Histological aspects of avocado embryo development and effect of developmental stages on germination

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Abstract

Anatomical differentiation and storage product accumulation were studied during avocado (*Persea americana* Mill.) zygotic embryo development using histological techniques. The results were correlated with the germination capacity under in vitro conditions. The histodifferentiation phase, characterized by active cell division and differentiation, was accomplished by 100 d after pollination (DAP), when embryos reached 16-18mm in length. After a phase in which there were no morphological changes, the maturation phase commenced at 125 DAP in embryos 24–26 mm in length. Accumulation of starch granules and the appearance of protein bodies occurred at this time. Germination was significantly influenced by the embryo developmental stage. Different parameters used as indicators of germination quality (germination percentage, germination speed and recovery of complete plantlets) showed a positive correlation with the age of the embryos. However, germination in vitro was not achieved fully until the end of zygotic embryo development, when the embryo reached physiological maturity.

Keywords: germination, *Persea americana* Mill., protein bodies, reserve products, starch granules, zygotic embryogenesis

1. Introduction

Embryogenesis is a complex and highly organized process during which the zygote undergoes successive changes that result in the formation of an embryo capable of germinating and growing into a healthy and vigorous seedling. While avocado (*Persea americana* Mill.) zygotic embryogenesis has been studied previously (Schroeder, 1952; Tomer and Gazit, 1979; Heo et al., 1998), emphasis was on the first few divisions of the zygote, from the fertilization of the egg to the establishment of the early cotyledonary stage embryo. Avocado embryos are considered to be recalcitrant. Recalcitrant embryos are larger and fleshier (Pliego- Alfaro et al., 1996), and have a more prolonged maturation period (Whiley, 1992), than embryos of the orthodox type. Moreover, recalcitrant seeds cannot tolerate maturation drying, generally lack dormancy (Pence, 1992), and reserve accumulation appears to be controlled differently (Alemanno et al., 1997). Therefore, it is important to determine changes occurring in the avocado zygotic embryo from the beginning of the early cotyledonary stage to the attainment of physiological maturity. The objective of this investigation was to establish a pattern of avocado zygotic embryogenesis using histological techniques.

2. Material and methods

Plant material

Zygotic embryos of avocado (*Persea americana* Mill.) cv. Hass, were studied at different developmental stages. After full bloom (Day 0), avocado fruits of uniform size were periodically harvested at random from different trees. Zygotic embryos, excised from these seeds, were classified according to their length, and used immediately for histological or in vitro germination studies. Sampling covered all stages of zygotic embryo development, from the early cotyledonary stage to the fully mature stage. Samplings were carried out from May 1999 to February 2000 and from May 2001 to February 2002.

Histology

The histological study was carried out from 48 to 305 d after pollination (DAP), using zygotic embryos ranging from 2 to 38-40mm in length. Following fruit harvesting and dissection, embryos corresponding to specific developmental stages were processed according to Johansen (1940). Samples were fixed in FAA [5 % formalin, 5 % acetic acid and 90 % ethanol (v/v/v)] for at least 24 h. The fixed samples were dehydrated in a TBA (tertiary butyl alcohol) and an ethanol series (70 %, 85 %, 95 % and 100 %), and embedded in paraffin. Longitudinal sections, 10 mm thick, were cut with a microtome (Leitz, Wetzlar, Germany), mounted on slides with several drops of a 3% (v/v) formalin solution and dried at 36 ºC on a hot plate. Preparations were observed with a fluorescence optical microscope (Laborlux 12, Leitz), and photomicrographs were taken with a Photoatomat Wild MPS 45 (Wild, Heerbrugg, Switzerland). For anatomical analysis, sections were stained by the Gerlach procedure (Gerlach, 1969). Accumulation of starch granules was monitored using periodic acid-Schiff reagent (PAS) (Herrero, 1979; Arbeloa, 1986), and protein bodies were stained with PAS- Amido Black (Jensen, 1962; Fisher, 1968). At least three sections from different embryos were used per developmental stage.

In vitro germination

The capacity for *in vitro* germination was tested in embryos corresponding to five developmental stages: 64 DAP (5mm in length), 95 DAP (15mm in length), 125 DAP (25mm in length), 204 DAP (35mm in length) and 305 DAP (38-40mm in length).

Avocado fruits were peeled and surface sterilized by immersion in a 0.5 % (v/v) sodium hypochlorite solution, containing Tween 20 (1 drop/100 ml), for 10 min and subsequently rinsed three times with sterilized distilled water. Sterilized fruits were bisected longitudinally under sterile conditions, and isolated embryos were induced to germinate using the procedure of Perán-Quesada (2001). Embryos with partially removed cotyledons (from 15mm and longer embryos) were cultured on M1 medium (Skene and Barlass, 1983), consisting of the MS (Murashige and Skoog, 1962) formulation at half strength supplemented with 0.5mgl^{-1} benzyladenine (BA) and solidified with 1.7 g l^{-1} Gelrite (Kelco, San Diego, California, USA). The pH of the medium was adjusted to 5.74 with 1N NaOH or 1N HCl before warming to melt the gelling agent. Then 25 ml were dispensed into 25 mm wide x 150 mm long test tubes (Bellco Glass, Vineland, New Jersey, USA), which were covered with polypropylene caps (Bellco Glass) and autoclaved at 121 ºC for 15 min at 0.1 MPa. Cultures were kept at 25 ± 1 ºC under a 16 h light photoperiod (40 µmolm^{-2} s^{-1} PAR) provided by Grolux lamps (Sylvania, Erlangen, Germany). Germination was carried out for 15 weeks, with reculturing onto fresh medium at 5-week intervals.

The germination experiment was repeated twice. For each experiment, 20 and 30 zygotic embryos, respectively, were used for each developmental stage. Embryos were considered germinated when shoot and/or root elongation was ≥ 2mm. Germination percentage, type of germination (shoot, root, or shoot and root) and percentage of embryos producing callus or rosette shoots were recorded at the end of each reculture (5, 10 and 15 weeks). In rosette shoots, only development of twisted, thick and small albino leaves was observed, while virtually no elongation of the shoot apical meristem could be detected.

3. Statistical analyses

Percentage data were subjected to a frequency analysis with the RxC test of independence (Sokal and Rohlf, 2003). The significance level was 0.05.

4. Results

Morphology

In addition to progressive weight and length increases, morphological changes were observed during avocado zygotic embryo development (Fig. 1), e.g. while small embryos were white and narrow, larger embryos gradually turned beige and became wider.

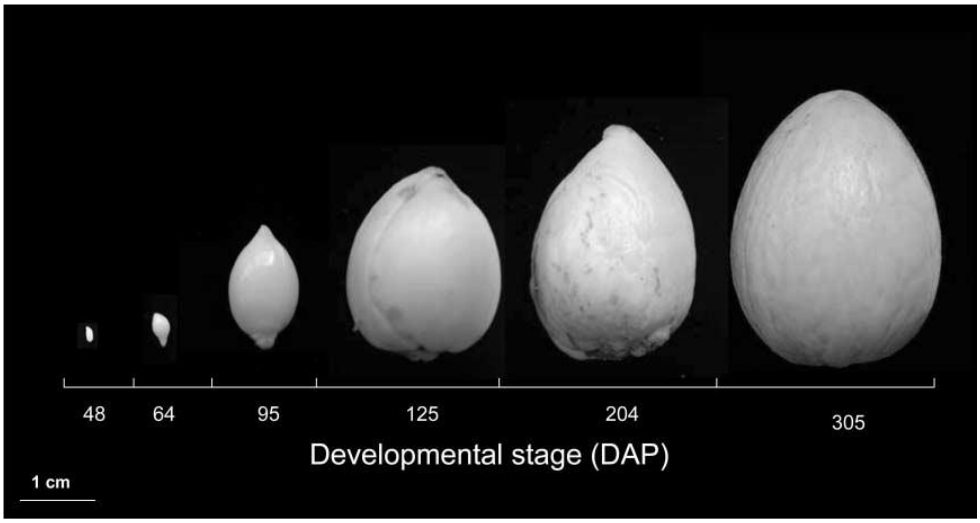

Figure 1. Avocado zygotic embryos at different developmental stages, from the early cotyledonary stage [48 d after pollination (DAP)] to full maturity (305 DAP).

Histology

Anatomical study

This study was started with zygotic embryos at approximately 48 DAP, when they were 2 mm long, white and opaque. The only visible feature in the embryonic axis was a group of cells with stained nuclei in an area of active division, which corresponded to the root meristem (Fig. 2a). Bipolarity in the embryonic axis was well defined at 60 DAP, when embryos reached 4mm

in length (Fig. 2b). At this stage, the shoot and root meristems began to develop as relatively small isodiametric cells. Procambial tissue differentiated as a group of elongated cells resulting from longitudinal divisions and cellular enlargement. Strands of procambial tissue extended in an acropetal direction towards the developing cotyledons. At approximately 74 DAP, when embryos were 7-8 mm long (Fig. 2c), the shoot meristem was well defined and became cone-shaped. Procambial cells developed basipetally towards the root apex, where the root cap was clearly distinguishable. At 89 DAP (embryos 12-13mm long) (Fig. 2d), the procambium was completely developed, forming a continuous cylinder. The procambium defined the central core of the embryonic axis where the pith cells began to enlarge, giving rise to the hypocotyl-shoot axis. The first leaf primordia were initiated on the shoot meristem. At approximately 100 DAP, the embryos were 16-18 mm in length and appeared completely differentiated (Fig. 2e), having all the structures and tissue types present in a fully developed embryo. In the central zone of the embryonic axis, the pith contained large cells and cytoplasm of low density. This zone was surrounded by the procambium, which in turn was surrounded by the cortex formed of small isodiametric parenchyma cells, and to the outside, the epidermis. In the shoot apical meristem (Fig. 2f), the most exterior region formed the tunica through an active anticlinal division with one layer of meristematic cells that were similar in shape and size. The subtending cells, which were larger, and in a random arrangement as a result of divisions in different planes, constituted the corpus. The transition zone where the procambium started to differentiate could be observed. In the outermost zone of the root apical meristem (Fig. 2g), the root cap was observed next to the columella, composed of cells arranged in files as a result of transverse divisions, and the quiescent centre, a centrally located spherical group of cells. On both sides of the quiescent centre, procambial strands started to develop in an acropetal direction. From this stage onwards, the appearance of new leaf primordia was linked to a gradual increase in size, which was the only morphological change observed until the embryo completed development at 305 DAP, its size being 38-40 mm in length; details of the embryonic axis are shown in Fig. 2h.

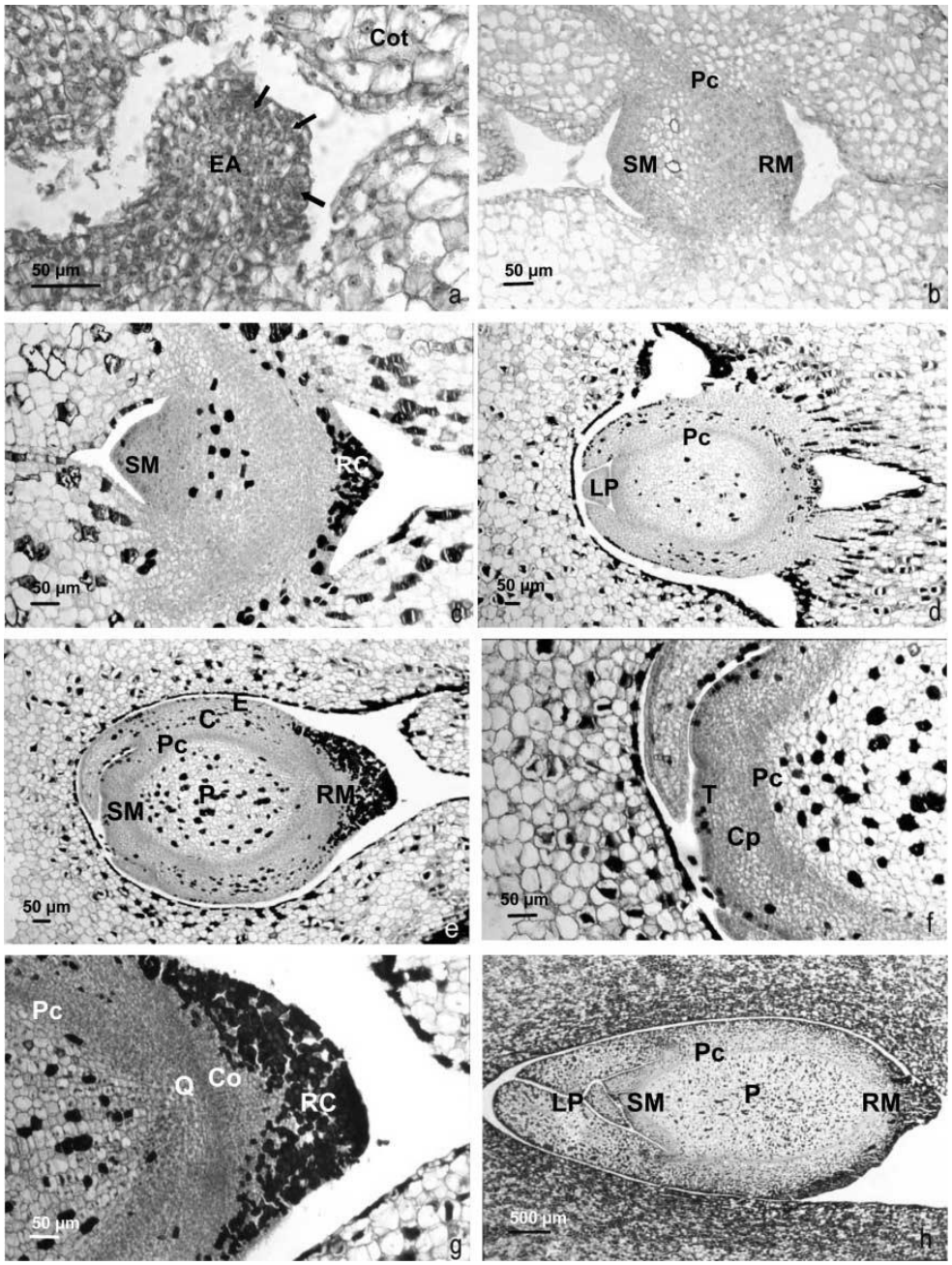

Figure 2. Light micrographs of longitudinal sections of avocado zygotic embryos at different developmental stages, stained with the Gerlach reagent. Micrographs include details of the embryonic axis and a small part of the cotyledons. (a) Zygotic embryo at 48 d after pollination (DAP) (2 mm long), showing the embryonic axis (EA) and part of the cotyledons (Cot). (b) Embryo at 60 DAP (4 mm long) with a shoot meristem (SM), root meristem (RM) and procambial strands (Pc). (c) Embryo at 74 DAP (7-8 mm long) showing a well-defined shoot meristem (SM) and root cap (RC). (d) Embryo at 89 DAP (12-13 mm long) with leaf primordia (LP) and the procambium (Pc) forming a cylinder. (e) Embryo at 100 DAP (16-18 mm long) showing complete histodifferentiation. A shoot meristem (SM), the pith (P), procambium (Pc), cortex (C), epidermis (E) and root meristem (RM) can be distinguished. (f) Shoot meristem at 100 DAP (16-18 mm in length) showing the tunica (T), the corpus (Cp) and the procambium (Pc). (g) Root meristem at 100 DAP (16-18mm in length) showing the procambium (Pc), a well-differentiated quiescent centre (Q), the columella (Co) and the root cap (RC). (h) Embryonic axis from a fully mature embryo at 305 DAP (38- 40 mm long) showing leaf primordia (LP), shoot meristem (SM), the pith (P), the procambium (Pc) and root meristem (RM). The embryonic axis is very small compared to the large and fleshy cotyledons. Photomicrographs are representative of three independent stainings carried out with samples from different embryos.

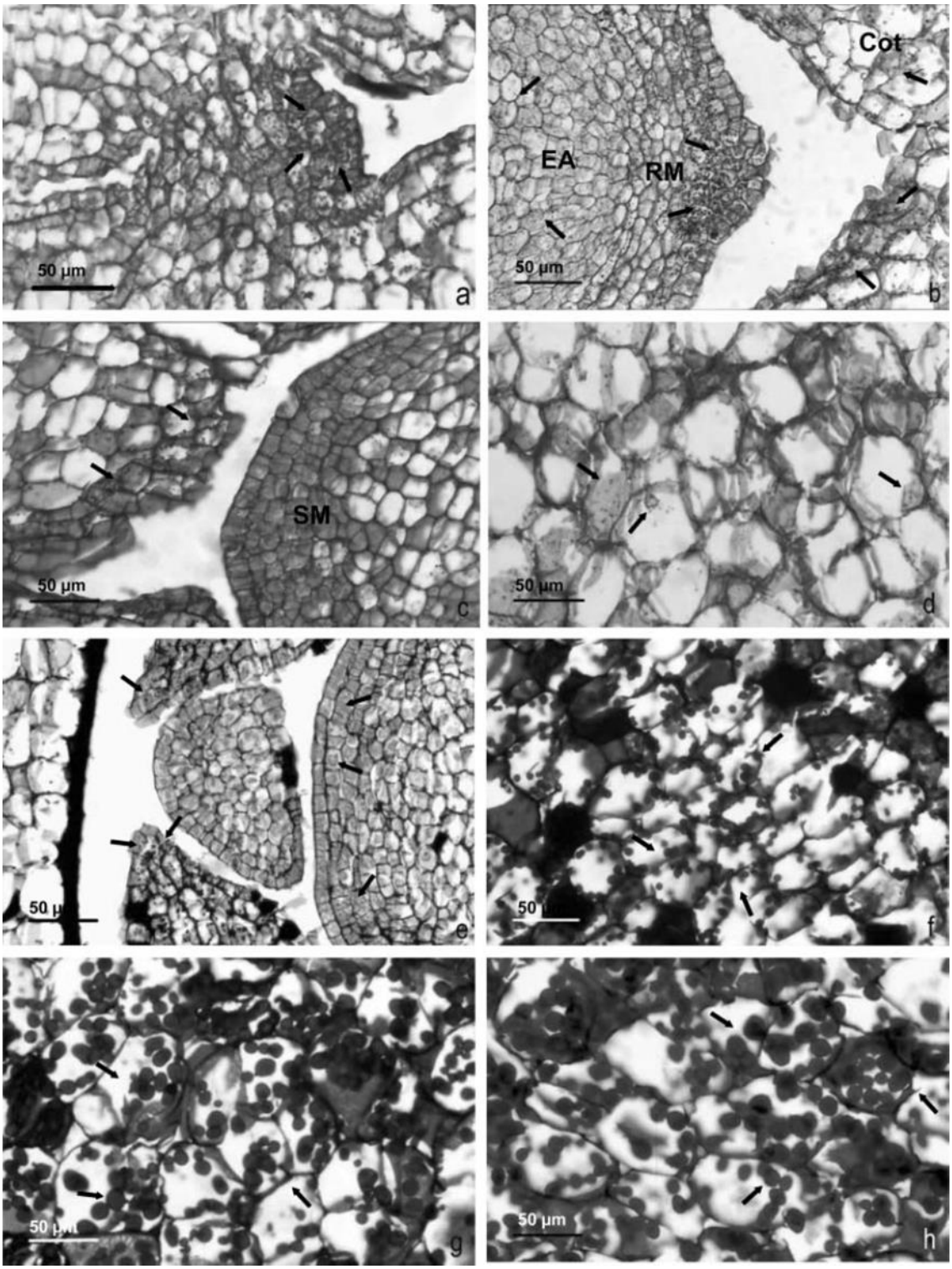

Figure 3. Light micrographs of longitudinal sections stained with PAS of avocado zygotic embryos at different developmental stages. Arrows indicate starch granules. (a) Embryo at 48 d after pollination (DAP). (b) Root region at 60 DAP, showing the central part of the embryonic axis (EA) and part of the cotyledons (Cot). (c) Apical meristemic region at 60 DAP. (d) Cotyledon mesophyll at 60 DAP. (e) Apical meristem of an embryo at 89 DAP. (f) Central zone of the embryonic axis in embryo at 125 DAP. (g) Cotyledon mesophyll in embryo at 125 DAP. (h) Cotyledonal tissue in a fully mature embryo (305 DAP). Photomicrographsare representative of three independent stainings carried out with samples from different embryos.

Storage product deposition

At 48 DAP, embryos contained small starch granules in the lower part of the embryonic axis, the root meristem (Fig. 3a), and starch increased in embryos collected at 60 DAP (Fig. 3b). In addition to the root meristem, starch granules could be observed in the central part of the axis and in the cotyledons. No starch was present in cells of the shoot apex (Fig. 3c). Within the cotyledons, starch granules appeared very small and sparse (Fig. 3d) and were located in parenchyma cells proximal to the root and shoot meristems (Fig. 3b, 3c). The formation of starch granules showed a slight, but gradual, increase throughout embryo development, and starch granules were also present in embryos at 89 DAP in the leaf primordia and in the shoot meristem, although in the latter they were very small and scarce (Fig. 3e). These changes became more evident as embryo development progressed. Embryos at 125 DAP contained many starch granules in the pith tissue of the embryonic axis (Fig. 3f) and the cotyledons (Fig. 3g). During the rest of the maturation phase, a progressive increase in starch granules was observed, mainly in cotyledons (Fig. 3h).

Protein bodies were first detected at 125 DAP, when embryos were 24-26 mm in length. At this stage, protein bodies could be observed in the cotyledonal tissue (Fig. 4a), where starch accumulation was also taking place. A gradual increase in protein bodies was observed as maturation progressed, until embryos reached the fully mature stage at 305 DAP (38-40 mm long) (Fig. 4b). No protein bodies were observed in the embryonic axis at any developmental stage.

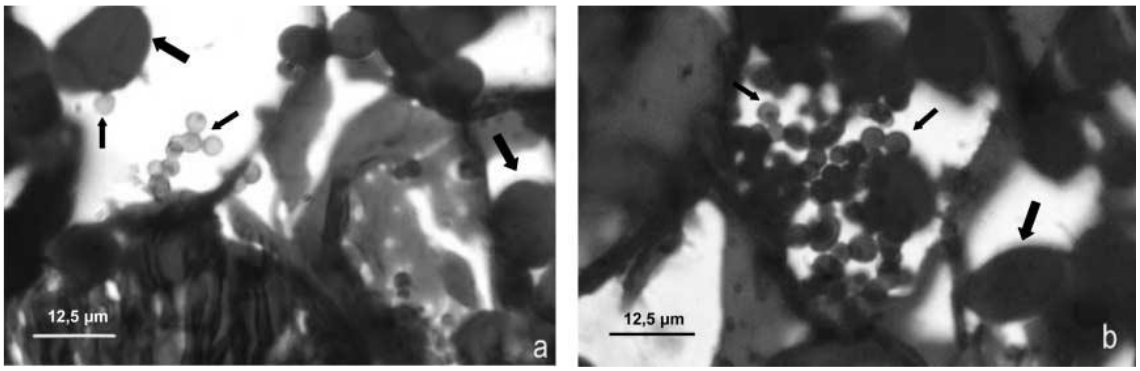

Figure 4. Light micrographs of longitudinal sections of cotyledons from avocado zygotic embryos at different developmental stages stained with PAS–Amido Black. Thin arrows indicate protein bodies and thick arrows indicate starch granules. (a) Embryo at the beginning of the maturation stage [125 d after pollination (DAP)]. (b) Embryo at a fully mature stage (305 DAP). Photomicrographs are representative of three independent stainings carried out with samples from different embryos.

In vitro germination

The capacity for in vitro germination increased gradually during avocado zygotic embryo development (Fig. 5). Immature embryos at 64 DAP seldom germinated, but changes were evident at 95 DAP. An increase was observed at 125 DAP, when 66 % of embryos gave rise to shoots and/or roots; at 204 DAP no changes were found, while virtually full germination was achieved at the end of embryo development (305 DAP).

The type of germination obtained (shoot, root or shoot and root) was also influenced by embryo developmental stage. Whereas in young and small embryos germination produced shoots or roots, as their maturity and size increased, a general trend in the recovery of complete plantlets (with shoot and root) was observed. At the fully developed stage (305 DAP), 74 % of embryos produced complete plantlets.

Callus development was high in immature embryos, with 58 % and 40 % producing callus at 64 and 95 DAP, respectively. The proportion of embryos developing callus decreased significantly as the age of the embryos increased. No callus production was observed from embryos at 204 DAP onwards. In some cases, embryos produced abnormal rosette shoots. The production of rosette shoots was significantly influenced by the embryo developmental stage, with higher values being observed at 125 (1 8 %) and 204 DAP (48 %).

The kinetics of germination were also influenced by embryo age. Whereas immature embryos germinated in successive recultures, almost 100 % of embryos at 305 DAP germinated during the first 5 weeks on M1 medium.

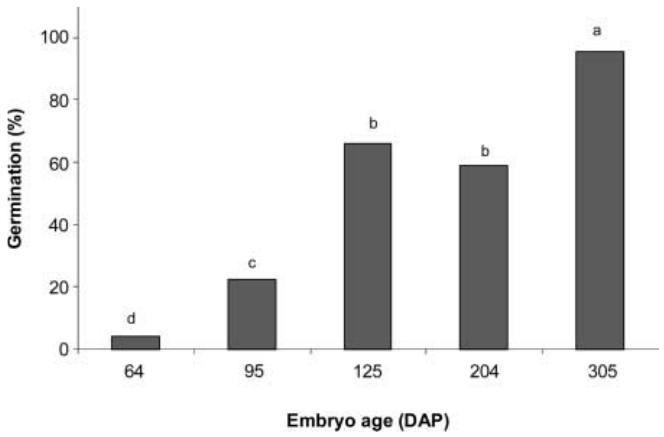

Figure 5. Germination of avocado zygotic embryos at different developmental stages. Data are means of two different experiments. In each experiment, 20 and 30 embryos, respectively, were used for each developmental stage. Different letters indicate significant differences obtained by frequency analysis, with a significance level of 0.05.

Discussion

In the avocado zygotic embryo, the cell division phase takes place from the formation of the zygote until the embryo attains a 0.2-0.3mm diameter, approximately 15-20 DAP (Tomer and Gazit, 1979), when cotyledonary differentiation becomes well marked (Schroeder, 1952). Our study was initiated at 48 DAP, with embryos in the histodifferentiation phase, 2 mm in length. During this phase, all the structures and tissue types present in a fully mature embryo are formed. In avocado embryos, histodifferentiation lasts until 100 DAP, when they reach 16-18mmin length. After a phase in which there are no noticeable morphological changes, the maturation phase begins at 125 DAP with 24-26 mm long embryos. It is marked by starch granule accumulation and the appearance of protein bodies. Both observations coincide with other maturation events previously reported in the avocado seed by our research group (Sánchez-Romero et al., 2002), e.g. a large increase in fresh and dry weights, significant decline in water content and a switch in the sugar status from high hexose to high sucrose levels. At the end of embryo development, physiological maturity is achieved. This stage is reached at approximately 305 DAP when embryos are 38-40 mm in length, falling within the range (180-365 days) stated by Whiley (1992) for avocado embryos.

Storage product accumulation is a key event in the maturation phase as it provides the food material that will be utilized by the germinated embryo when giving rise to the new plant (Merkle et al., 1995). Starch deposited as starch granules, and storage proteins accumulated in protein bodies, are the main reserve products in avocado embryos. Starch granules and protein bodies show different temporal and spatial accumulation patterns during avocado embryogenesis.

As in other non-endospermic seeds (Harada, 1997), starch begins to accumulate at early developmental stages in avocado, and starch granules are visible in the root meristematic zone by 48 DAP. In the next developmental stages, starch granule distribution increases and appears mainly in the root and shoot meristematic zones, as well as in the surrounding cotyledonary tissue. This location of starch, in areas where rapid storage material breakdown is anticipated, suggests that starch is the preferred short-term storage form (Krasowski and Owens, 1993). Thorpe (1993) also proposed that the accumulation of starch in meristematic zones at earlier developmental stages may serve as a readily available source of energy for mitotic activity. The progressive accumulation of starch granules observed during embryo development is in accordance with previous results obtained by our research group (Sánchez-Romero et al., 2002); starch content increased throughout avocado embryo growth, with the highest value observed in fully developed mature embryos [10.24 mg $(g\ fw)^{-1}$].

Protein bodies are visible for the first time in embryos at 125 DAP. However, previous investigations by our research group (Sánchez-Romero et al., 2002) have shown that storage proteins begin to accumulate in embryos of smaller size (7-8 mm in length), although most protein is deposited at the end of the growth period. At the fully mature stage, proteins extracted from protein bodies represent 83 % of total proteins in the avocado embryo (Sánchez- Romero et al., 2002). The presence of storage proteins when protein bodies are not discernible could be explained, according to Tewes et al. (1991) and Ross and Murphy (1992), by the fact that accumulation of storage proteins in immature embryos can occur in smaller vesicles and vacuoles, which evolve to protein bodies when an embryo reaches the maturation phase.

Cotyledons are the primary site of storage product accumulation in avocado embryos, as in other non-endospermic seeds (Harada, 1997). Interestingly, while starch granules are present in the cotyledons and embryonic axis, protein bodies are present only in cotyledons. This result

confirms previous observations in non-endospermic seeds by Müntz (1996) and is indicative of an organ-specific control of storage protein synthesis (Motto et al., 1997).

The stage of development is a critical factor influencing germination. Avocado embryos at early developmental stages fail to germinate, probably due to incomplete histodifferentiation and the absence of storage products. Immature avocado embryos at 64 and 95 DAP develop frequently into callus under in vitro germination conditions; the cytokinin present in the germination medium could be inducing this callus proliferation, rather than enhancing the growth of the incompletely differentiated shoot meristem.

Between 125 and 204 DAP, germination was observed in 60 % of the embryos; however, while only 18 % of embryos at 125 DAP formed rosette shoots, this value was 48 % for embryos at 204 DAP. This trait has been associated with physiological dormancy (Tukey, 1938).

Approximately 100 days later, at 305 DAP, moisture content had decreased, embryo development ended and physiological maturity was achieved. Only at this developmental stage was a very high percentage germination obtained, as well as optimal germination speed and recovery of complete plantlets. These results are consistent with those of Ellis et al. (1987) for legumes, which suggested that embryos achieve maximum viability and vigour at physiological maturity

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