryo development takes place over a large period of time giving rise to the formation of a large embryo with fleshy, massive cotyledons filled with reserve products (Perán-Quesada et al., 2005). Although the length of this period ranges from 6 to 12 months depending on the genotype (Whiley, 1992), Perán-Quesada et al. (2005) established that physiological maturity in cultivar 'Hass' was achieved approximately 305 DAP, when embryos were 40 mm in length.

Storage products accumulated during zygotic embryo development play a key role in its subsequent germination and conversion into a plant as they provide energy as well as the carbon and nitrogen sources needed for the synthesis of new components in the growing seedling (Raghavan, 1997). Previous investigations carried out throughout the development of avocado zygotic embryos under *in vivo* conditions showed that reserve products were mainly accumulated at the end of the developmental period, during the maturation phase (Sánchez-Romero et al., 2002; Perán-Quesada et al., 2005). Generally, maturation is considered a transitory but frequently indispensable stage between the embryo development and germination phases (Quatrano, 1987). In avocado, Perán-Quesada et al. (2005) reported that this phase represents an important part of embryo development.

Furthermore, Sánchez-Romero et al. (2007) have found that when zygotic embryos at different developmental stages are

Abbreviations: ABA, abscisic acid; cw, coconut water; DAP, days after pollination; PAS, periodic acid-Schiff.

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doi:10.1016/j.scienta.2009.06.018

germinated under *in vitro* conditions, they show different culture requirements, e.g., very immature embryos, <10 mm, germinate better in liquid media while more developed embryos require solid texture media. As expected, very immature embryos give rise to hyperhydric shoots due to the liquid medium texture in which they are usually cultured.

The aim of this investigation was to develop a protocol for maturation of very immature, <10 mm, embryos, under *in vitro* conditions, in such a way that subsequent germination could be carried out in a medium with solid texture. Changes in embryo anatomical differentiation and accumulation of storage products at the end of the maturation period were also evaluated.

2. Material and methods

2.1. Plant material

Avocado (*Persea americana* Mill.) fruits, cv. 'Hass', were collected at random from open-pollinated trees growing within a monovarietal orchard at La Mayora Experimental Station (Algarrobo Costa, Spain). This genotype was selected because it is the most extensively grown cultivar all around the world, and a parental line in all avocado breeding programs, hence, most selections from directed crosses will have a 'Hass' genetic background. Very immature embryos, 68–80 days after pollination (DAP), were utilized for embryo rescue experiments and histological analysis. Each stage included embryos within a 2–3 mm interval around the stage-representative length (Perán-Quesada et al., 2005).

2.2. In vitro embryo rescue

After harvesting, fruits were surface sterilized by immersion in a 0.5% (v/v) sodium hypochlorite solution containing 10 drops l^{-1} of Tween 20 for 10 min, followed by three rinses in sterile distilled water. Sterilized fruits were cut lengthwise under aseptic conditions and the immature zygotic embryos carefully excised.

The basal maturation medium (B5m) consisted of major salts of the B5 formulation (Gamborg et al., 1968), MS minor salts and vitamins (Murashige and Skoog, 1962) and 88 mM sucrose. M1 germination medium consisted of half strength MS formulation supplemented with 2.22 μM benzyladenine and gelled with 1.7 g l^{-1} gelrite (G-1910, Sigma Chemical Co., St. Louis, MO, USA).

In all media preparations, the pH was adjusted to 5.74 before autoclaving. Media were warmed 7 min to melt the gelling agent and subsequently, 25 ml were dispensed into 25 mm × 150 mm test tubes (Bellco Glass Inc., NJ, USA) or 50 ml into 85 mm × 80 mm cylindrical glass jars. Finally, media were autoclaved at 121 °C and 0.1 MPa for 15–20 min. Coconut water (cw) (C-5915, Sigma Chemical Co., St. Louis, MO, USA) and (±)-cis,trans-abscisic acid (ABA) (A-1049, Sigma Chemical Co., St. Louis, MO, USA) were filter-sterilized and added to the cooled sterilized media.

Maturation phase was carried out during 10 weeks with reculture onto fresh medium after 5 weeks. Subsequently, embryos were induced to germinate by partial removal of the cotyledons and culture on M1 medium (Skene and Barlass, 1983). Germination was carried out during 15 weeks with recultures onto fresh medium at 5-week intervals.

In the first experiment, 68 DAP embryos, averaging 7 mm in length, were utilized for testing the effect of the gelling agent (gelrite versus agar) on maturation medium. Two treatments were included: B5m basal medium solidified with 1.7 g l^{-1} gelrite and B5m basal medium solidified with 6 g l^{-1} agar (A-1296, Sigma Chemical Co., St. Louis, MO, USA). After maturation, embryos were transferred to M1 medium for germination.

In the second experiment, the effects of extra supplements of sucrose, amino acids and ABA were studied using 80 DAP embryos (9.5 mm in length). Eight treatments were included: B5m, B5m supplemented with the Jensen's amino acids (Jensen, 1977) (B5m + aa), B5m supplemented with an extra 88 mM sucrose (B5m + suc) and B5m supplemented with the Jensen's amino acids as well as extra 88 mM sucrose (B5m + aa + suc). To determine the effect of ABA on the maturation process, all media were prepared with and without 30 μM ABA. All media were solidified with 6 g l^{-1} agar. After maturation, embryos were transferred to M1 medium for germination.

Finally, in the third experiment, the organic supplements treatment selected in the second assay was compared with coconut water (cw), a nutrient addendum previously used for embryo culture (Bhojwani and Razdan, 1996). Coconut water at 10% (v/v) was added to B5m basal medium (B5m + cw). Very immature embryos, 68 DAP and 7 mm in length, were utilized in this experiment. Three treatments were included: B5m, B5m + cw and B5m + aa + suc. All culture media included in this experiment were solidified with 6 g l^{-1} agar. As in previous experiments, following maturation treatment, embryos were transferred to M1 medium for germination.

In the three maturation experiments, control treatment included embryos that were directly placed in germination medium (after partial excision of their cotyledons) without having undergone a maturation treatment.

Cultures were maintained in a growth chamber at 25 ± 1 °C and incubated in darkness during the maturation treatments, while germination was carried out under a 16 h light photoperiod, provided by Grolux lamps (Sylvania, Germany) (40 μmol m^{-2} s^{-1}).

2.3. Histological study

The effect of *in vitro* maturation at optimum conditions on embryo morphological development and accumulation of storage products was studied by means of histological techniques. For this purpose, 68 DAP embryos, prior to and after an *in vitro* maturation cycle (10 weeks) in the presence of B5m + aa + suc medium, were processed according to Johansen (1940). Samples were fixed in FAA (5% formalin, 5% acetic acid and 90% ethanol (70%, v/v)), dehydrated through a TBA and ethanol series and embedded in paraffin. Longitudinal 10 μm sections were cut and mounted on slides.

For anatomical studies, sections were stained with the Gerlach's procedure (Gerlach, 1969). Starch was monitored using the periodic acid-Schiff's (PAS) reagent (Herrero, 1979; Arbeloa, 1986) and protein bodies were visualized with the PAS-Amido Black staining (Jensen, 1962; Fisher, 1968).

At least three sections from different embryos were observed using light microscopy.

2.4. Data taken and statistical analysis

For *in vitro* plant recovery experiments, 30–50 zygotic embryos were used per treatment. A single embryo was considered to be a replicate. Fresh weight increases as well as number of precociously germinating embryos were recorded at the end of the maturation phase.

Embryos were considered as germinated when shoot and/or root elongation was ≥2 mm. Data on percentage of germinated embryos, recovery of complete plants as well as length of the shoots and/or roots obtained, were recorded at the end of each germination reculture.

Experiments using percentage data were subjected to frequency analysis with an R×C test of independence. Length and weight increase data were analysed by ANOVA and differences

among means were tested by the LSD (Sokal and Rohlf, 2003). The significance level used was 0.05 in all cases.

3. Results

3.1. In vitro embryo rescue

3.1.1. Effect of gelling agent (gelrite versus agar) in the maturation medium, on subsequent embryo rescue

Fresh weight increase during the maturation phase was significantly affected by the gelling agent ($P = 0.0064$) (Fig. 1a). Bigger size embryos developed in maturation medium solidified with agar. In these conditions, embryos went from 0.04 g average initial weight to 1.07 g at the end of the maturation period. Moreover, embryos matured on agar-gelled media showed a much better general aspect than those cultured on gelrite-gelled media.

Precocious germination (12% in gelrite-gelled medium versus 20% in agar-gelled medium) was observed during the maturation period, although differences between treatments were not statistically significant (data not shown).

Final germination rate was increased with maturation treatments ($P = 2.10 \times 10^{-5}$) (Fig. 1b); e.g., germination rates in *in vitro* matured embryos ranged between 30% (gelrite) and 48% (agar) although differences were not statistically significant between both treatments; however, in embryos directly cultured on M1 medium significantly lower values for germination rate were obtained (8%). Significant differences of maturation treatments with respect to the control could also be found regarding recovery of complete plantlets ($P = 0.0001$) and shoot length ($P = 0.0224$) (Table 1). *In vitro* matured embryos gave rise to longer shoots, with values ranging from 1.78 cm (gelrite) to 2.96 cm (agar) versus 1.05 cm in control, non-matured, embryos. Roots developed only in *in vitro* matured embryos.

Table 1
Effect of gelling agent in maturation medium, on subsequent recovery of complete plantlets and seedling growth from avocado zygotic embryos collected 68 DAP. Different letters within each column indicate significant differences obtained by frequency analysis or ANOVA ($\alpha = 0.05$).

Maturation treatment	Recovery of complete plantlets (%)	Seedling growth	
		Shoot length (cm)	Root length (cm)
Control	0 b	1.05 b	–
B5m gelrite	12 a	1.78 ab	2.93 a
B5m agar	24 a	2.96 a	3.52 a

3.1.2. Effect of carbon (sucrose), nitrogen (Jensen's amino acids) and ABA supplements in the maturation medium, on subsequent embryo rescue

Embryo weight increases recorded at the end of the maturation phase were significantly affected by the maturation treatments ($P = 0.0000$) (Fig. 2a). The main effect could be attributed to the organic supplements ($P = 0.0000$) whereas no significant influence could be detected for ABA.

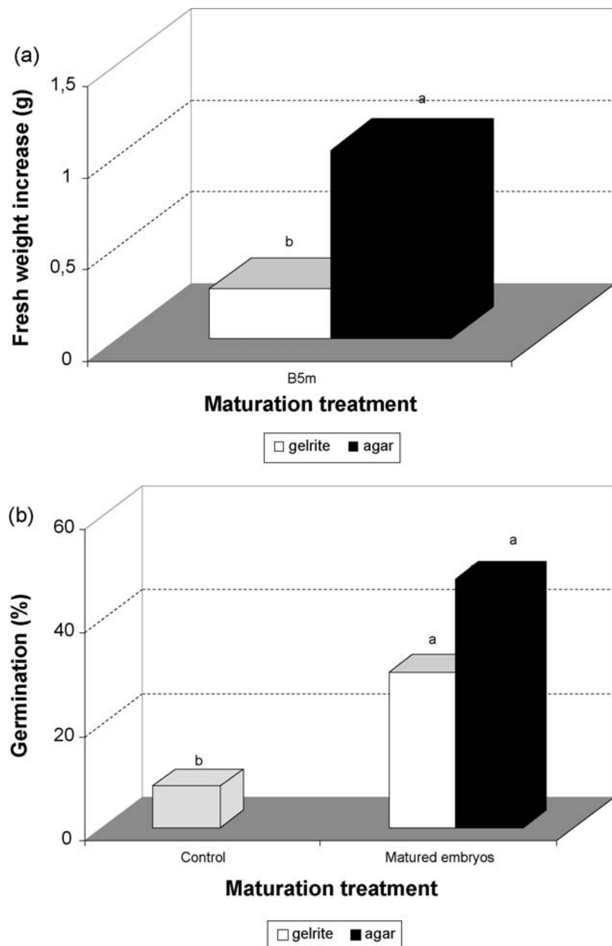

Fig. 1. Effect of gelling agent in the maturation medium on (a) fresh weight increase and (b) germination rate of avocado zygotic embryos excised 68 DAP. Different letters indicate significant differences obtained by ANOVA or frequency analysis ($\alpha = 0.05$).

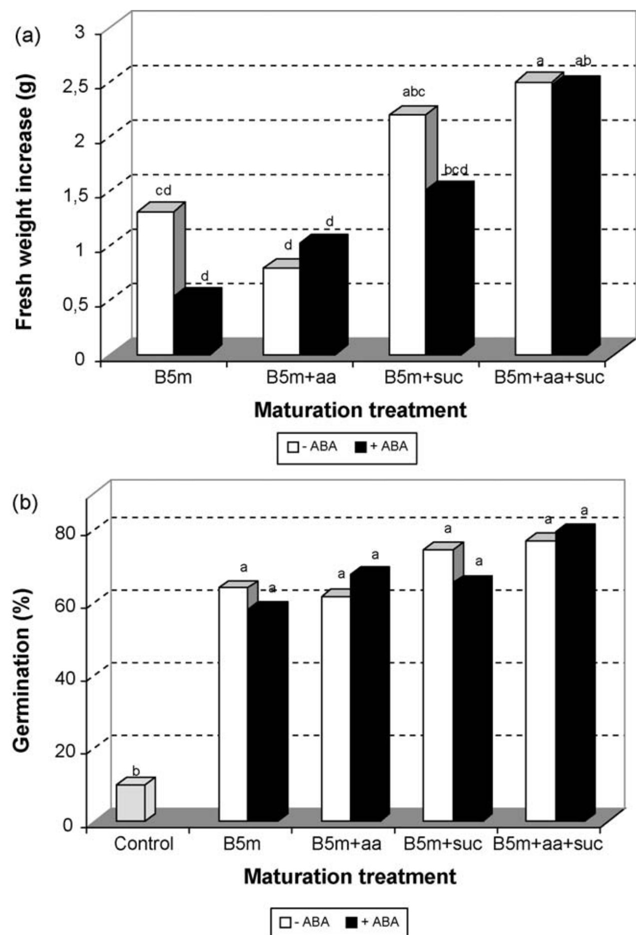

Fig. 2. Effect of carbon, nitrogen and ABA supplements in the maturation medium on (a) fresh weight increase and (b) germination rate of avocado zygotic embryos excised 80 DAP. Different letters indicate significant differences obtained by ANOVA or frequency analysis ($\alpha = 0.05$).

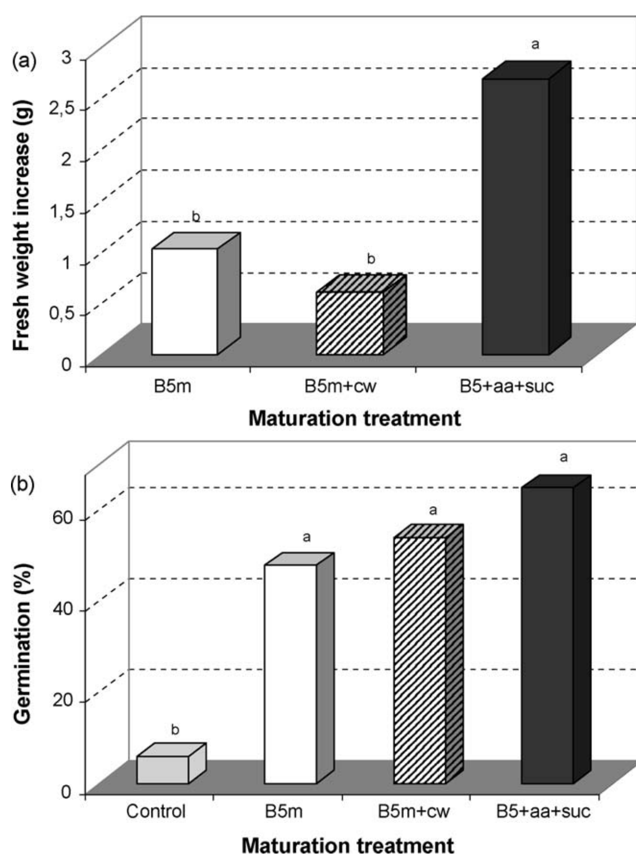

Fig. 3. Effect of Jensen's amino acids with sucrose or coconut water in the maturation medium on (a) fresh weight increase and (b) germination rate of avocado zygotic embryos excised 68 DAP. Different letters indicate significant differences obtained by ANOVA or frequency analysis (α = 0.05).

Fig. 4. Plantlet recovery from avocado zygotic embryos excised 68 DAP. Seedlings developed from (a) control, non-matured embryo, and embryos following *in vitro* maturation in (b) B5m + cw medium and (c) B5m + aa + suc medium.

Table 2
Effect of carbon and nitrogen supplements and ABA in maturation medium, on subsequent recovery of complete plantlets and seedling growth from avocado zygotic embryos collected 80 DAP. Different letters within each column indicate significant differences obtained by frequency analysis or ANOVA (α = 0.05).

Maturation treatment		Recovery of complete plantlets (%)	Seedling growth	
			Shoot length (cm)	Root length (cm)
Control		0 b	1.60 c	–
B5m	−ABA	38.72 a	5.36 bc	3.90 a
	+ABA	7.74 b	5.15 bc	5.75 a
B5m + aa	−ABA	37.86 a	6.87 ab	3.72 a
	+ABA	42.86 a	5.49 bc	4.17 a
B5m + suc	−ABA	46.93 a	5.46 bc	3.92 a
	+ABA	42.86 a	7.25 ab	4.61 a
B5m + aa + suc	−ABA	43.69 a	7.65 a	4.20 a
	+ABA	47.95 a	6.56 ab	5.76 a

Precocious germination during the maturation period was significantly inhibited by ABA ($P = 2.54 \times 10^{-5}$), with an average of 25.42% embryos germinating in absence of this hormone versus only 4.27% on ABA-containing media. Virtually in all cases, precociously germinated embryos only produced roots (data not shown).

In relation to final germination, the inclusion of the maturation period significantly improved germination percentage ($P = 2.79 \times 10^{-10}$) (Fig. 2b). Moreover, roots only developed in *in vitro* matured embryos (Table 2). Although no significant differences in terms of germination percentage could be found among the different maturation treatments, plantlets formed following maturation on media with both supplements and without ABA were the most vigorous and better developed, with shoots averaging 7.65 cm in length while in the rest of the treatments shoot lengths ranged from 5.15 to 7.25 cm (Table 2).

Generally, addition of ABA to maturation media affected plantlets growth; e.g., a general increase in root growth could be observed in all cases (Table 2); however, the differences were not statistically significant.

3.1.3. Effect of organic supplements (Jensen's amino acids with sucrose versus coconut water) in maturation medium, on subsequent embryo rescue

Fresh weight increase was significantly affected by the maturation treatment ($P = 0.0000$) with considerably larger

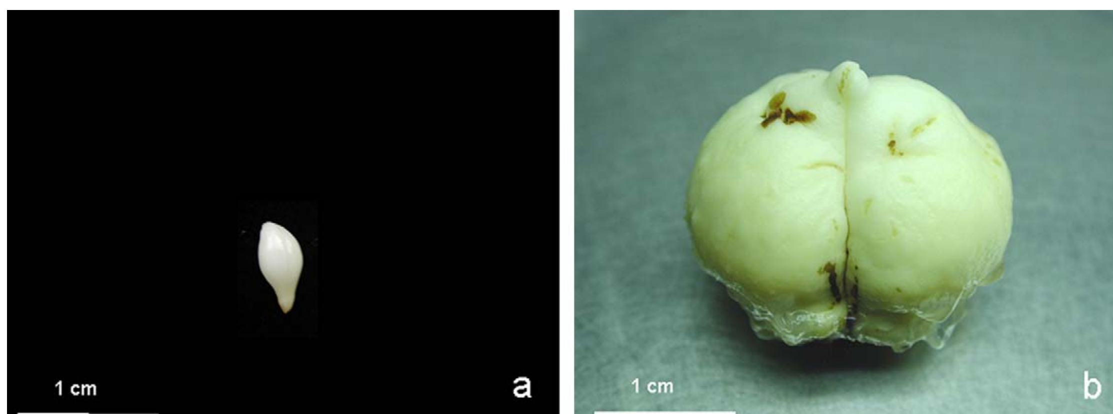

Fig. 5. Avocado zygotic embryos excised 68 DAP (a) prior and (b) after *in vitro* maturation on B5m + aa + suc medium during two recultures of 5 weeks each.

embryos developing in basal maturation medium supplemented with the Jensen's amino acids and additional sucrose (B5m + aa + - suc) (Fig. 3a).

Precocious germination was observed during the maturation period, with values ranging between 12.50% and 20% in the different treatments (data not shown).

Important germination parameters, such as germination rate ($P = 4.78 \times 10^{-11}$) and recovery of complete plantlets ($P = 1.81 \times 10^{-7}$), were again significantly improved with maturation (Fig. 3b and Table 3). However, no significant differences could be observed between the maturation treatments tested, although general aspect and appearance of plants were superior on the treatment including both, Jensen's amino acids and additional sucrose (B5m + aa + suc) (Fig. 4).

3.2. Histological study

3.2.1. Morphology

During the *in vitro* maturation period, embryo morphology considerably changed, e.g., following excision from the fruit, embryos were white and elongated, while *in vitro* matured embryos significantly increased in weight and size, turned beige and acquired an irregular round form (Fig. 5).

3.2.2. Anatomical study

The histological study revealed that histodifferentiation was not accomplished in the avocado embryos used in this investigation (Fig. 6a). Although bipolarity in the embryonic axis was evident, root and shoot meristems were only partially developed.

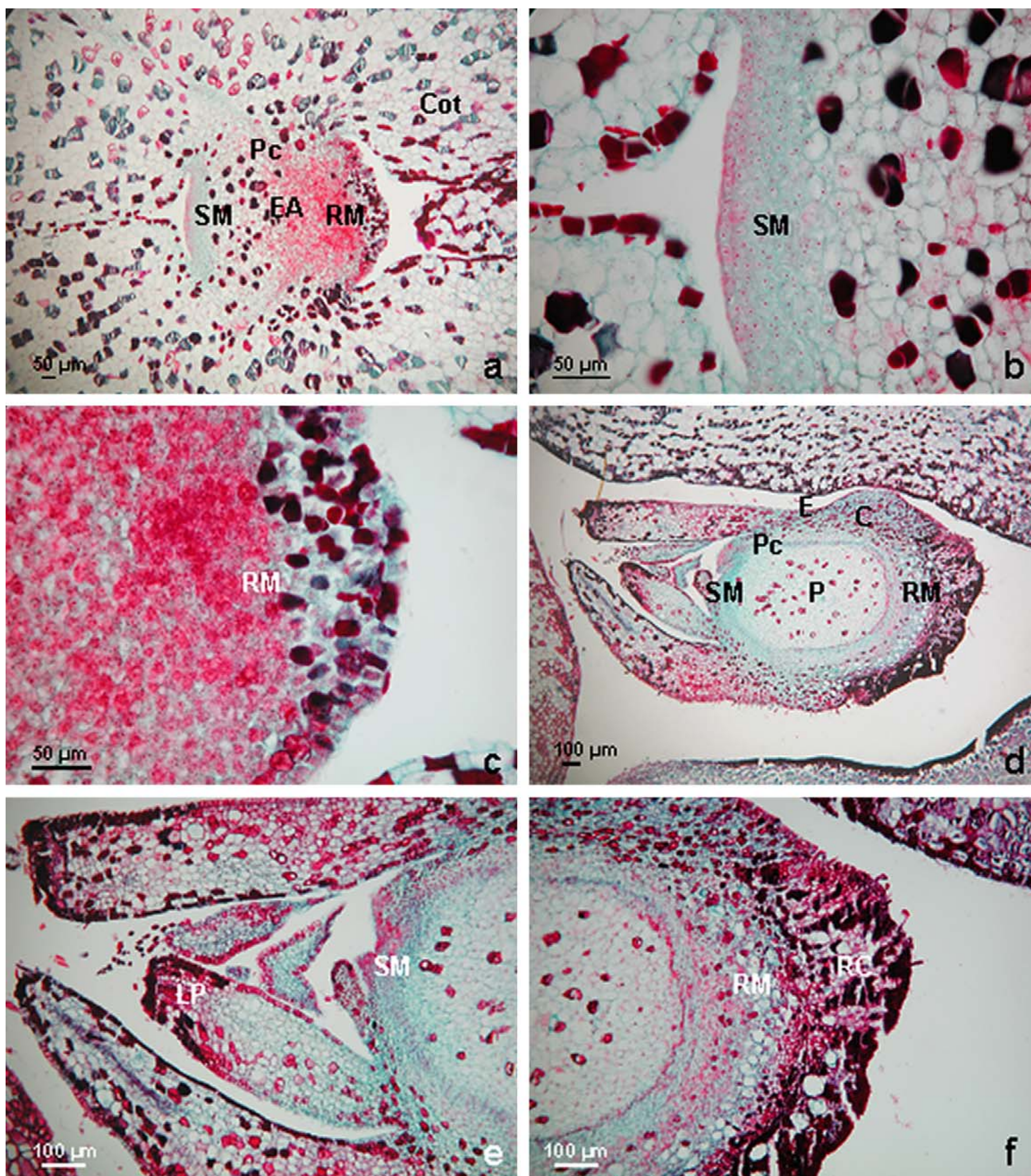

Fig. 6. Longitudinal sections of avocado zygotic embryos excised 68 DAP (a–c) and after 70 days *in vitro* maturation treatment (d–f), stained with the Gerlach's reagent. (a) Control, non-matured, embryo showing part of the cotyledons (Cot) and the embryonic axis (EA) including shoot meristem (SM), root meristem (RM) and procambial traces (Pc). (b) Shoot meristem (SM) of control embryo. (c) Root meristem (RM) of control embryo. (d) *In vitro* matured embryo showing the shoot meristem (SM), the pith (P), procambium (Pc), cortex (C), epidermis (E) and root meristem (RM). (e) Shoot meristem (SM) of *in vitro* matured embryo, with several leaf primordial (LP). (f) Root meristem (RM) of *in vitro* matured embryo, showing the root cap (RC).

Table 3
Effect of Jensen's amino acids and sucrose or coconut water in maturation medium, on subsequent recovery of complete plantlets and seedling growth from avocado zygotic embryos collected 68 DAP. Different letters within each column indicate significant differences obtained by frequency analysis or ANOVA ($\alpha = 0.05$).

Maturation treatment	Recovery of complete plantlets (%)	Seedling growth	
		Shoot length (cm)	Root length (cm)
Control	0 b	1.83 a	–
B5m	24.00 a	2.96 a	3.52 a
B5m + cw	26.00 a	2.14 a	3.20 a
B5m + aa + suc	37.50 a	3.11 a	4.22 a

In the shoot meristem, differentiation was not observed and it could only be distinguished by the occurrence of typically meristematic cells (Fig. 6b). In the root meristem, however, a group of intensely stained cells revealed the root cap (Fig. 6c). Provascular traces appeared in the hypocotyl region indicating initial stages of the procambium development.

Throughout culturing in B5m + aa + suc medium, embryos underwent extensive anatomical development and, at the end of the maturation period, appeared completely differentiated (Fig. 6d). The procambium appeared fully developed delimiting the central core of the embryonic axis where the pith, formed by large cells with cytoplasm of low density, was clearly visible. The shoot apex appeared highly differentiated showing several leaf

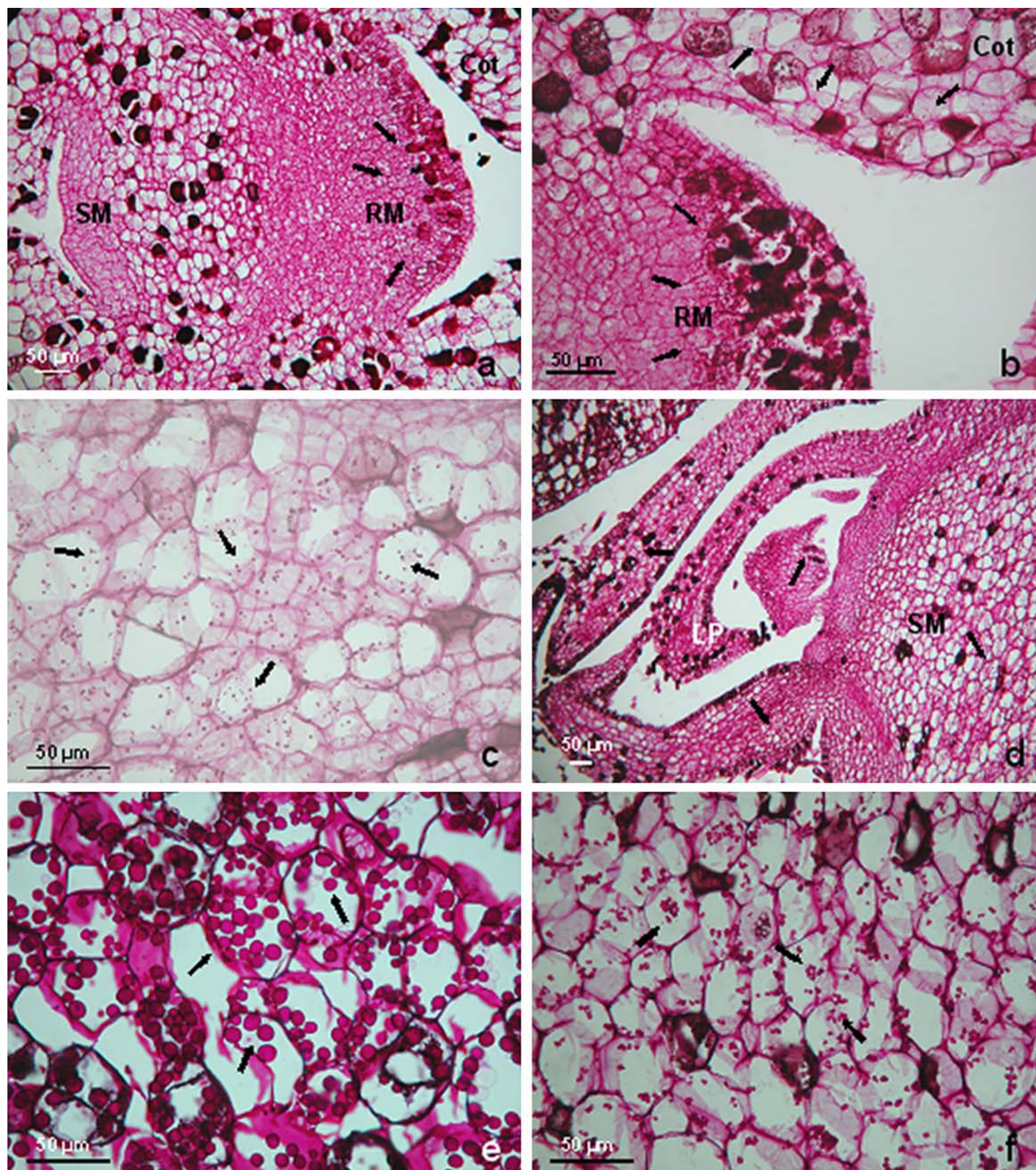

Fig. 7. Longitudinal sections of avocado zygotic embryos excised 68 DAP (a–c) and after 70 days *in vitro* maturation treatment (d–f), stained with PAS. Arrows indicate starch granules. (a) Control, non-matured, embryo showing embryonic axis, with shoot (SM) and root (RM) meristems, and part of the cotyledons (Cot). (b) Root region of control embryo showing part of the root meristem (RM) and cotyledons (Cot). (c) Cotyledon mesophyll of control embryo. (d) Shoot meristem (SM) of *in vitro* matured embryo with several leaf primordia (LP). (e) Cotyledon mesophyll and (f) embryonic axis of *in vitro* matured embryo.

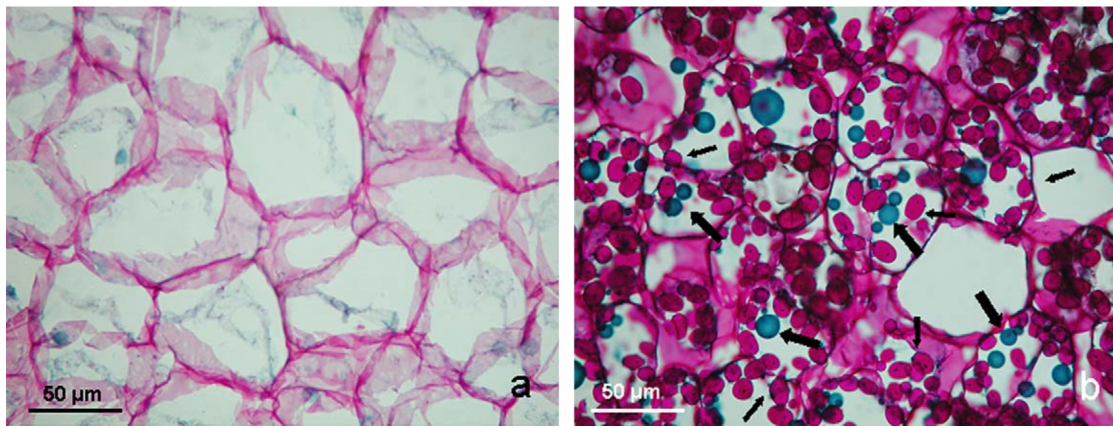

Fig. 8. Longitudinal sections of cotyledons from avocado zygotic embryos excised 68 DAP and stained with PAS-Amido Black. Thick arrows indicate protein bodies and thin arrows indicate starch granules. (a) Control, non-matured, embryo. (b) Embryo following *in vitro* maturation in B5m + aa + suc medium.

primordia (Fig. 6e). In the root meristem, despite the advanced developmental stage, structures such as the quiescent centre or the columella, were not clearly defined (Fig. 6f).

3.2.3. Storage products deposition

Prior to culture, embryos contained starch granules both, in the embryonic axis and the cotyledons, although they were small and scarce and appeared at specific locations. Starch granules in the embryonic axis were only visible in the root meristem (Fig. 7a) while in the cotyledons starch was mainly deposited in the parenchyma cells next to this region (Fig. 7b and c).

Following *in vitro* maturation, an important increase in starch accumulation could be observed with numerous starch granules visible in the embryonic axis as well as in the cotyledons. Organelles distribution was widespread and, after maturation, they were present in the shoot meristem, leaf primordia and the pith (Fig. 7d). Nevertheless, cotyledons constituted the main places of starch accumulation. Starch granules that accumulated in the embryonic axis (Fig. 7f) were smaller and less abundant than those present in the cotyledonal cells (Fig. 7e).

As revealed by PAS-Amido Black staining, although protein bodies were not visible in embryos before culture (Fig. 8a), they were abundant after the *in vitro* maturation period (Fig. 8b). Protein bodies appeared as spherical organelles showing, in some cases, a size similar to that of starch granules. Their presence was limited to cotyledonal cells which also contained a great amount of starch granules.

4. Discussion

Developmental stage has been revealed, in avocado, as a critical factor influencing embryo germination and recovery of complete plants (Perán-Quesada et al., 2005). Under *in vitro* conditions, the inclusion of an embryo maturation phase, prior to germination, shows a positive effect as it significantly increases both, germination rate and plant recovery. Using the developed protocol, the obtained germination rates are similar to those reported by Perán-Quesada et al. (2005) for avocado embryos of similar age matured on the tree.

In relation to the maturation factors influencing subsequent germination, our results show that an important role can be attributed to the gelling agent. Agar appears to be more adequate for avocado since culturing immature embryos on agar-gelled medium gave rise to well-developed embryos with higher weight increases and, finally, higher germination rates. Although differences between gelling agents were not statistically significant, the better quality of the plants obtained in agar-gelled media could be due to the lower water availability to the explants under these conditions (Beruto et al., 1999). This fact could have decisive importance as the water relations between the embryo and its environment, *in vivo* and *in vitro*, play a determinant regulatory role in embryo development and, in particular, in the maturation phase (Adams and Rinne, 1980). In agar-gelled media, it can be observed that, among all the maturation treatments tested, the main influence was due to the basal maturation medium (B5m).

A strategy repeatedly used for inducing embryo development and maturation is the addition to the culture medium of different nutritious supplements or plant growth regulators, such as ABA. Although ABA has been found to induce the expression of maturation genes (Morris et al., 1990) and the accumulation of storage proteins (Koornneef et al., 2002), in avocado embryos, ABA did not show any improvement in the final embryo rescue rate despite its clear influence, previously reported in other species (George et al., 2008), in decreasing precocious germination.

In agar-gelled media, organic carbon and nitrogen supplies improved germination quality by enhancing the vigour of the plants obtained, although no significant differences in germination rate could be observed between the treatment where both supplements were jointly added and the basal maturation medium. The quality effect of carbon and nitrogen supplements could be explained by a direct role as the main substrates for storage products deposition. Nevertheless, sucrose and nitrogen compounds may also exert a regulatory role on storage activities (Weber et al., 1997). Along this line, Koch (1996) and Scheible et al. (1996) pointed out that the signals necessary to coordinate the expression of genes involved in carbohydrate and protein pathways can be provided directly by sugars and nitrogen compounds.

Coconut water has been repeatedly used for improving development of very immature embryos (Bhojwani and Razdan, 1996). However, the results obtained when culturing immature avocado zygotic embryos in a cw-supplemented medium did not improve those obtained when carbon and nitrogen supplements were jointly used.

The histological study revealed that poor germination observed in embryos directly cultured on M1 medium, could be due to incomplete histodifferentiation and lack of reserve products. *In vitro* culture of immature avocado embryos in B5m + aa + suc medium allowed the maintenance of embryonic development and stimulated the accumulation of storage products, e.g., starch and storage proteins. Both, anatomical differentiation and reserve products deposition, resemble the pattern previously established for *in situ* matured avocado embryos (Perán-Quesada et al., 2005),

The presence of protein bodies in embryos cultured in B5m + aa + suc medium confirmed the occurrence of maturation.

These reserve organelles have been proposed as morphological markers of embryo developmental age (Koltunow et al., 1996) and, in the case of avocado embryos, their presence has been clearly associated with the maturation phase (Perán-Quesada et al., 2005).

5. Conclusions

Very immature avocado zygotic embryos can be successfully rescued by *in vitro* culture obtaining high germination rates and vigorous, well-developed plants. The inclusion into the *in vitro* culture protocol of a maturation phase is crucial for the obtainment of these results and represents a significant advance with respect to previous investigations on avocado embryo rescue, overcoming the development of hyperhydric plants when germinating very immature embryos (Skene and Barlass, 1983; Sánchez-Romero et al., 2007). Moreover, germination conditions previously developed for mature avocado embryos (Pliego-Alfaro, 1988) are genotype independent, hence, it is fairly possible that once avocado embryos are matured *in vitro*, they will show a similar behavior. Our findings also demonstrate, for the first time in this species, that embryos undergoing *in vitro* maturation are capable of storing starch and reserve proteins in a similar manner to that of embryos matured under *in vivo* conditions.

Acknowledgements

The authors wish to thank Luis Alberto Romero Talavera for providing technical assistance. This work was supported by the Comisión Interministerial de Ciencia y Tecnología, Spain (Grant no. AGL 2004-07028 C03-03/AGR). The authors would like to express their gratitude to Dr. J.M. Farré and J.M. Hermoso (La Mayora Experimental Station (Algarrobo Costa, Spain)) for providing the plant material used in this investigation.

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