Journal of Plant Growth Regulation
https://doi.org/10.1007/s00344-019-10039-6

Fruit Development and ABA Biosynthesis During *Caryocar brasiliense* (Caryocaraceae) Seed Maturation

Iara Veloso Rodrigues[1] · Paulo Sérgio Nascimento Lopes[1] · Leonardo Monteiro Ribeiro[2] · Maria Olívia Mercadante-Simões[2] · Cristina de Paula Santos Martins[1] · Felipe Ramos Vieira[1] · Ane Patrícia Cacique[1] · Demerson Arruda Sanglard[1] · Flaviano Oliveira Silvério[1]

Received: 21 January 2019 / Accepted: 7 October 2019
© Springer Science+Business Media, LLC, part of Springer Nature 2019

Abstract

Little is currently known about the interactions between structural and physiological factors in Neotropical fruit development. We sought to characterize fruit development and the dynamics of ABA biosynthesis during seed maturation in *Caryocar brasiliense*, a tree species endemic to the Cerrado biome of Brazil. Biometric evaluations were made of both the pericarp and seed during fruit development. ABA1, NCED4, and NCED9 expression were evaluated in embryonic tissues during seed maturation, as well as anatomical, histochemical, and ultrastructural evaluations, in vitro and ex vitro embryo cultures, and ABA quantifications. Fruit development demonstrated three stages: histodifferentiation (0–40 days after anthesis—DAA); endocarp maturation (40–60 DAA); and the conclusion of mesocarp and seed maturation (60–90 DAA). The embryos showed early degrees of differentiation: hypocotyl expansion, development of the leaf primordia, and vascularization. Seed maturation occurred (from 40 DAA) concomitantly with the development of endocarp aculei in four phases influenced by ABA dynamics. The beginning of reserve deposition and the acquisition of dehydration tolerance were related to increases in extra-embryonic ABA. ABA biosynthesis at the radicular pole was related to increased reserve deposition and dehydration tolerance (which was not associated with the occurrence of a desiccation phase). The maturation of *C. brasiliense* seeds is influenced by embryonic and extra-embryonic ABA, and the dormancy of the pyrenes results from the interaction of the effects of that hormone and pericarp tissue.

Keywords Abscisic acid · Embryo · Reserve deposition · Germination · Pericarp · Desiccation tolerance

Introduction

Caryocar brasiliense Camb. (Caryocaraceae) is an endemic and widely distributed tree species in the highly seasonal Brazilian Cerrado (Neotropical savanna) biome (Giroldo and Scariot 2015; Flora do Brasil 2016). Its oleaginous fruits are important to the regional fauna and provide income for traditional human populations that used them to produce an edible oil as well as preserves, liqueurs, and ice cream (Araújo 1995; Barradas 1973; Ascari et al. 2013). The fruits also have potential for use in the pharmaceutical and cosmetics industry due to their anti-inflammatory, antioxidant, and antimicrobial properties (Amaral et al. 2014; Machado et al. 2013; Roesler et al. 2008).

The drupaceous fruits of *C. brasiliense* demonstrate a number of peculiarities, such as a mesocarp divided into two regions in mature fruits, and the presence of aculeate endocarps, as well as the development of seeds with rich reserves and highly differentiated embryos (Barradas 1973). The seeds are dispersed with high water contents but are resistant to dehydration (Sousa et al. 2017a)—an unusual trait (Bewley et al. 2013). The diaspores demonstrate pronounced dormancy resulting from the interaction between the low-growth potential of the embryo and resistance provided by the endocarp (Sousa et al. 2017a, b). That dormancy is

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00344-019-10039-6) contains supplementary material, which is available to authorized users.

✉ Leonardo Monteiro Ribeiro
leonardo.ribeiro@unimontes.br

[1] Instituto de Ciências Agrárias, Universidade Federal de Minas Gerais, Montes Claros, MG 39404-547, Brazil

[2] Departamento de Biologia Geral, Universidade Estadual de Montes Claros, Montes Claros, MG 39401-089, Brazil

considered an adaptation to the Cerrado environment, but it limits seedling production for commercial and/or conservation purposes (Sousa et al. 2017b).

Seed development usually involve stages of histodifferentiation, maturation, and desiccation (Bewley et al. 2013). There is considerable diversity, however, in the temporal distributions of phases among different species, what is related to differences in hormonal control (Werker 1997; Gillaspy et al. 1993; Bewley et al. 2013). Abscisic acid (ABA) is considered a key molecule in the regulation of the main physiological processes related to seed maturation: acquisition of desiccation tolerance, dormancy, and reserve deposition (Finkelstein et al. 2008). ABA biosynthesis in seeds is controlled by ABA1 and NCED gene expression, which are in turn associated with the synthesis of the rate-limiting enzyme 9-cis-epoxycarotenoid dioxygenase (Lefebvre et al. 2006; Nambara et al. 2010; Chao et al. 2014). Studies involving gene expression associated with that hormone during seed maturation have been scarce in neotropical species and there are no reports of works on the topic in the Caryocaraceae family. Such studies would contribute to our knowledge of fruit development patterns as well as the reproductive strategies of Cerrado species.

The objectives of the present work were to characterize the development of the fruits of *C. brasiliense* and evaluate the anatomical, ultrastructural, and physiological aspects of the seed maturation, with emphasis on ABA1, NCED4, NCED9 gene expression, and ABA quantification in the embryo by addressing the following questions: (i) What are the developmental stages of the fruit? (ii) When are germination capacity, desiccation tolerance, and dormancy established? (iii) What are the relationships between ABA synthesis and the physiological events associated with seed maturation?

Materials and Methods

Fruit Collection and Biometric Assessments

Twenty-nine *C. brasiliense* plants, each approximately 10 years old, were selected in an experimental orchard located on the campus of the Federal University of Minas Gerais, in the municipality of Montes Claros in northern Minas Gerais State, Brazil. The dates of floral anthesis were recorded starting in September 2017 (Fig. 1a). Weekly collections of developing fruits were made until 90 days after anthesis (DAA), when abscission occurred.

The exocarp and external mesocarp were manually removed using a knife. The seeds were also removed from the endocarp with the aid of a knife up until 40 DAA; after that, pliers, a bench grinder, and tweezers were used, following the methodology described by Mendes (2015). The lengths, widths, and thicknesses of the fruits and seeds were measured at each collection time (using four replicates of five fruits). The fresh and the dry masses of the exocarp + mesocarp, endocarp, and seed were determined, followed by dehydration at 105 °C, for 24 h to calculate their original water contents (Brasil 2009). The fruits of *C. brasiliense* can develop from one to five seeds (Barradas 1973); the evaluations were carried out only with monospermic fruits (which can be identified early by their spheriform shapes) to facilitate comparisons and better define the developmental phases.

Embryo Germination Capacity and Desiccation Tolerance

The in vitro germination capacities of the embryos were evaluated at 40, 50, 60, 80 and 90 DAA. The developing seeds were removed from the fruits as described above, disinfected in 2% sodium hypochlorite solution for 10 min, and rinsed three times in distilled water. The seed coats were subsequently removed using a scalpel, and the embryos placed in a solution of 100 mg L^{-1} of ascorbic acid (to avoid oxidation) and then disinfected in 0.5% sodium hypochlorite for 10 min, followed by triple rinses in sterile distilled water. The embryos were then placed into test tubes (20 × 3 cm) containing 20 mL of sterile culture medium containing MS salts (at 75% of its original concentration) (Murashige and Skoog 1962) supplemented with 0.5 mg L^{-1} thiamine, 1 mg L^{-1} pyridoxine, 0.5 mg L^{-1} nicotinic acid, 100 mg L^{-1} myo-inositol, 0.5 g L^{-1} hydrolyzed casein, 30 g L^{-1} sucrose, 3 g L^{-1} activated carbon, and 6 g L^{-1} agar (pH adjusted to 5.7) (Ribeiro et al. 2011). The embryos were then kept in a germination chamber at 30 °C, in the dark, for 30 days.

Embryos were also inoculated, under the same conditions described above, after dehydration for five days over silica gel. The silica had been dehydrated in an oven at 130 °C for 8 h, and was replaced when color changes were observed. An amount of silica was used equivalent to the mass of the containers plus the seeds. In both experiments, the embryos that emitted a primary root (> 5 mm), or a plumule (> 10 mm long), were considered germinated. Embryos that became softened, or were attacked by microorganisms and did not germinate, were classified as dead.

The germinative capacities of the embryos under ex vitro condition were also evaluated, during the same periods previously mentioned. The embryos were sown to germinate in transparent gerbox boxes (11 × 11 × 3.5 cm) on two sheets of sterile filter paper moistened with sterile distilled water (in the proportion of 2.5 times the mass of the substrate) (Brazil 2009). Both hydrated (taken directly from the seeds) and dehydrated embryos were used; the other procedures and evaluations were performed as previously described.

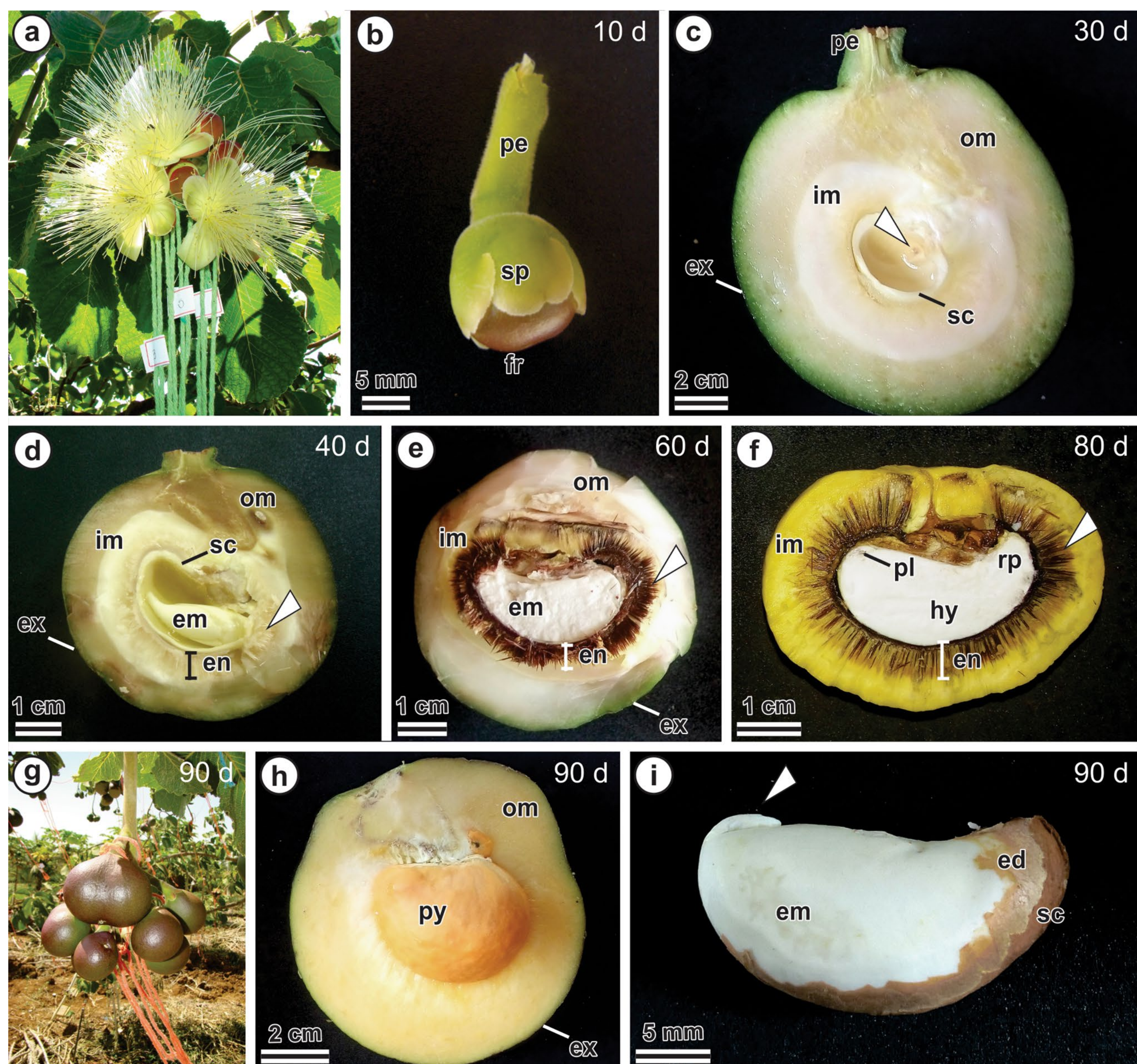


Fig. 1 Morphology of the flowers and fruits of *C. brasiliense*. Longitudinal sections (**c**–**f**, **h**–**i**). **a** Flowers in anthesis. **b** Fruit, 10 days after anthesis (DAA). **c** Fruit, 30 DAA, highlighting the embryo (arrowhead). **d** Fruit, 40 DAA, showing the non-lignified aculeus endocarp (arrowhead). **e** Fruit, 60 DAA, showing the already lignified aculei (arrowhead). **f** Fruit, 80 DAA, highlighting the fully developed aculei (arrowhead). **g**, **h** Fruits, 90 DAA. **i** Seed, with part of the tegument removed to allow visualization of the embryo, 90 DAA, highlighting the plumule (arrowhead). *ed* inner seed coat, *em* embryo, *en* endocarp, *ex* exocarp, *fr* fruit, *hy* hypocotyl, *im* internal mesocarp, *om* external mesocarp, *pe* peduncle, *pl* plumule, *py* pyrene, *rp* radicular pole, *sc* external seed coat, *sp* sepal

Both experiments (in vitro and ex vitro conditions) were established in a completely randomized design, considering a factorial scheme of 5 (times) × 2 (hydrated or dehydrated embryos) using four replicates of five embryos. The data (number of germinated embryos) were transformed into percentages and submitted to analysis of variance; the means were compared by the Tukey test, at a 5% level of probability.

Anatomy and Histochemistry

Embryos were removed from the fruits at 35, 40, 50, 60, 80 and 90 DAA, as previously described, and were selectioned (using a razor blade) into cubes (sides approximately 3 mm) in the following regions: the radicular pole and the median regions of the hypocotyl and plumule. The material was fixed in Karnovsky's solution (Karnovsky 1965) for 24 h

under a vacuum, dehydrated in an ethanol series, and embedded in (2-hydroxyethyl)-methacrylate (Paiva et al. 2011). Longitudinal sections (5 µm thick) were obtained using a rotary microtome (Atago, Tokyo, Japan). The sections were stained with 0.05% toluidine blue, pH 4.7 (modified from O'Brien et al. 1964) and mounted on slides with acrylic resin (Itacril, Itaquaquecetuba, Brazil). Histochemical tests were carried out on samples from the plumule using: lugol solution (Jensen 1962) to identify starch; periodic acid and Schiff's reagent (PAS) (Feder and O'brien 1968) to identify polysaccharides; sudan black (Pearse 1980) to identify lipids; and Xylidine-Ponceau (Vidal 1970) who identified proteins. The sections were subsequently analyzed and the images recorded using a photomicroscope (Axio Scope. A1/Axiocam 105 color, Zeiss, Jena, Germany).

Electron Microscopy

For examinations using scanning electron microscopy (SEM), samples of the plumule were obtained at 35, 40, 50, 60, 80 and 90 DAA and fixed in Karnovsky's solution (Karnovsky 1965) under a vacuum for 24 h, dehydrated in an ethanol series, dried to their critical point using CO_2 (CPD 020, Bal-Tec, Balzers, Liechtenstein), placed on aluminum supports, and metalized with gold (MED 010-Balzers, Balzers, Leichtenstein). The analyses were performed using a scanning electron microscope (Quantum 200, FEI Company, Eindhoven, Netherlands), with digital image capture at 12–20 kV (Robards 1978).

For examinations using transmission electron microscopy (TEM), samples of the cotyledonary node region of embryos with 40, 50 and 90 DAA were used. Cross-sections (approximately 0.4 mm thickness) were fixed in Karnovsky's solution for 24 h under a vacuum (Karnovsky 1965), post-fixed in 1% osmium tetroxide (0.1 M phosphate buffer, pH 7.2), dehydrated in acetone series, and embedded in Araldite resin. Ultra-thin section (50 nm) were contrasted with uranyl acetate and lead citrate and examined using a transmission electron microscope (Philips CM 100, Philips / FEI Corporation, Eindhoven, Netherlands) with digital image capture at 80 kV (Robards 1978; Roland 1978).

Quantitative Real-Time Reverse Transcription PCR (qPCR)

Total RNA was isolated from embryo tissue (plumule, radicle and hypocotyl region obtained at 40, 50, 60, 80 and 90 DAA) with the TriReagent® (Sigma-Aldrich, USA), according to the manufacturer's instructions. The purity and concentration of the isolated RNA samples were checked on 1% (w/v) agarose gels and in a spectrophotometer. Reverse transcription was carried out using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) and 10 µg of total RNA, according to the manufacturer's instructions. The cDNA concentration was determined spectrophotometrically.

All the qPCR reactions were performed on Real-Time PCR Systems 7500 Fast (Applied Biosystems, USA) according to the manufacturer's instructions. qPCR primers were designed to avoid the conserved regions from *Arabdopsis thaliana*. Primer sequences are shown in Table 1 (CbABA1, CbNCED4 and CbNCED9). GAPC2 and ACT2 was used as an internal reference gene to normalize expression (Table 1). Reactions were performed in triplicate, containing 10 ng of cDNA, 0.5 µL of each primer (10 pmol), 10 µL PowerUp™ SYBR® Green Master Mix (Applied Biosystems, USA), and sterile Milli-Q water for a final volume of 20 µl. The amplification reactions were performed under the following conditions: (1) activation of Taq DNA polymerase at 50 °C for 2 min (2) initial denaturation at 95 °C for 3 min (3) denaturation at 95 °C for 20 s (4) annealing/extension at 60 °C for 30 s. Steps 3 and 4 were repeated for 40 cycles.

The Dissociation Curve 1.0 program (Applied Biosystems, USA) was used to verify that only a single PCR product was generated by the amplification of transcripts. Non-cDNA control reactions were used in all experiments. Gene expression was quantified using the comparative methods Ct: $2^{-\Delta Ct}$ and $2^{-\Delta\Delta Ct}$, with data obtained from three

Table 1 Primers used in the qPCR analysis of *Caryocar brasiliense* embryos

Symbol	Gene	Primer	References
CbABA1	ABA deficient 1	F: 5′-AACCCGGAGGAGTATCTGG-3′ R: 5′-AACACCAATCCTCCGATTC-3′	Bentsink et al. (2006)
CbNCED4	Nine-cis-epoxycarotenoid dioxygenase 4	F: 5′-TCTCGCAACAGCTCTCTTCA -3′ R: 5′-GAGTGCCGTGGATGATTTC -3′	Wang et al. (2013)
CbNCED9	Nine-cis-epoxycarotenoid dioxygenase 9	F: 5′-ATCGACCGGAGAGATTCGAAAG-3′ R: 5′-TCACCTTCTCCTCGTCGTGAAC-3′	Liu et al. (2007)
CbACT2	Actin 2	F: 5′-CTCTCCTTGTACGCCAGTGGTC-3′ R: 5′-TAAGGTCACGTCCAGCAAGGTC -3′	Liu et al. (2007)
CbGAPC2	Glyceraldehyde-3-phosphate dehydrogenase	F: 5′-TTGGTGACAACAGGTCAAGCA-3′ R: 5′-AAACTTGTCGCTCAATGCAATC-3′	Gao et al. (2015)

biological replicates and two experimental replicates (Livak and Schmittgen 2001). Data were subjected to analysis of variance and means were compared by Tukey's test at 5% probability.

The transcripts were validated using the AB 3500 Genetic Analyzer automated sequencer equipped with 50 cm capillaries and POP7 polymer (Applied Biosystems, USA). Sequencing was performed using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The consensus sequence of 5´ and 3´UTR resulting submissions was searched from the Phytozome v12.1 database (https://phytozome.jgi.doe.gov/pz/portal.html) using BLAST and BLASTx. The results of this analysis are presented in Supplementary Material I.

ABA Quantification

The evaluations were performed using three replicates of the embryonic tissue samples (plumule, radicular pole, and median region of the hypocotyl) at 40, 50, 60, 80 and 90 DAA, according to the methodology adapted from Müller and Munné-Bosch (2011). The ABA analytical standard and the sample extracts were analyzed in an UPLC system coupled to a diode arrangement detector (1290 Infinity, Agilent Technologies, Santa Clara, USA). Twenty microliters of each sample were injected into the analytical column (15 cm × 4.6 mm, 5 µm) (Kinetex, Phenomenex, Torrance, USA). The elution gradient was run using ultrapure water acidified to 0.05% acetic acid (solvent A) and acetonitrile with 0.05% acetic acid (solvent B).The temperature was maintained at 30 °C and the flow of the mobile phase was 0.600 mL min^{-1}. Detection used the wavelength of 250 nm and the identification of the ABA chromatographic peak in the samples was performed by comparing its retention time with that of the analytical standard. Each evaluation was performed twice for each biological replicate. The results were expressed on a fresh weight basis. The data were submitted to analysis of variance and the means of three replicates were compared using the Tukey test, at a 5% level of probability.

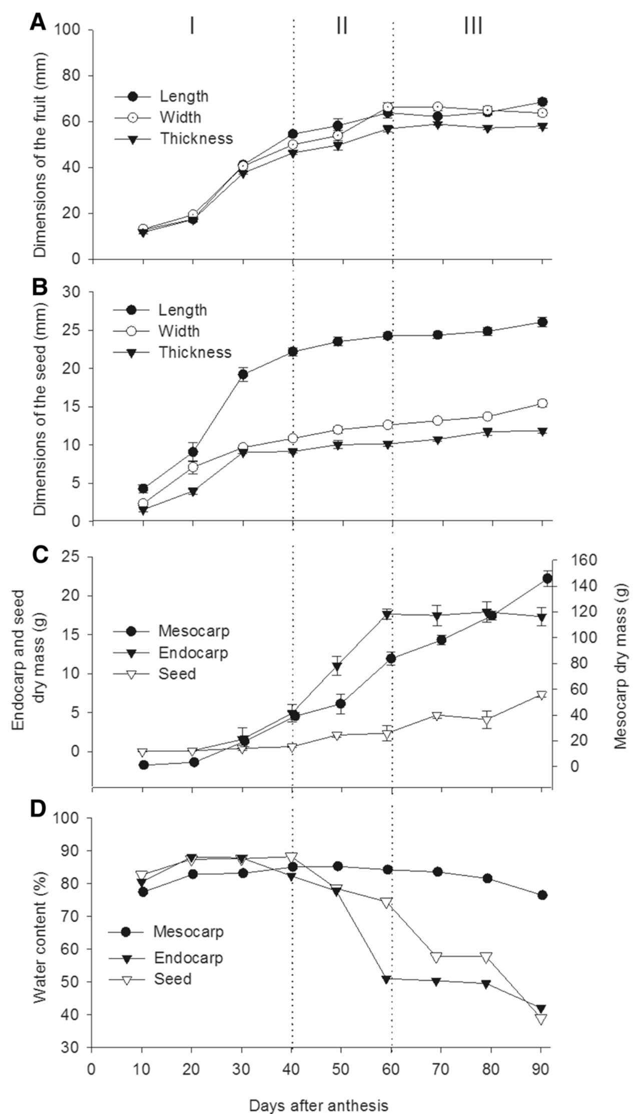


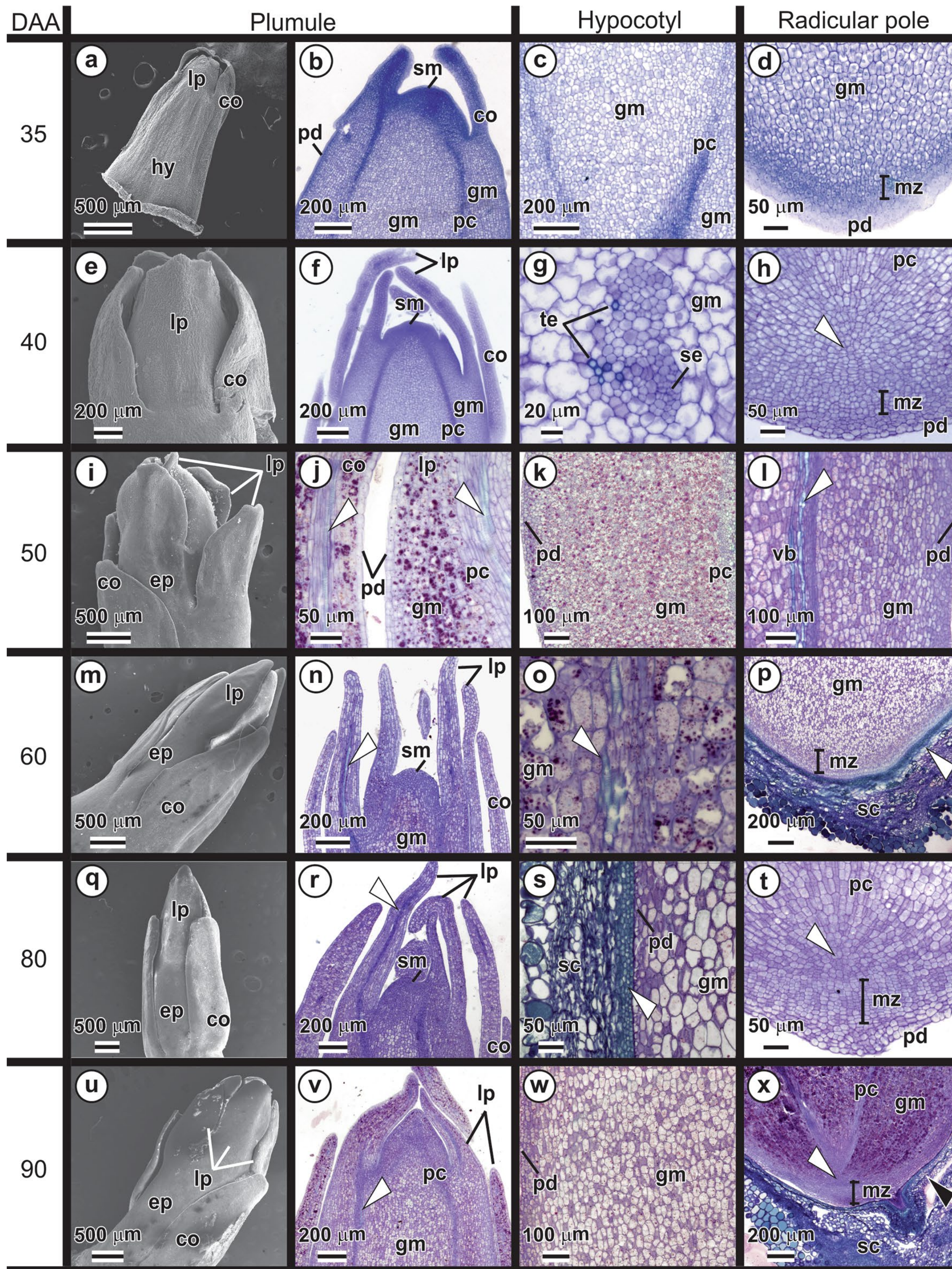
Fig. 2 Biometric characteristics of *C. brasiliense* fruits. **a** Dimensions of the fruits. **b** Dimensions of the seeds. **c** Dry masses of fruit and seed structures. **d** Water contents of fruit and seed structures. The dotted lines delimit the developmental stages: I—histodifferentiation; II—endocarp maturation; III—completion of mesocarp and seed maturation. Vertical bars indicate the standard errors of the means

Results

Fruit Development

Biometric evaluations showed that *C. brasiliense* fruit development took approximately 90 days, and could be divided into three phases: (i) histodifferentiation; (ii) maturation of the endocarp; and (iii) completion of maturation of the mesocarp and seed (Figs. 1a–i, 2a–d).

The histodifferentiation phase occurred up to 40 DAA, and was characterized by high water contents and most of the changes in the dimensions of the structures – all aspects associated with histogenesis (Figs. 1a–d, 2a–d). After fertilization, the abscission of most of the floral structures occurred in about 3 days. It was possible, at 10 DAA, to identify the fruit by the brown color of the exocarp (Fig. 1b). At 30 DAA, the exocarp coloration had changed to green and it was possible to visually identify some of the fruit structures by consulting the anatomical studies on pericarp development published by Barradas (1973) (Fig. 1c). The endocarp was still inconspicuous at that time, and the small embryo was restricted to the micropylar region. At 40 DAA, the beginning of aculei development (when it was not yet



lignified) was evident in the endocarp, and the embryo occupied approximately half of the seminal cavity (Fig. 1d).

The endocarp maturation phase occurred between 41 and 60 DAA, and was characterized by maximum dry mass values of that structure, associated with significant reductions in water content (Fig. 2a–d). By the end of that period, the dimensions of the fruit and the seed had reached values close to maximum, the endocarp was woody and rigid (with numerous lignified aculei), and the embryo occupied the entire seminal cavity (Figs. 1e, 2a, b).

Seed and mesocarp maturity (maximum dry mass) were only reached at about 90 DAA, with fruit abscission (Fig. 1g,

◂**Fig. 3** Morphology of the plumule and the anatomy of *C. brasiliense* embryos at different developmental stages. **b–d**, **f**, **h**, **j**, **l**, **n**, **p**, **r**, **t**, **v**, **x** Longitudinal sections. **g**, **k**, **o**, **s**, **w** Cross-sections. **a** Plumule. **b** Epicotyl, highlighting the cotyledons and apical meristem. **c** Hypocotyl. **d** Radicular pole, showing the meristematic zone. **e**, **f** Plumule, highlighting cotyledons and leaf primordia. **g** Hypocotyl, showing differentiated tracheary elements. **h** Radicular pole, highlighting the root promeristem (arrowhead). **i** Plumule, highlighting increases in the numbers of leaf primordia. **j** Plumule, evidencing differentiated vascular bundles (arrowhead). **k** Hypocotyl, showing voluminous ground meristem cells with accumulated reserves. **l** Radicular pole, highlighting the differentiated vascular bundles (arrowhead). **m** Plumule with epicotyl, cotyledons, and elongated foliar primordia. **n**, **o** Plumule and hypocotyl, showing differentiated tracheary elements (arrowhead). **p** Radicular pole, demonstrating the inner seed coat adhered to the embryo (arrowhead) and meristematic zone. **q** Plumule with epicotyl, cotyledons, and elongated leaf primordia. **r** Plumule, evidencing differentiated vascular bundles in the leaf primordia (arrowhead). **s** Hypocotyl, highlighting the inner seed coat adhered to the embryo (arrowhead). **t** Radicular pole, showing the promeristem (arrowhead). **u** Plumule, showing epicotyl thickening. **v** Plumule, evidencing differentiated vascular bundles (arrowhead). **w** Hypocotyl, showing voluminous ground meristem cells. **x** Radicular pole, highlighting the inner seed coat adhered to the embryo (black arrowhead) and the promeristem (white arrowhead). *co* cotyledon, *ec* epicotyl, *gm* ground meristem, *hy* hypocotyl, *lp* leaf primordia, *mz* peripheral meristematic zone, *pc* procambium, *pd* protoderm, *sc* seed coat, *se* sieve elements, *sm* shoot meristem, *te* tracheary elements, *vb* vascular bundle, *DAA* days after anthesis

h, Fig. 2c, d). At the end of that phase, the internal mesocarp (soft and aromatic, with a yellowish coloration) adhered to the endocarp, covering the pyrene (Fig. 1f, h). The seed did not show a typical desiccation phase (it's water content being close to 40% at the time of fruit abscission) and was reniform, with a brownish tegument, an inconspicuous radicle, and an evident plumule (Figs. 1i, 2d).

Embryo Anatomy

At 35 DAA, the embryos had plumules with a meristematic dome, and cotyledon primordia (Fig. 3a, b). The embryonic tissues could be distinguishable based on the arrangements and shapes of their cells: protoderm, with a peripheral arrangement; procambium, organized in concentric strands; ground meristem, predominant and with larger cells, and an apical meristem with small, juxtaposed cells (Fig. 3b). The cells of the ground meristem in the median region of the hypocotyl were large and highly vacuolated (Fig. 3c), and the procambial strands formed a cone that tapered towards the radicular pole, where a meristematic zone was present adjacent to the protoderm (Fig. 3d). At 40 DAA, the plumule showed elongated cotyledons and numerous leaf primordia (Fig. 3e, f).

Tracheary and sieve elements in the early phase of differentiation were identified between the procambial strands in the median region of the hypocotyl (Fig. 3g). The promeristem at the radicular pole had differentiated, and there was an active and expanded peripheral meristematic zone (Fig. 3h). At 50 DAA, the cotyledons were more elongated, and there were new leaf primordia overlying the apical meristem (Fig. 3i). The differentiation of vascular bundles and the accumulation of reserve compounds in the cells of the ground meristem were observed throughout the plumule (Fig. 3j). The median region of the hypocotyl had greatly expanded, mainly by the proliferation and expansion of the cells of the ground meristem, which showed accumulations of reserve compounds (Fig. 3k); vascular bundle differentiation was also evident in the radicular pole (Fig. 3l). At 60 DAA, the cotyledons had reduced their development; there were no indications of the emission of new leaf primordia, but those that had previously been emitted showed significant elongation (Fig. 3m, n). Differentiated vascular bundles were seen throughout the hypocotyl (Fig. 3o). The cells produced by the peripheral meristematic zone in the radicular pole showed accumulations of phenolic compounds and were juxtaposed to the seed coat, which also showed phenolic accumulations (Fig. 3p). At 80 DAA, the continued leaf primordia growth was evident, associated with the deposition of reserves in the ground meristem cells (Fig. 3q, r). The entire hypocotyl could be seen adhering to the seed coat, which had layers of small, juxtaposed cells as well as layers of peripherally distributed expanded cells; all of the cells of the seed coat showed significant accumulations of phenolic compounds (Fig. 3s). In the radicular pole, both the apical meristem and the peripheral meristematic zone showed high cell division activity (Fig. 3t). At the time of fruit abscission, cellular expansion in the plumule had resulted in increases in the diameter and the cylindrical shape of that structure (Fig. 3u, v). The hypocotyl cells likewise showed significant expansion (compare Fig. 3k, w). The cells produced by apical meristem activity at the radicular pole had differentiated into ground meristem cells with accumulated reserves, and procambial cells organized into conical strands (Fig. 3x). The peripheral cell layers of the radicular pole were juxtaposed to the seed coat, and both showed significant accumulations of phenolic compounds.

Deposition of Reserves

There was no deposition of protein and starch reserves at 40 DAA (Supplementary Material II a–c), and only discrete accumulations of cell wall polysaccharides and lipids (Supplementary Material II b, d). The beginnings of protein and starch accumulation and increases in lipid reserves were seen that 50 DAA (Supplementary Material II e, h). Between 60 and 90 DAA, there was a gradual increase in the numbers and volumes of protein and lipid bodies as well as starch grains (Supplementary Material II i–t).

The cells of the cotyledonary node region at 40 DAA had thin cell walls, voluminous nuclei, and dense

cytoplasm rich in organelles (especially mitochondria and endoplasmic reticles); small amounts of lipid bodies and rare plastids with starch accumulation were observed (Fig. 4a, b). At 50 DAA, the cells showed significant expansion, cell wall thickening, and lipid body proliferation in the cytoplasm; the formation of plastids with starch deposits became amplified, and the proliferation of small protein bodies surrounded by membranes was observed (Fig. 4c, d). At the end of the development, at 90 DAA, the cells were packed with lipid bodies, protein bodies of varying dimensions with inclusions, and starch-containing plastids (Fig. 4e, f).

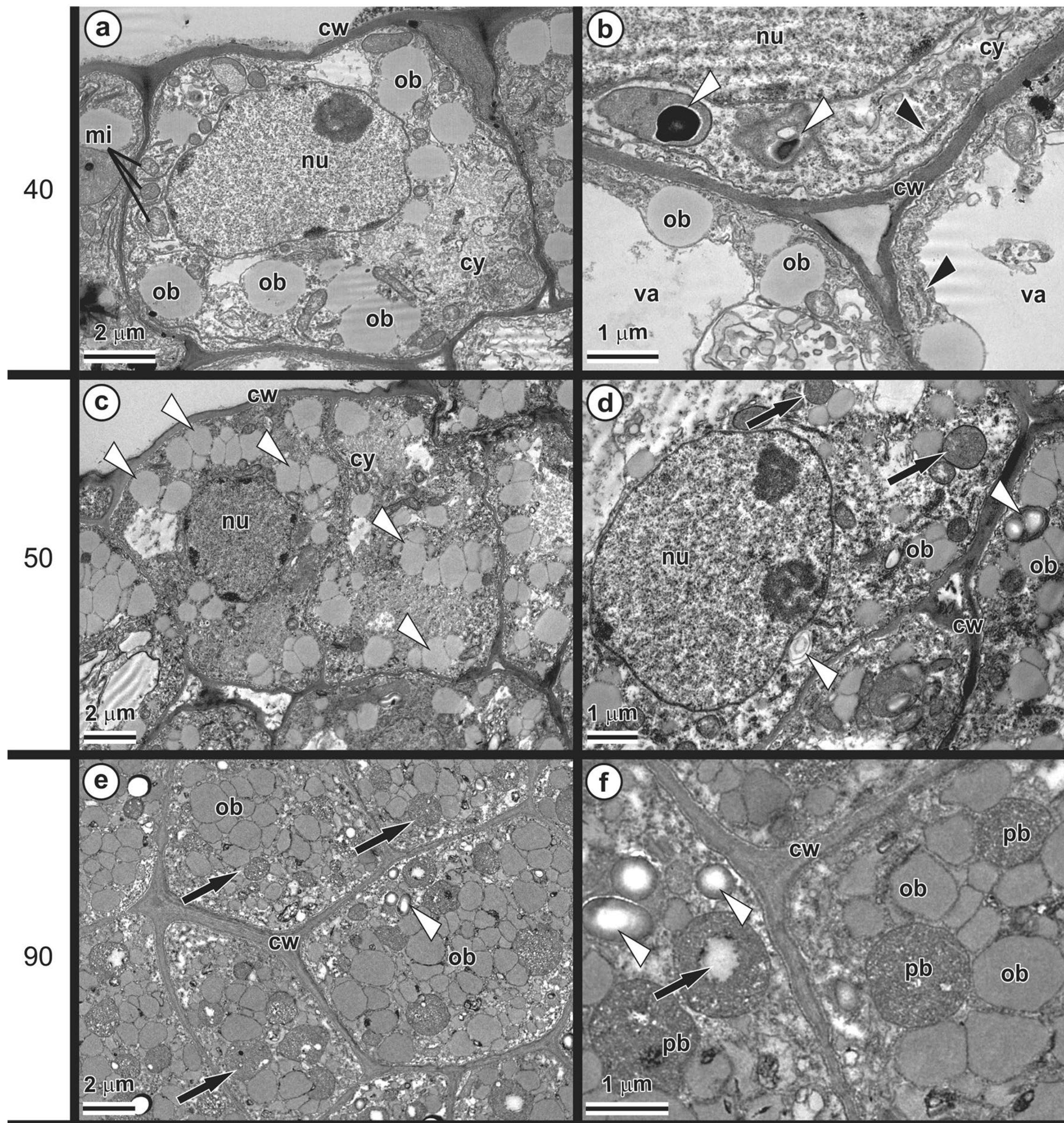
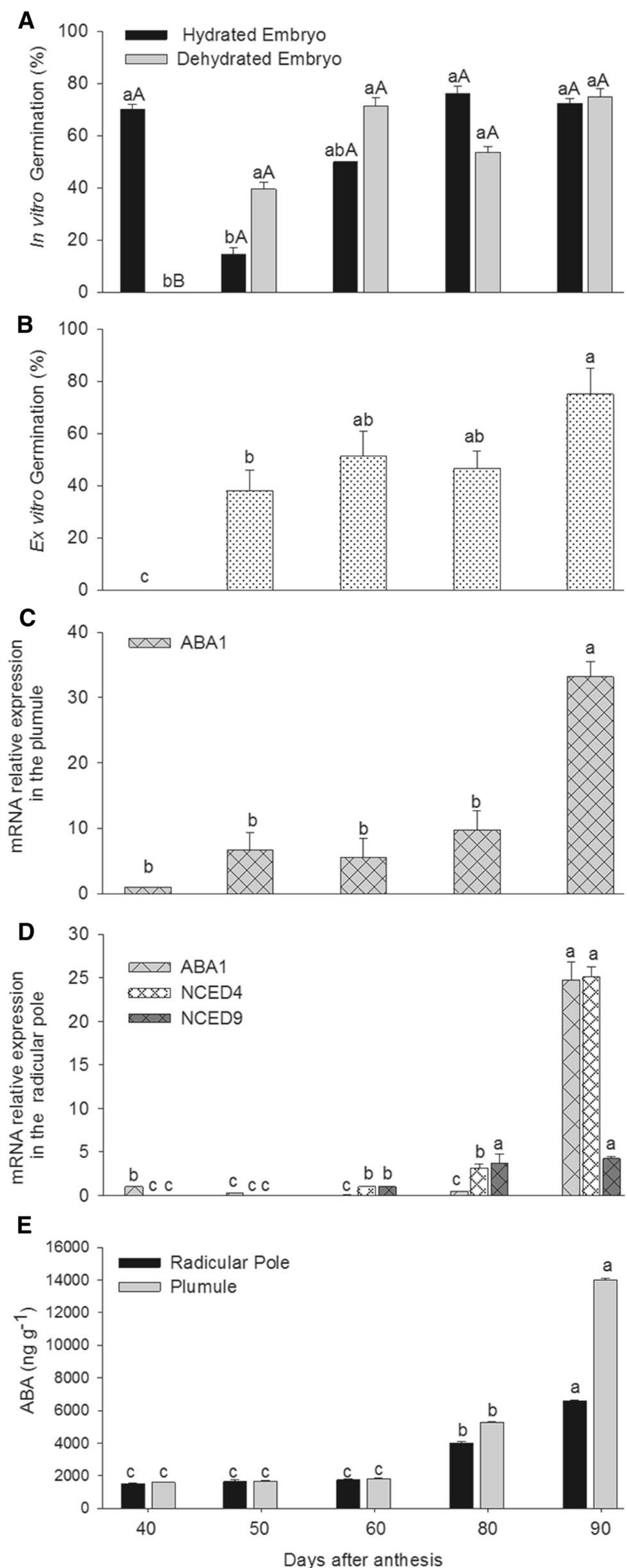


Fig. 4 Images obtained by transmission electron microscopy of the ground meristem cells of the cotyledonary node region of *C. brasiliense* embryos in different developmental stages. The numbers shown on the left indicate days after anthesis. **a** Cell in the initial phase of lipid reserve deposition showing lipid bodies. **b** Interface region between adjacent cells, showing plastids with starch grains (white arrowhead) and endoplasmic reticulum (black arrowhead). **c** Cell with a proliferation of lipid bodies (arrowheads). **d** Cells with protein bodies (black arrow) and plastids containing starch grains (white arrowhead). **e** Cell containing numerous lipid bodies, protein bodies of various sizes (black arrow), and plastids containing starch grains (white arrowhead). **f** Detail of the periphery of adjacent cells, including protein bodies with inclusions (black arrow), and plastids containing starch grains (white arrowheads). *cy* cytoplasm, *cw* cell wall, *ob* lipid bodies, *pb* protein bodies, *mi* mitochondria; nucleus: *va* vacuole

Fig. 5 Germinability of isolated embryos, expression of ABA biosynthesis genes, and the quantification of that hormone throughout the maturation period of the *C. brasiliense* seeds. **a** Germinability of cultured embryos in vitro, hydrated or after dehydration. **b** Germinability of ex vitro cultured embryos. **c**, **d** Gene expression analysis in different regions of the embryo (plumule and radicular pole), as measured by qPCR. **c** ABA1 expression pattern in the plumule (NCED4 and NCED9 were not expressed). **d** ABA1, NCED4, and NCED9 expression pattern in the radicular pole. GAPC2 and ACT2 were used as endogenous controls. **e** ABA content in the plumule and radicular pole. Equal letters indicate the absence of differences between means by the Tukey test, at a 5% level of probability. In (**a**), lowercase letters compare the means of embryo development times; upper case letters compare the means of hydrated and dehydrated conditions. Vertical bars indicate the standard errors of the means



Embryo Germination Capacity and Desiccation Tolerance

The embryos that were cultured in vitro showed an early germinative capacity at 40 DAA, at the end of the histodifferentiation phase (Fig. 5a). At 50 DAA, embryo germinability decreased, returning to values above 70% from 80 DAA onward. Embryos submitted to desiccation at 40 DAA did not show any in vitro germinative capacity, with desiccation tolerance only starting to be acquired from 50 DAA, but present in most embryos from 60 DAA onward.

Embryos cultured under ex vitro conditions showed no significant differences in their germinative capacities as a function of their hydration conditions (Fig. 5b). They did not show any germination capacity at 40 DAA, and were not able to withstand desiccation (as evidenced by the in vitro culture) and did not have enough reserves to germinate. At later times, some of the embryos germinated, with the highest percentages being reached at the end of development.

Gene Expression Related to ABA Biosynthesis and ABA Quantification in the Embryo

ABA1, NCED4, and NCED9 genes were not amplified in the hypocotyl region. ABA1 was expressed in the plumule and in the radicular pole from 40 DAA, with a significant increase in its induction at the end of the seed maturation phase (Fig. 5c, d). NCED4 and NCED9 were not expressed in the plumule. The expression of those genes could be detected from 60 DAA in the radicular pole region, and increasing during the final evaluation periods (Fig. 5d).

The ABA was not detected in the hypocotyl, but was detected in both the plumule and radicular pole at concentrations of 1700 to 2000 ng g^{-1} between 40 and 60 DAA (Fig. 5e). Significant increases in hormone contents were observed after 80 DAA, being higher in the plumule at 90 DAA.

Discussion

We used an integrated approach in the present study, involving evaluations of the structural aspects of fruit development

and the physiological aspects of seed maturation in *C. brasiliense*, a neotropical fruit species, identifying peculiarities in its pattern of fruit development and in the structure and physiology of the seeds as adaptations to the Cerrado environment.

Fruit Development

Fruit development in *C. brasiliense* required approximately 90 days, and could be divided into three phases: (i) histodifferentiation; (ii) completion of endocarp maturation; and (iii) completion of mesocarp and seed maturation (Fig. 2). The first, histodifferentiation, phase is the stage of fruit development and is characterized by a high water content, and intense cell division activity and differentiation resulting in tissue formation (Bewley et al. 2013; Dante et al. 2014). Histodifferentiation in *C. brasiliense* required almost half the total development time of the fruit, which is not a common pattern (Gillaspy et al. 1993) and may be related to the diversity of tissues produced. Endocarp maturation (with the formation of numerous aculei) was precocious and occurred prior to the phase of intense deposition of embryonic reserves. Early differentiation of the endocarp was likewise observed in *Acrocomia aculeata* (Arecaceae), a typical species of the Cerrado biome, apparently to protect the embryo against predation (Mazzottini-dos-Santos et al. 2015). The mesocarp of *C. brasiliense* showed a progressive increase in dry mass up until the time of dispersal, without any marked reduction in its water content—which is in agreement with the proposal that its fruits are climacteric and have a ripening phase after dispersal (Oliveira et al. 2006, 2017).

The seed of *C. brasiliense* demonstrated several peculiarities in their development. The embryo occupied almost the entire volume of the seed, which is considered a derived characteristic associated with the absorption of the endospermic reserves by the embryonic tissues (Forbis et al. 2002; Linkies et al. 2010). The hypocotyl was the most developed region of the embryo, and most of the reserves were deposited in that structure. The cotyledons were rudimentary and the radicle undifferentiated, as reported by Barradas (1973) and Sousa et al. (2017b). The embryo developed numerous leaf primordia, indicating a high level of differentiation. Vascularization occurred early, which was peculiar, as differentiated vascular systems are rarely seen in embryos (Werker 1997). The establishment of vascularization before embryo maturation may facilitate reserve translocation and deposition, which will be investigated in future studies. The seed coat accumulated large amounts of phenolic compounds and is known to have roles in both the protection of the embryo and germination restriction (Bewley et al. 2013)—thus contributing to the interactions of the seeds with their environment.

Relationships Between ABA and Physiological Aspects of Seed Maturation

The integrated analysis of ABA1, NCED4, and NCED9 gene expression, ABA quantification, reserve deposition, desiccation tolerance, and the germinative capacity of the embryos allowed the identification of four phases of *C. brasiliense* seed maturation, which are summarized in Fig. 6.

In the first phase of seed maturation (40–60 DAA), the isolated embryos demonstrate a germinative capacity, although embryo germination at 40 DAA was only possible in nutrient medium under in vitro conditions, as reserve deposition is only in its initial phase at that time (with only lipid deposition), and the embryos show no desiccation tolerance. Normally, embryos have the ability to germinate after the histodifferentiation phase (Ren and Bewley 1999; Taylor et al. 2005; Kermode 1990; Bewley et al. 2013). The seed environment, however, including the presence of ABA, maintains the embryo in a development mode, and prevents germination (Kermode 1990; Kermode and Bewley 1988; Kermode 2005). Embryo isolation and its exposure to the atmosphere, on the other hand, commonly promote ABA degradation (or reduces tissue sensitivity to the hormone) in processes induced by reactive oxygen species (ROS) (Bailly 2004; Finch-Savage and Leubner-Metzger 2006; Nambara et al. 2010). We quantified ABA in the embryos of *C. brasiliense* in the early stage of seed maturation in both the plumule and the radicular pole—but ABA1 expression was identified only in the plumule. ABA1 encodes zeaxanthin epoxidase (the first enzyme in ABA biosynthesis) and also regulates ABA accumulation during seed development in several species (Seo and Koshiba 2002; Chao et al. 2014). No NCED4 and NCED9 expressions were identified in the embryos of *C. brasiliense* at this stage. These genes control the final phase of the ABA synthesis pathway, and the absence of their expression indicates that the hormone present had extra-embryonic origin. ABA synthesized in the

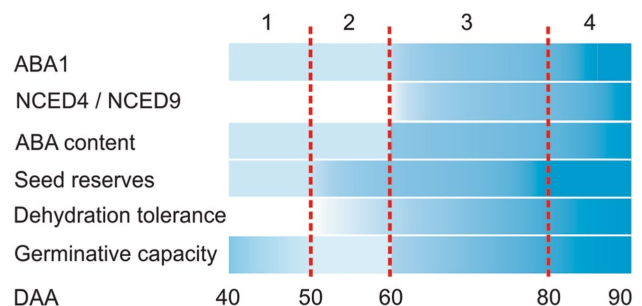


Fig. 6 Scheme illustrating the association between ABA quantification in the embryo, gene expression related to hormone biosynthesis, and physiological aspects of *C. brasiliense* seed maturation. Darker tones indicate greater intensity. Dashed lines indicate the delimitations of the stages of seed maturation. *DAA* days after anthesis

embryo normally controls germination, although the tissues surrounding the seeds and/or maternal tissue can play key roles in supplying that hormone and or controlling its biosynthesis (Frey et al. 2004; Kermode 2005; Bewley et al. 2013).

In the second phase of seed maturation (50–60 DAA), there were increases in reserve deposition and the acquisition of desiccation tolerance in approximately 40% of the embryos, with germination under ex vitro conditions. The acquisition of desiccation tolerance is a process mediated by ABA in most seeds, and is related to the synthesis of LEA proteins in the final phase of embryogenesis (Bewley et al. 2013). In the case of *C. brasiliense,* it is peculiar that this occurs during the initial phase of seed maturation, when reserve deposition is still incipient. At 50 DAA, there was a decrease in the germinability of the hydrated and in vitro cultured embryos, although without any reduction in ABA content, possibly due to variations in the sensitivities of the embryonic tissue—important modulators of hormonal action, even in seeds with high ABA contents (Hilhorst 1995; Weyers and Paterson 2001).

In the third stage of seed maturation (60–80 DAA), ABA1, NCED4, and NCED9 were expressed in the radicular pole, associated with an increase in the ABA content in that region and in the plumule, with increasing seed reserve deposition. The genes NCED4 and NCED9 encode for the synthesis of enzymes responsible for the final and most critical steps of ABA synthesis (especially 9-cis-epoxycarotenoid dioxygenase), and its expression in embryos has been associated with seed maturation and dormancy induction (Lefebvre et al. 2006; Frey et al. 2004). The joint expression of those three genes at the radicular poles indicated them as the main sites of the hormone synthesis in *C. brasiliense* seeds. That increase in ABA content was related to increases in protein and carbohydrate deposition and the increasing dry matter contents of the seeds.

In the fourth phase of seed maturation (80–90 DAA) there was an increase in ABA1 and NCED4 expression at the radicular pole, and of ABA1 in the plumule, all related to high ABA contents in both structures and the deposition of abundant of protein, starch, and lipid reserves. At this stage, more than 70% of the isolated embryos germinated when dehydrated or in ex vitro cultures. Those results illustrate interesting peculiarities in relation to the physiology of *C. brasiliense* seeds. The seeds do not undergo a severe dehydration phase at the end of maturation and are dispersed with water contents above 40%—even though they are highly dehydration tolerant, as already described by Mendes (2015) and Sousa et al. (2017a). In most seeds, desiccation tolerance is acquired (during controlled desiccation) at the end of the maturation phase, associated with the synthesis of proteins and carbohydrates that act to protect cell membranes, in processes mediated by ABA (Kermode and Bewley 1988; Bewley et al. 2013; Mcatee et al. 2016). It is possible to suggest that the high hormone contents in *C. brasiliense* seeds make it possible to establish protection systems even under hydration conditions and/or during the post-dispersion dehydration process, which should be elucidated in future studies. The pyrenes of *C. brasiliense* show pronounced dormancy (Sousa et al. 2017a; b). It is interesting that the germinative capacity of isolated embryos is high in spite of their high ABA contents. That situation indicates that dormancy in the species depends on the interactions of ABA and pericarp tissues that tend to limit the oxygen supply and exert restrictive mechanical effects on embryo growth, as shown by Sousa et al. (2017b).

The protection of the rigid and aculeus endocarp and the desiccation tolerance and dormancy of the seeds all favor the survival of *C. brasiliense* embryos in seed banks. The accumulation of abundant reserves in the hypocotyl and the development of vascularization and leaf primordia in the plumule, on the other hand, contribute to rapid post-germinative development once dormancy is overcome. Together, those characteristics are important factors in the adaptation of the species to the environmental conditions of the Cerrado biome, with its seasonal climate and irregular precipitation (Rajjou et al. 2012; Ribeiro et al. 2012).

This is the first work involving physiological aspects of fruit development in the Caryocaraceae family. Future studies involving other species of the family, besides the investigation of the role of hormones (such as auxins and gibberellins) and genes (such as NCED3, NCED6, ABA2, or DOG1), not studied in the present work, may contribute to our knowledge about the reproduction of this important family of tropical plants.

Conclusions

The development of *C. brasiliense* fruits can be divided into three phases: histodifferentiation (0–40 days DAA); completion of endocarp maturation (40–60 DAA); and the conclusion of mesocarp and seed maturation (60–90 DAA). Early maturation of the aculeus endocarp contributes to the protection of the developing embryo. The mesocarp and the seed show increasing dry matter contents throughout fruit development. The embryos demonstrate early degrees of differentiation: hypocotyl expansion, leaf primordia development, and vascularization. The seeds do not have a desiccation phase, and are dispersed with water contents > 40%. Seed maturation occurs in four phases (from 40 DAA) influenced by ABA dynamics. The beginning of reserve deposition and the acquisition of dehydration tolerance are related to extra-embryonic ABA. ABA biosynthesis at the radicular pole is associated with increased hormone contents, the deposition of abundant reserves, and dehydration tolerance, which are all maximal at the time of abscission. The germinative

capacity of the isolated embryos is not affected by a high-ABA content during the final stages of maturation. The maturation of *Caryocar brasiliense* seeds is influenced by embryonic and extra-embryonic ABA levels, and the dormancy of its pyrenes results from interactions between that hormone and pericarp tissues.

Acknowledgements The authors thank the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the masters scholarship awarded to IVR; and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the postdoctoral fellowship to CPSM and for the research productivity Grants awarded to FOS, LMR, MOMS and PSNL.

Author Contributions PSNL and LMR conceived and designed the research; IVR, CPSM, FRV collected and prepared the plant material and conducted the experiments; IVR prepared the histological slides; LMR and MOMS analyzed the anatomical and histochemical data; MOMS analyzed the ultrastructural data; FOS and APC performed ABA quantification and data interpretation; CPSM and DS performed gene expression analysis; LMR and PSNL analyzed the physiological and biometric data; IVR wrote the initial text; and PSNL and LMR prepared the final text. All authors read and approved the manuscript.

Compliance with Ethical Standards

Conflict of interest The authors have no conflicts of interest to declare.

References

Amaral LFB, Moriel P, Foglio MA, Mazzola PG (2014) *Caryocar brasiliense* supercritical CO_2 extract possesses antimicrobial and antioxidant properties useful for personal care products. BMC Complement Altern Med 14:73

Araújo FD (1995) A review of *Caryocar brasiliense* (Caryocaraceae): an economically valuable of central Brazilian Cerrados. Econ Bot 49:40–48

Ascari J, Takahashi JA, Boaventura MAD (2013) The phytochemistry and biological aspects of Caryocaraceae family. Rev Bras Pl Med 15:293–308

Bailly C (2004) Active oxygen species and antioxidants in seed biology. Seed Sci Res 14:93–107

Barradas MM (1973) Morfologia do fruto e da semente de *Caryocar brasiliense* (pequi) em várias fases de desenvolvimento. Rev Biol 9:69–84

Bentsink L, Jowett J, Hanhart CJ, Koornneef M (2006) Cloning of *DOG1*, a quantitative trait locus controlling seed dormancy in *Arabidopsis*. Proc Natl Acad Sci 103:17042–17047

Bewley JD, Bradford KJ, Hilhorst HWM, Nonogaki H (2013) Seeds: physiology of development, germination and dormancy. Springer, New York

Brasil (2009) Regras para análise de sementes. Ministério da Agricultura, Pecuária e Abastecimento. Secretaria de Defesa Agropecuária. DF: Mapa/ACS, Brasília

Chao WS, Doğramaci M, Anderson JV, Foley ME, Horvath DP (2014) The resemblance and disparity of gene expression in dormant and non-dormant seeds and crown buds of leafy spurge (Euphorbia esula). BMC Plant Biol 14:216

Dante RA, Larkins BA, Sabelli PA (2014) Cell cycle control and seed development. Front Plant Sci 5:493

Feder N, O'Brien TP (1968) Plant microtechnique: some principles and new methods. Am J Bot 5:123–142

Finch-Savage WE, Leubner-Metzger G (2006) Seed dormancy and the control of germination. New Phytol 171:501–523

Finkelstein R, Reeves W, Ariizumi T, Steber C (2008) Molecular aspects of seed dormancy. Annu Rev Plant Biol 59(1):387–415

Flora do Brasil. Lista de espécies da Flora do Brasil. https://floradobrasil.jbrj.gov.br/reflora/listaBrasil/ConsultaPublicaUC/. Accessed 15 June 2016

Forbis TA, Floyd SK, Queiroz A (2002) The evolution of embryo size in angiosperms and other seed plants: implications for the evolution of seed dormancy. Evolution 56:2112–2125

Frey A, Godin B, Bonnet M, Sotta B, Marion-Poll A (2004) Maternal synthesis of abscisic acid controls seed development and yield in Nicotiana plumbaginifolia. Planta 218:958–964

Gao Y, Zhang Y, Zhang D, Dai X, Estelle M, Zhao Y (2015) Auxin binding protein 1 (ABP1) is not required for either auxin signaling or *Arabidopsis* development. Proc Natl Acad Sci 112:2275–2280

Gillaspy G, Ben-Divid H, Gruissem W (1993) Fruits: a developmental perspective. Plant Cell 5:1439–1451

Giroldo AB, Scariot A (2015) Land use and management affects the demography and conservation of an intensively harvested Cerrado fruit tree species. Biol Conserv 191:150–158

Hilhorst HWM (1995) A critical update on seed dormancy. 1 Primary dormancy. Seed Sci Res 5:61–73

Jensen W (1962) A botanical histochemistry: principles and practice. W. H. Freeman & Company, San Francisco

Karnovsky MJ (1965) A formaldehyde—glutaraldehyde fixative of high osmolality for use in electron microscopy. J Cell Biol 27:137–138

Kermode AR (1990) Regulatory mechanisms involved in the transition from seed development to germination. Crit Rev Plant Sci 9:155–195

Kermode AR (2005) Role of abscisic acid in seed dormancy. J Plant Growth Regul 24:319–344

Kermode A, Bewley JD (1988) The role of maturation drying in the transition from seed development to germination. J Exp Bot 39:487–497

Lefebvre V, North H, Frey A, Sotta B, Seo M, Okamoto M, Nambara E, Marion-Poll A (2006) Functional analysis of Arabidopsis NCED6 and NCED9 genes indicates that ABA synthesized in the endosperm is involved in the induction of seed dormancy. Plant J 45:309–319

Linkies A, Graeber K, Knight C, Leubner-Metzger G (2010) The evolution of seeds. New Phytol 186:817–831

Liu Y, Koornneef M, Soppe WJJ (2007) The absence of histone H2B monoubiquitination in the *Arabidopsis hub1* (*rdo4*) mutant reveals a role for chromatin remodeling in seed dormancy. Plant Cell 19:433–444

Livak KJ, Schmittgen TD (2001) Analysis de relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Method 25:402–408

Machado MTC, Mello BCBS, Hubinger MD (2013) Study of alcoholic and aqueous extraction of pequi (*Caryocar brasiliense* Camb.) natural antioxidants and extracts concentration by nanofiltration. J Food Eng 117:450–457

Mazzottini-dos-Santos HC, Ribeiro LM, Mercadante-Simões MO, Sant'anna-Santos BF (2015) Ontogenesis of the pseudomonomerous fruits of Acrocomia aculeata (Arecaceae): a new approach to the development of pyrenarium fruits. Trees 29:199–214

Mcatee P, Karim S, Schaffer R, David K (2016) A dynamic interplay between phytohormones is required for fruit development, maturation, and ripening. Front Plant Sci 4:79

Mendes DS (2015) Dormência, germinação e longevidade em sementes de pequizeiro (*Caryocar brasiliense Camb.*) 2015, p 50. Dissertation, Universidade Federal de Minas Gerais

Müller ML, Munné-Bosch S (2011) Rapid and sensitive hormonal profiling of complex plant samples by liquid chromatography coupled to electrospray ionization tandem mass spectrometry. Plant Methods 7:37

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

Nambara E, Okamoto M, Tatematsu K, Yano R, Seo M, Kamiya Y (2010) Abscisic acid and the control of seed dormancy and germination. Seed Sci Res 20:55–67

O'Brien TP, Feder N, Mccully ME (1964) Polychromatic staining of plant cell walls by toluidine blue O. Protoplasma 59:368–373

Oliveira MNS, Mercadante-Simões MO, Gusmão E, Lopes PSN, Ribeiro LM, Dias BAS (2006) Estádio de Maturação dos frutos e fatores relacionados aos aspectos nutritivos da polpa do pequi (*Caryocar brasiliense* Camb.). Rev Bras Frutic 28:380–386

Oliveira MNS, Lopes PS, Mercadante-Simões MO, Pereira EG, Ribeiro LM (2017) Post-harvest quality of pequi (Caryocar brasiliense CAMB.) collected from the plant or after naturally falling off and subjected to slow and quick freezing. Rev Bras Frutic 39:768

Paiva EAS, Pinho SZ, Oliveira DMT (2011) Large plant samples: how-to process for GMA embedding. In: Chiarini-Garcia H, Melo RCN (eds) Light microscopy: methods and protocols. Humana Press, Totowa, pp 37–49

Pearse AGE (1980) Histochemistry theoretical and applied: preparative and optical technology. Churchill Livingston, Edinburgh

Rajjou L, Duval M, Gallardo K, Catusse J, Bally J, Job C, Job D (2012) Seed germination and vigor. Annu Rev Plant Biol 63:507–533

Ren C, Bewley JD (1999) Developmental and germinative events can occur concurrently in precociously germinating Chinese cabbage (*Brassica rapa* ssp. Pekinensis) seeds. J Exp Bot 50:1751–1761

Ribeiro LM, Neves SC, Silva PO, Andrade IG (2011) Germinação de embriões zigóticos e desenvolvimento in vitro de coquinho-azedo. Ceres 58:133–139

Ribeiro LM, Oliveira TGS, Carvalho VS, Silva PO, Neves SC, Garcia QS (2012) The behaviour of macaw palm (*Acrocomia aculeata*) seeds during storage. Seed Sci Technol 40:345–353

Robards AW (1978) An introduction to techniques for scanning electron microscopy of plant cells. In: Hall JL (ed) Electron microscopy and cytochemistry of plant cells. Elsevier, New York, pp 343–403

Roesler R, Catharino RR, Malta LG, Eberlin MN, Pastore G (2008) Antioxidant activity of *Caryocar brasiliense* (pequi) and characterization of components by electrospray ionization mass spectrometry. Food Chem 110:711–717

Roland AM (1978) General preparations and staining of thin sections. In: Hall JL (ed) Electron microscopy and cytochemistry of plant cells. Elsevier, New York, pp 1–62

Seo M, Koshiba T (2002) Complex regulation of ABA biosynthesis in plants. Trends Plant Sci 7:41–48

Sousa AMS, Lopes PSN, Ribeiro LM, Santiago TA, Lacerda VR, Martins CPS (2017a) Germination and storage of *Caryocar brasiliense* seeds. Seed Sci Technol 45:557–569

Sousa AMS, Lopes PSN, Ribeiro LM, Andrade MS, Mercadante-Simões MO (2017b) Structural aspects of germination control in pyrenes of *Caryocar brasiliense* (Caryocaraceae). Trees 31:1–16

Taylor IB, Sonneveld K, Bugg TDH, Thompson AJ (2005) Regulation and manipulation of the biosynthesis of abscisic acid, including the supply of xanthophyll precursors. J Plant Growth Regul 24:253–273

Vidal BC (1970) Dichroism in collagen bundles stained with Xylidine-Ponceau 2R. Ann Histochim 15:289–296

Wang Z, Cao H, Sun Y, Li X, Chen F, Carles A, Li Y, Ding M, Zhang C, Deng X, Soppe WJ, Liu YX (2013) *Arabidopsis* paired amphipathic helix proteins SNL1 and SNL2 redundantly regulate primary seed dormancy via abscisic acid-ethylene antagonism mediated by histone deacetylation. Plant Cell 25:149–166

Werker E (1997) Seed anatomy. Gebrüder Borntraeger, Berlin

Weyers JDB, Paterson NW (2001) Plant hormones and the control of physiological processes. New Phytol 152:375–407

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.