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

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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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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

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