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ORIGINAL ARTICLE

Structural aspects of germination control in pyrenes of *Caryocar brasiliense* (Caryocaraceae)

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Abstract

Key message **Germination in oleaginous tree*Caryocar brasiliense*is controlled by complex interactions between pyrene components, contributing to the reproductive success of the species in the seasonal environment of the Cerrado biome.**

Abstract *Caryocar brasiliense* is an arboreal species widely distributed in the highly seasonal Cerrado (neotropical savanna) biome. The oleaginous fruits of this species are important to the local fauna and as a source of income for traditional human populations. As the diaspores have peculiar structures and pronounced dormancy, we sought to examine the interactions between the morphoanatomy of the pyrene (the seed enveloped by the endocarp) and physiological aspects of germination. We describe here the morphology of the pyrene, the anatomy, histochemistry, and ultrastructure of the seed during the germination process, and evaluate the effect of gibberellic acid (GA_3) on the germination of isolated seeds or those still contained within the endocarp. The hard and aculeate endocarp protects the embryo and retards water absorption—thereby controlling germination under natural conditions. The seed coat is thin, unlignified, and rich in phenolic compounds. The embryo is hypocotyledonary, has differentiated vascular bundles, and abundant proteic and lipidc reserves. The physiological dormancy of this species is related to the low growth potential of its embryo and mechanical restraints imposed by the endocarp. Germination is associated with hypocotyl growth through cell expansion (stimulated by GA_3)—which promotes the cracking of the endocarp, and thus allows radicle and plumule protrusion. The anatomical characteristics and physiological dormancy of *C. brasiliense* pyrenes restrict germination to the most propitious occasions and favor the establishment of seedlings in Cerrado environments.

Keywords Dormancy · Embryo · Endocarp · Oilseeds · Reserve mobilization

Introduction

The Caryocaraceae family is originally from South America, with *Caryocar brasiliense* Camb. (“pequizeiro”) being an arboreal species endemic to, but widely distributed in, the Cerrado (neotropical savanna) biome (Fig. 1a) (Ascari et al. 2013). The species produces numerous oleaginous fruits (Fig. 1b) that are important to the regional fauna and also serve as a source of income for traditional human populations as a raw material for food, medicinal, and cosmetic industries (Araújo 1995; Giroldo and Scariot 2015). The species is tolerant of water deficits and has potential for commercial agricultural use in areas with dry tropical climates. *C. brasiliense* is threatened, however, by deforestation and the intensive harvesting of its fruits (Giroldo and Scariot 2015). The seeds demonstrate dormancy, which results in erratic and low germination levels that limit seedling production for both the conservation of natural populations and commercial cultivation (Carvalho et al. 1994; Dombroski et al. 2010).

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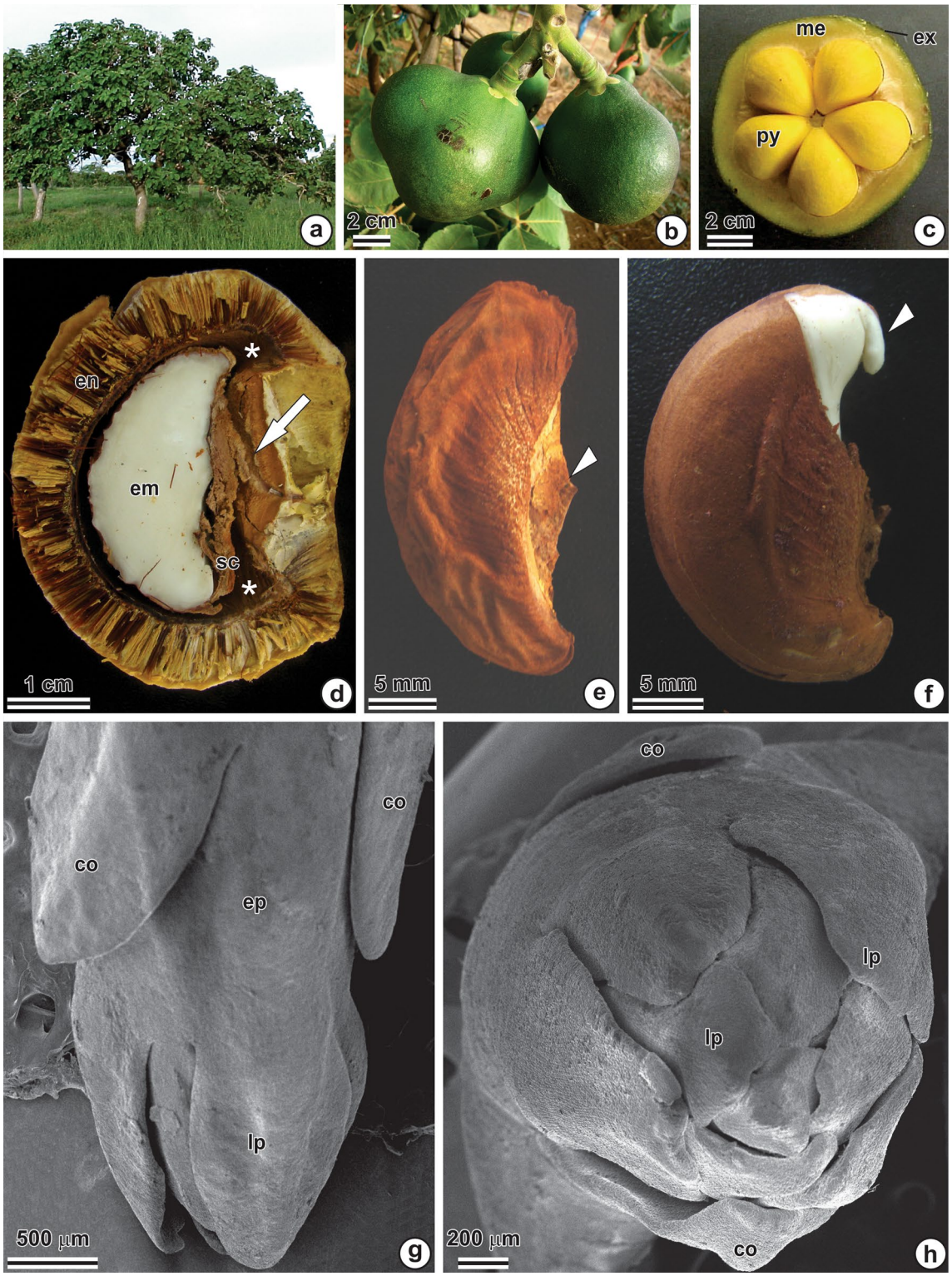


Fig. 1 Morphology of the plant and fruit of *Caryocar brasiliense*. **a***C. brasiliense* in its natural habitat. **b** Fruits. **c** Fruit cross-section. **d** Pyrene, showing cortical tissue near the hilar region (*arrow*) and empty spaces between the seed and the endocarp (*asterisks*). **e** Dry seed, indicating the hilum (*arrowhead*). **f** Imbibed seed, indicating the plumule (*arrowhead*). **g** Detail of the plumule by SEM imagery. **h** Detail of the leaf primordia. *co* cotyledon, *em* embryo, *en* endocarp, *ep* epicotyl, *ex* exocarp, *lp* leaf primordia, *me* mesocarp, *py* pyrene (inner mesocarp, endocarp, and seed), *sc* seed coat

Dormancy is a blockage of germination, even under favorable conditions, by intrinsic factors (Baskin and Baskin 2014) and is associated with diaspore (dispersal unit) structure in several Cerrado species (Da Silva et al. 2007; Neves et al. 2013; Carvalho et al. 2015). The fruits of *C. brasiliense* are characterized by their specialized pyrenes, with the seeds being enclosed by a hard and aculeate endocarp (Fig. 1b–d). The embryo also has unique characteristics, such as a very well-developed hypocotyl, vestigial cotyledons, and an undifferentiated radicle (Fig. 1f–g) (Barradas 1973), which makes it difficult to define morphological germination criteria.

There have been preliminary reports that the pyrene structure restricts embryo development, causing dormancy, which can be partially overcome by exogenous application of gibberellic acid (GA_3) (Dombroski et al. 2010). There have been no studies, however, addressing the interactions between the structural and physiological aspects controlling germination in this species that could contribute to our knowledge concerning reproduction in the Caryocaraceae family.

In addition to germination, the mobilization of seminal reserves and the pattern of seedling development are essential to the reproductive success of the species (Nonogaki et al. 2010; Bewley et al. 2013), especially in areas with a seasonal climate, such as the Cerrado. *C. brasiliense* seeds are rich in lipidic and proteic reserves (Lima et al. 2007; Ascari et al. 2013), but no detailed information is available concerning the mobilization of those seminal reserves or the patterns of early seedling development in this species.

We, therefore, evaluated the morphological, anatomical and physiological aspects of the pyrenes and seedlings of *C. brasiliense* to address the following questions: (1) what is the role of the pyrene in controlling germination? (2) Which histological and cytological features are related to seed germination and seedling establishment?

We also sought to relate our results to ecological aspects associated with the adaptation of this species to the Cerrado environment.

Materials and methods

Collections and preliminary procedures

Ripe fruits of *C. brasiliense* were collected after abscission (recent abscission was identified by the integrity of the exocarp and mesocarp) from more than 100 plants in a natural population in the municipality of São João da Lagoa in northern Minas Gerais State, Brazil (16°46′42″S × 44°18′24″W). The exocarp and mesocarp were removed using a knife, and the pyrenes were stored in the shade for 38 days until the seeds (initially adhering to endocarp) reached a dehydration level that allowed their extraction with minimal damage. The seeds were then extracted from the pyrenes according to the methodology described by Mendes (2015), using an emery grinder to scarify the endocarp, a vise to open it, and pliers to remove the seed.

Morphology

Twenty fruits and 20 pyrenes were cross- and longitudinally sectioned with the aid of a guillotine, and their internal structures recorded using a digital camera (DSC-H9; Sony, Osaka, Japan). Five replicates of 20 isolated seeds were soaked in deionized water for 48 h, and ten replicates of ten pyrenes were soaked for 14 days in tap water (the soaking times required for seeds with original water contents of approximately 7% to reach phase II of germination, with water contents of approximately 40%) (Mendes 2015). The seeds and pyrenes were then disinfected, planted in polyethylene containers (17×12×6 cm) containing vermiculite moistened with deionized water to 80% of its retention capacity, and placed in a germination chamber at 30 °C under a photoperiod of 12 h of light. Observations of the isolated seeds and pyrenes were conducted for 30 and 120 days, respectively, and the morphologies of their structures were recorded and described.

Germination and morphometric analyses

Seeds were extracted from the endocarps (as described above) and subjected to three pre-germinative treatments: (1) no pre-hydration (control); (2) soaking in deionized water for 48 h (pre-hydration); and, (3) imbibition in a 50 mg/L gibberellic acid solution (Sigma–Aldrich, St Louis, USA) for 48 h (GA_3). After these treatments, the seeds were immersed in a commercial fungicide composed of 100 g/L of carboxanilide and 100 g/L dimethyl dithiocarbamate (Vitavax-tiram® 200 SC, Chemtura, Rio Claro, Brazil) and subsequently held in a germination chamber under the conditions described above. Before sowing, and after 0.5; 1; 2; 3; 4; 5; 10; 15; 20 and 30 days, five replicates of ten seeds from each treatment were evaluated in terms of their lengths, widths, and thicknesses (using a caliper). The seed fresh and dry weights (after drying in an oven at 105 °C for 24 h) were determined and their water contents calculated (Brasil 2009). Five replicates of 20 seeds (for each treatment) were placed in a germination chamber for 30 days under the conditions described above. We evaluated the rupture of the seed coat and germination on a daily basis. Seeds were considered germinated when showing root protrusion and/or plumule growth of 3 mm in relation to the average of their initial condition. At the end of the experiments, seeds that showed no indication of

germination were classified as deteriorated if they became softened or were attacked by microorganisms.

The experiments were conducted using a completely randomized design. Germination data were obtained by counting and then converting the results into percentages. The germination speed index (GSI) was calculated by summing the number of germinated seeds per day and dividing that by the number of days after sowing (Maguire 1962). Data were submitted to analysis of variance, and the means were compared by Tukey test at a 5% level of probability. The correlation between the occurrence of seed coat rupture and germination was determined using the Pearson coefficient.

The pyrenes were subjected to three pre-germinative treatments: (1) no pre-hydration (control); (2) soaking in water for 14 days (pre-hydration); and (3) soaking in water for 10 days followed by immersion 4 days in a 125 mg/L GA_3 solution (Progibb 400, Abbott Laboratories, North Chicago, USA) (GA_3). After these treatments, the pyrenes were incubated under the conditions described above. Before sowing, and after 0.5; 1; 2; 3; 5; 20; 40; 60; 120 and 180 days, five replicates of ten seeds from each treatment were extracted from their endocarps and their dimensions and water contents were evaluated. Ten repetitions of ten pyrenes from each treatment were accommodated in a germination chamber for 196 days, under the conditions described above, and germination was evaluated every week, considering the seeds that cracked their endocarps and emitted root or plumule as germinated. The experimental design and statistical analyses were the same as the experiments with isolated seeds.

Seed anatomy

Dry seeds (6.4% water content), isolated seeds (control treatment) after 1 day of sowing, and newly germinated seeds were sectioned under stereomicroscope (7667 L, Motic, Hong Kong, China), using a razor blade, at the plumule, root pole, and central region of hypocotyl—yielding cubes with edges of approximately 3 mm. These fragments were then fixed in Karnovsky solution (1965) under vacuum (500 mmHg) for 24 h, dehydrated in an ethanol series (Jensen 1962), and incorporated into historesin (Technovit® 7100, Heraeus Kulzer, Wehrheim, Germany) (Paiva et al. 2011). Cross- and longitudinal section (5 µm) were obtained using a rotary microtome (Atago, Tokyo, Japan) and stained with 0.05% toluidine blue, pH 4.7 (O'Brien et al. 1964). The slides were mounted in acrylic resin (Itacril®, Itacril Indústria e Comércio de Resinas, Itaquaquecetuba, Brazil), and described and documented using a photomicroscope (AI/AxionCamIcc 3, Zeiss, Jena, Germany).

Histochemistry

The materials used for histochemical analyses were the same as those used for the anatomical analysis. Sections (10 µm thick) were stained with lugol solution (Jensen 1962) to detect starch, with Xylidine Ponceau (Vidal 1970) and bromophenol blue (Mazia et al. 1953) to detect proteins, and with Sudan IV (Pearse 1980) to detect lipids. To characterize the vascularization of the hypocotyl, fragments obtained from cross sections of the seeds in the median region (2 mm thick) were stained with 1% basic fuchsin to identify lignins and subjected to diaphanization (Fuchs 1963). The images were documented as described for the morphological evaluations.

Electron microscopy

Samples of the plumule and root pole from dry seeds and newly germinated seeds were obtained for scanning electron microscopy (SEM) by sectioning under a stereomicroscope. The samples were fixed in Karnovsky's solution (1965) for 24 h, dehydrated in an ethanol series, dried to the critical point using CO_2 (CPD 020, Bal-Tec, Balzers, Liechtenstein), metalized with gold (MED.010-Balzers, Balzers, Leichtenstein), and analyzed in a scanning electron microscope (Quantum 200, FEI Company, Eindhoven, The Netherlands), with digital image capture, at 12–20 kV (Robards 1978).

For ultrastructural analyses, dry and newly germinated seeds were dissected in the hypocotyl region (adjacent to the insertion of the cotyledons) into sections approximately 0.4 mm thick. These samples were then fixed in Karnovsky's solution (1965) for 24 h, post-fixed in 1% osmium tetroxide (0.1 M phosphate buffer, pH 7.2), dehydrated in an acetone series, and infiltrated in Araldite resin. Ultrathin section (50 nm) were contrasted with uranyl acetate and lead citrate and examined in a transmission electron microscope (Philips CM 100, Philips/FEI Corp., Eindhoven, Netherlands) at 80 kV (Robards 1978; Roland 1978).

Results

Morphology

Caryocar brasiliense fruits are drupaceous and have variable lengths (approximately 10 cm) (Fig. 1b). The outer pericarp region and pyrenes (from one to five) could be seen in the sectioned fruits (Fig. 1c). The outer region of the pericarp includes the exocarp (with a corky consistency and green colored) and the thick, fleshy mesocarp (with a whitish to yellow color) (Fig. 1b–c). The pyrenes are covered with a yellow, fleshy, oleaginous, and aromatic

pulp (Fig. 1c), with a woody endocarp with numerous fine aculeus and, internally, the seed (Fig. 1d). The inner region of the endocarp is less resistant and has a corky texture, which apparently favors seed hydration. The seeds have a reniform shape, and brown and papyraceous seed coats that are thicker and with lighter pigmentation near the hilum, being 25 mm long, 13 mm wide, and 10 mm thick, on the average (Fig. 1d, e). The embryo is whitish, has an oleaginous aspect, a very developed hypocotyl, an undifferentiated radicle, a plumule with two small scaly cotyledons, and a slightly elongated epicotyl (Fig. 1d–g) with many overlapping leaf primordia at the end (Fig. 1h).

On the ninth day after sowing, 44% of pre-hydrated isolated seeds appeared swollen, with cracks in their seed coats (Fig. 2a). On the tenth day after sowing, protrusion of the tap root was observed in 9% of the seeds, with early emissions of lateral roots (Fig. 2b, c). 2% of the germinated seeds showed plumule growth before radicle protrusion (Fig. 2d). In some cases, early degeneration of the tap root was observed, with proliferation of adventitious roots from the hypocotyl (Fig. 2e).

The completion of germination from pyrenes was preceded by the expansion of the hypocotyl and rupturing of the endocarp (Fig. 2f). At 40 days after sowing, the emission of the root and/or plumule from the crack in the endocarp began (Fig. 2g, h), although seedling development was very variable. Some seeds were strongly attacked by fungi, but in many cases seedling development was not limited by those infections.

Germination and morphometric analyses

The water contents of the isolated seeds in the control treatment (initially 6.3%) increased rapidly until the second day after sowing, reaching 38.5%—which marked the end of phase I of germination (Fig. 3a). Phase II was characterized by a tendency for stabilization of the water content. At 10 days after sowing, a further increase in the water content of the seeds occurred, marking phase III of germination, which was associated with root emission. Pre-hydration and the application of GA_3 to isolated seeds anticipated phase II of germination. Treatment with GA_3 promoted significant increases in the water contents of the seeds at most times of evaluation as compared to the control treatment. Seeds still held within the endocarps in the control treatment absorbed water only slowly (in relation to isolated seeds) and reached the phase II of germination 20 days after sowing, when they attained water contents of 39.7% (Fig. 3b). Phase III was reached 40 days after sowing, associated with root emission or plumule growth. The water contents of the pre-hydrated seeds and the seeds treated with GA_3 were significantly higher than those of the control treatment until 40 days, and all times after sowing, respectively.

On the ninth day after sowing isolated seeds, pre-hydrated seeds, and seeds treated with GA_3 showed ruptures in their seed coats (Fig. 2a), an event that was highly correlated ($r=0.82$; $P<0.0001$) with root protrusion (Fig. 2b–d). After 30 days, the germination percentage of seeds treated with GA_3 was higher than the control (Fig. 4a). The GSI demonstrated the same behavior, with values of 1.29 and 0.46 for the GA_3 and control treatments, respectively, while the pre-hydrated seeds showed intermediate values. The germination of seeds still contained within their endocarps started 40 days after sowing, with the application of GA_3 resulting in higher germination rates as compared to the pre-hydration treatment (Fig. 4b). The GSI of the GA_3 treated seeds (1.16) was greater than both the control (0.58) and pre-hydration (0.43) treatment.

The germination of isolated seeds demonstrated a tendency to stabilization 24 days after sowing, while the germination of seeds still contained within their endocarps stabilized after 133 days (Fig. 4a, b). The germination of isolated seeds was considerably higher than seeds still contained within their endocarps, even considering a shorter evaluation period. There was no significant effect of the treatments (control, pre-hydration and GA_3 application) on the deterioration percentages of both isolated seeds and seeds inserted in the endocarps, with averages of 35 and 60%, respectively.

The dimensions of the isolated seeds in the control treatment in relation to their initial condition increased over time (Fig. 5a, c, e). GA_3 treatment promoted increases in the lengths and thicknesses of the seeds in relation to the control 10 days after sowing. There were increases in the dimensions of the seeds still held within their endocarps in all treatments over time (Fig. 5b, d, f). Treatment with GA_3 provided significant increases in seed dimensions as compared to the control treatment at most evaluation times, while pre-hydration gave intermediate results.

Anatomy of dry seeds

The seeds of *C. brasiliense* are highly specialized in terms of their various cell types and the diversity of tissues that compose them. The leaf primordia in the plumule involve apical meristem, which is composed of compactly arranged isodiametric cells (Fig. 6a, b). The leaf primordia have a protoderm formed by a layer of tabular cells and a ground meristem with several layers of globular and voluminous cells with dense contents (indicative of reserve accumulation) (Fig. 6b, c). Differentiated vascular bundles are found in the more developed leaf primordia (Fig. 6c). The cotyledon tissues have characteristics similar to those of the leaf primordia, except for their protoderm, with cells on the abaxial face that are voluminous, elongated in anticlinal direction, and with secretory appearances (Fig. 6d). The

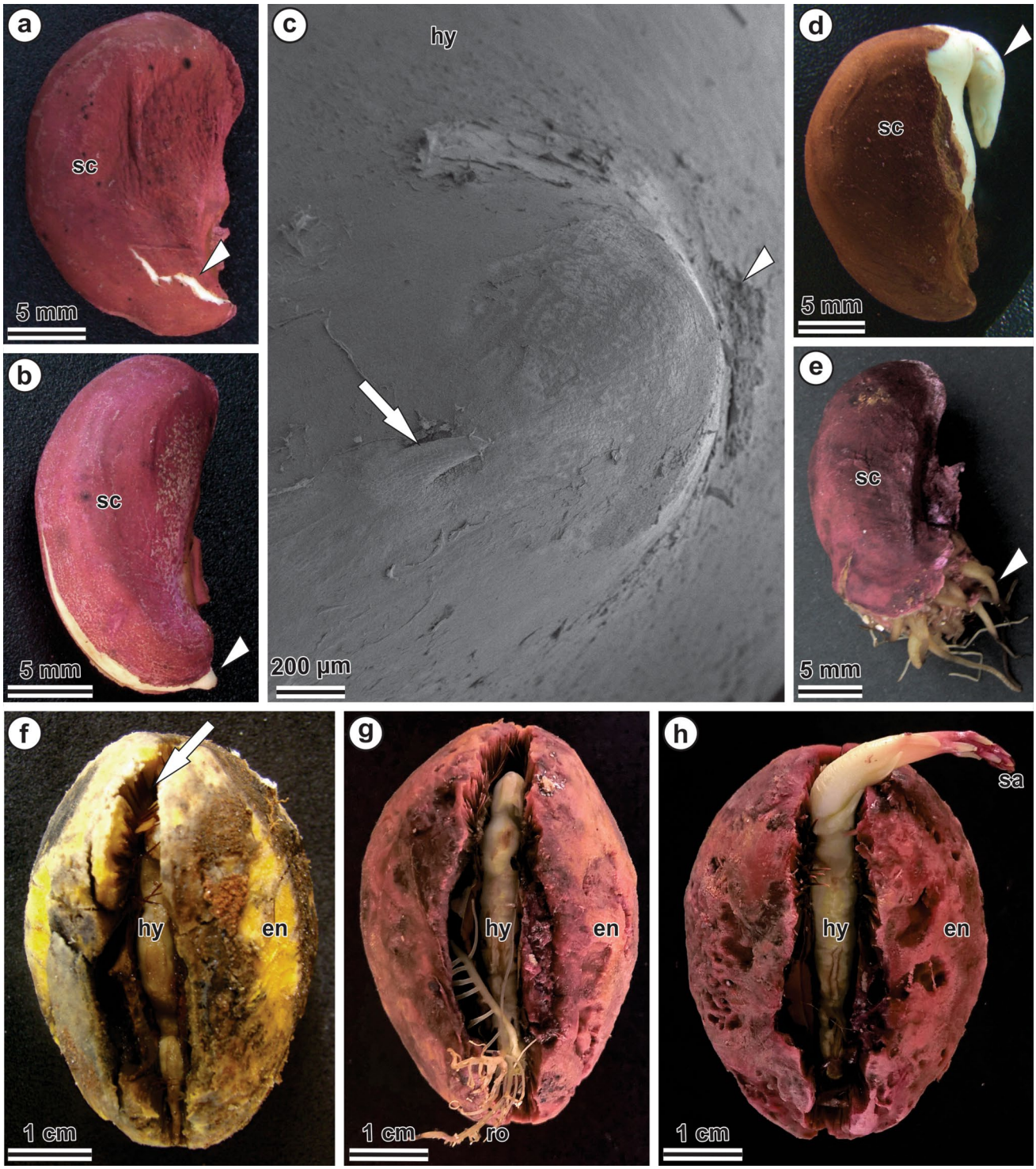


Fig. 2 Morphology of the seed, pyrene, and seedling of *Caryocar brasiliense*. Isolated seeds (**a**–**e**). Pyrenes (**f**–**h**). **a** Nine days after sowing, indicating the ruptured seed coat (*arrowhead*). **b** Ten days after sowing, indicating the protrusion of the taproot (*arrowhead*). **c** Taproot primordium rupturing the seed coat tissue (*arrowhead*) by SEM imagery; the *arrow* indicates the emerging lateral root. **d** Ten days after sowing, showing elongation of the plumule (*arrowhead*). **e** Germinated seed, showing emission of adventitious roots from the hypocotyl (*arrowhead*). **f** 40 days after sowing, indicating the rupturing of the endocarp (*arrow*), **g** emission of roots and **h** plumule. **a, b, e, g, h** Pink tinge due to the fungicide. *en* endocarp, *hy* hypocotyl, *sc* seed coat

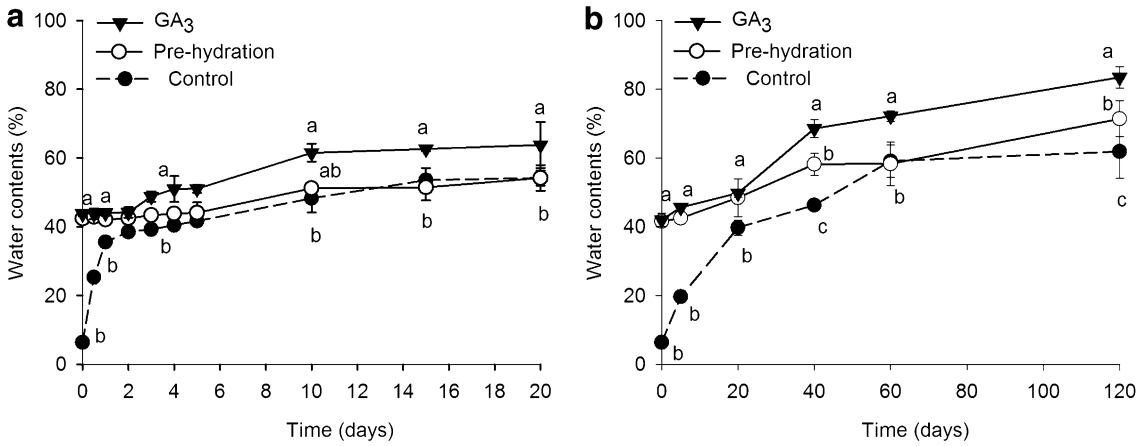


Fig. 3 Water contents of isolated *Caryocar brasiliense* seeds (**a**), and those still enveloped by the endocarp (**b**), submitted to pre-germinative treatments, sown onto vermiculite, and held in a germination chamber at 30 °C. Within each time interval, *different letters* indicate significant differences between the treatments by the Tukey test, at a 5% level of probability. *Vertical bars* indicate the standard errors of the means

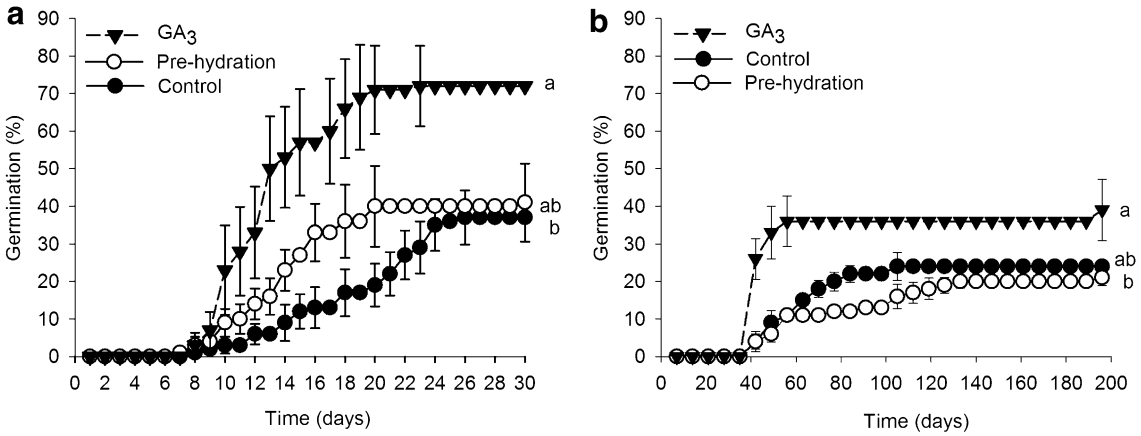


Fig. 4 Germination percentages of *Caryocar brasiliense* seeds isolated (**a**), and those still enveloped by the endocarp (**b**), submitted to pre-germinative treatments, sown onto vermiculite, and held in a germination chamber at 30 °C. Within each time interval, *different letters* indicate significant differences between the treatments by the Tukey test, at a 5% level of probability. *Vertical bars* indicate the standard errors of the means

epicotyls show a protoderm with a layer of tabular cells, and a differentiated vascular system consisting of four to six bundles inserted into a procambium cylinder (Fig. 6e). The ground meristem is thus divided into cortical and medullar regions, both formed by globular and vacuolated cells, with larger cells located in the central region.

The seed coat, in contact with most of the hypocotyl, shows collapsed cells with accumulations of phenolic compounds (Fig. 6f). In the region of the hilum, and near the plumule, are the remains of the endosperm (previously identified in ontogenetic studies by Barradas 1973) forming folded layers in some regions whose cell walls are rich in pectic substances. The ground meristem in the hypocotyl shows globoid and voluminous cells with accumulations of reserve compounds (Fig. 6g). The vascular system connected to the plumule has from 36 to 40 bundles with peripheral dispositions inserted in a procambial cylinder (Fig. 6h). The seed coat near the radicular pole has cells rich in phenolic compounds; the remains of the endosperm were not observed in this region (Fig. 6i). The radicle is not differentiated, and only the root promeristem (with a peripheral disposition) is conspicuous (Fig. 6j). The procambium forms a cone at the root pole and shows a wide medullar region formed by a ground meristem composed of globular and vacuolated cells. Differentiated vascular bundles are inserted in some regions of the procambial cone, and their tracheal elements show scalariform deposits of secondary walls (Fig. 6k).

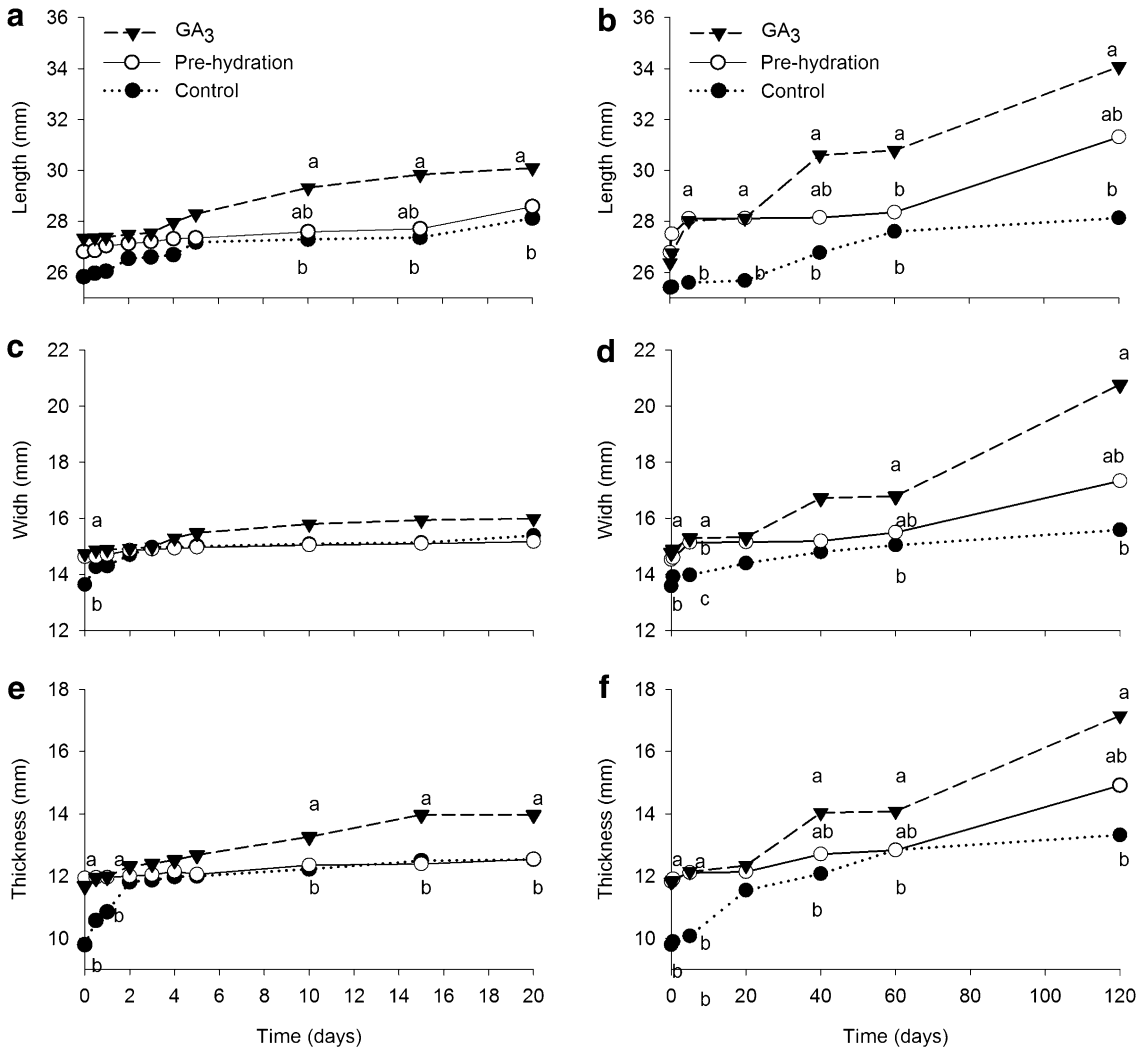


Fig. 5 Lengths, widths, and thicknesses of *Caryocar brasiliense* seeds isolated (**a, c, e**), and those still enveloped by the endocarp (**b, d, f**), submitted to pre-germinative treatments, sown onto vermiculite, and held in a germination chamber at 30 °C. Within each time interval, *different letters* indicate significant differences between the treatments by the Tukey test, at a 5% level of probability. *Vertical bars* indicate the standard errors of the means

Seminal reserves and their mobilization

The predominant reserve compounds in the dry seeds are proteins and lipids. These reserves are the main components of the protoplasts in most cells, and are present in the entire embryo. Globular protein bodies of variable sizes and densities, with dense matrices and enclosed by membranes, are stored in the cells of the protoderm and ground meristem (Figs. 7a, b, 8a). Within the protein bodies numerous globular inclusions, possibly globoids (phytin storage sites), are observed. Lipids are stored in globoid lipid bodies of variable sizes that are also enclosed by membranes, and can be seen in clusters around the protein bodies (Figs. 7b, c, 8c). Starch grains can be seen in the ground meristem cells of the hypocotyl and plumule, especially surrounding the vascular bundles (Fig. 8d).

After sowing, seed reserve mobilization was initiated in the regions near the plumule and radicular pole, spreading then to the central and inner regions of the hypocotyl after completing germination. Evidence of protein mobilization in the cells of the ground meristem of the plumule was observed starting from the first day after sowing (Fig. 8e). The proteins were being consumed, there was an emptying and fusion of protein bodies—forming large vacuoles in newly germinated seeds (Figs. 7e, 8 f), simultaneous with the formation of many small vacuoles due to consumption of the content of the inclusions (Fig. 7e, f). Lipid mobilization was observed slightly later, associated with the coalescence of lipid bodies (Fig. 7f), being first observed in the

cotyledonary node region after root protrusion. The mobilization of cell wall components was also observed in freshly germinated seeds by the irregular outlines and the loosened aspects of the cell walls (Fig. 7e).

Anatomy of germinated seeds

The cells of the epidermis, epicotyl, and cotyledon showed Casparian-like strips in the anticlinal walls of newly germinated seeds (Fig. 9a). The parenchymal cells along the plumule had expanded and now contained large hyaline vacuoles, indicating reserve mobilization, especially near the vascular bundles (Fig. 9b, c). Cambial activity was observed in the interfascicular region in the dorsal peripheral region of the hypocotyl, indicating early secondary growth (Fig. 9c). Root protrusion caused the rupturing of the seed coat, the remaining layers of the endosperm, and the embryo protoderm, simultaneous with the differentiation of the root protoderm (Fig. 9d). Seed coat cells did not exhibit high accumulations of phenolic compounds as seen in dry seeds (compare Fig. 6i with Fig. 9d). A wide root cap developed, covering the promeristem, composed of cells with dense nuclei and exhibiting intense cell division activity (Fig. 9d). The parenchyma cells were voluminous, with large vacuoles and accumulations of phenolic compounds. The differentiation of procambium with central arrangement initiated the formation of a protostelic vascular system. The procambial cells showed pronounced elongation, voluminous vacuoles, and accumulations of phenolic compounds. The histochemical tests showed starch accumulation in the hypocotyl near the root differentiation region, and in the root cap (Fig. 9e). Proteic reserves were consumed in the transition region between the hypocotyl and the root, and there were accumulations of proteins in the cell division region of the root and root cap (Fig. 9f).

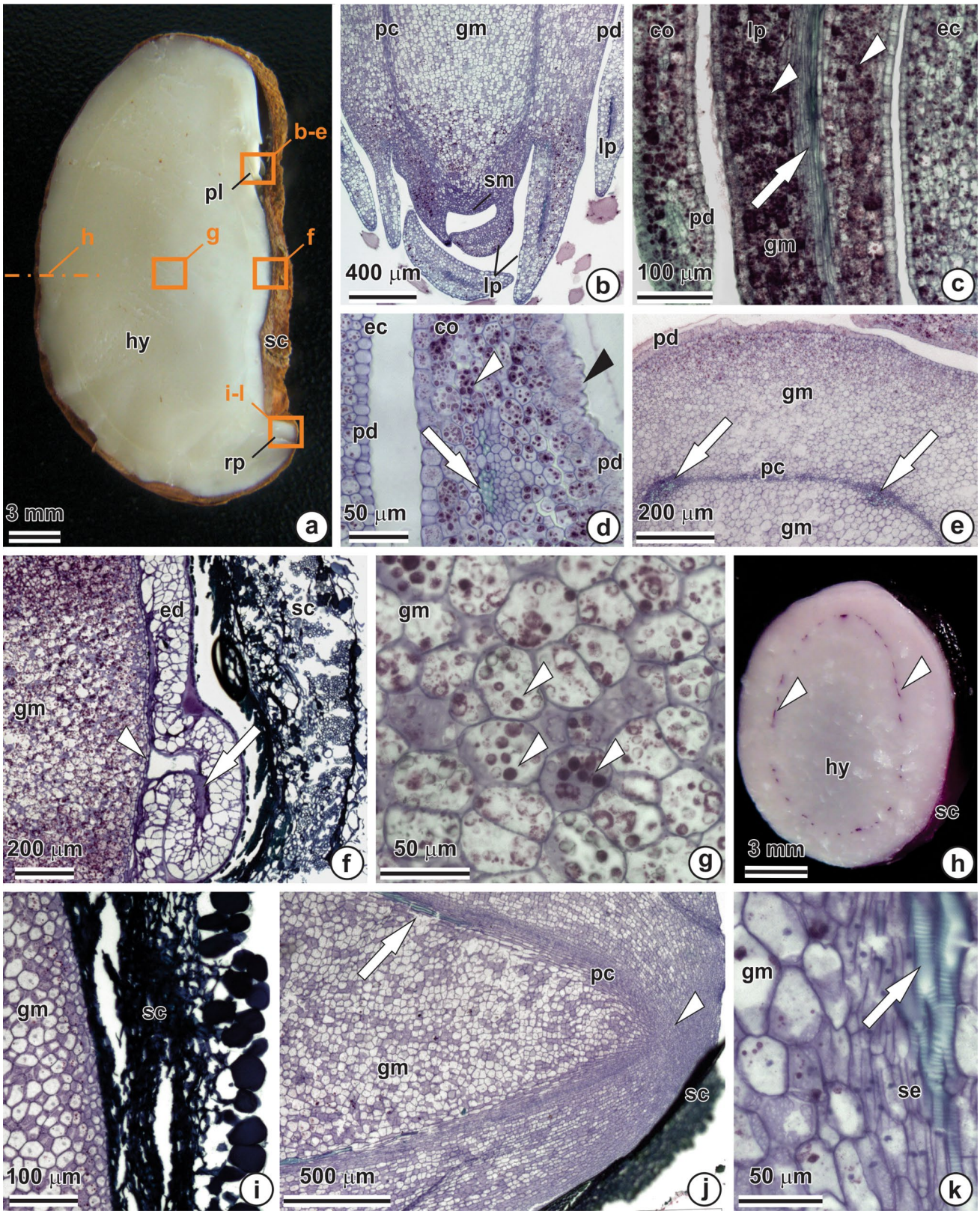
Discussion

The roles of pyrene structures in controlling germination

Germination of *C. brasiliense* diaspores is controlled by interactions between the pyrene structure and physiological aspects of the seed. The endocarp retards water absorption, thereby establishing a control on germination. As water absorption is not fully constrained by the endocarp, there is no strict physical dormancy (a criteria of Baskin and Baskin 2014). We have shown, however, that seeds enveloped by the endocarp require approximately 20 days after sowing to achieve water levels above the 35% required for germination (phase II of germination, Bewley et al. 2013)—while isolated seeds reach this phase in only 2 days. On the other hand, the germination rate of isolated seeds 30 days after sowing was about twice that of seeds still contained within the endocarp after 200 days, which indicates that there is also an effect (probably mechanical) of the endocarp restricting embryo growth.

Hypocotyl growth plays a crucial role in breaking dormancy, as it promotes the cracking of the hard endocarp that allows the completion of germination. The growth of the hypocotyl is associated with reserve mobilization and embryonic cell expansion possibly mediated by gibberellins. It is known that reserve mobilization diminishes the osmotic potential and increases cell turgor (Tan-Wilson and Wilson 2012; Bewley et al. 2013; Rosental et al. 2014). We observed protein reserve mobilization 1 day after sowing (associated with vacuolization) in seeds not subjected to GA_3 treatment. The increased turgidity of the cells was associated with significant increases in the sizes of the seeds still enveloped in the endocarp in the control treatment after completing imbibition. This event alone was not sufficient, however, to crack the endocarps of most pyrenes, and did not result in high germination levels.

The significant effect of GA_3 on the germination of *C. brasiliense* seeds, whether isolated or still enveloped by the endocarp, indicates the physiological nature of dormancy related to hormonal balance as reported by some workers (Dombroski et al. 2010; Mendes 2015). In this study, treatment with GA_3 promoted greater imbibition, increases in the sizes of the seeds, and increases in germination percentages relative to the control, which were not observed in pre-hydration treatments. As endocarp tissues are nonliving and rigid, one can rule out any role of that phytoregulator in weakening tissues adjacent to the embryo, as has been reported in several species (Pritchard et al. 2002; Koornneef et al. 2002; Kucera et al. 2005). The positive effects of GA_3 are probably related to the role of gibberellins in embryo cell wall loosening, which promotes cell expansion (Bewley et al. 2013) and, in association with increased turgidity, provides the additional force necessary to crack the endocarp. The pronounced cell expansion observed in the hypocotyls of the newly germinated seeds is evidence of the importance of this process in *C. brasiliense*—as had already been well-established in several species (Debeaujon et al. 2000; Finch-Savage and Leubner-Metzger 2006; Nonogaki et al. 2010). On the other hand, the significant effects of GA_3 on the germination of isolated seeds suggests that gibberellins may also act directly on root and plumule growth, and that the physiological dormancy in this species is of the intermediate type (according to classification of Baskin and Baskin 2014). Further work should contribute to addressing these questions.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◂**Fig. 6** Morphology and anatomy of the dry seeds of *Caryocar brasiliense*. Longitudinal sections (**a–c, j–k**). Cross-sections (**d–i**). Sections stained with toluidine blue (**b–g, i–k**). Section stained with basic fuchsin (**h**). **a** Morphology, highlighting regions represented in **b–k. b** Plumule. **c** Detail of the plumule, showing leaf primordium ground meristem cells with abundant reserves (*arrowhead*) and vascular bundles (*arrow*). **d** Detail of the cotyledon, highlighting vascular bundles (*arrow*), ground meristem cells with storage reserves (*white arrowhead*), and protoderm cells with secretory appearances (*black arrowhead*). **e** Epicotyl, showing the eustelic vascular cylinder and differentiated vascular bundles (*arrows*). **f** Periphery of the seed adjacent to the ventral region of the hypocotyl, highlighting folds in the remaining layers of the endosperm (*arrow*) and embryo protoderm (*arrowhead*). **g** Central region of the hypocotyl, showing reserve accumulation in the ground meristem cells. **h** Median region of the hypocotyl, highlighting vascular bundles with lignified tracheary elements (*arrowheads*). **i** Seed coat near the radicular pole, showing the accumulation of phenolic compounds. **j** Radicular pole, highlighting vascular bundles (*arrow*) and the root promeristem (*arrowhead*). **k** Detail of differentiated tracheary elements with scalariform depositions on secondary walls (*arrow*) in the radicular pole. *co* cotyledon, *ec* epicotyl, *ed* endosperm, *gm* ground meristem, *hy* hypocotyl, *lp* leaf primordia, *pc* procambium, *pd* protoderm, *pl* plumule, *rp* radicular pole, *sc* seed coat, *se* sieve elements, *sm* shoot promeristem

Anatomical and cytological characteristics of the seeds as related to germination and seedling establishment

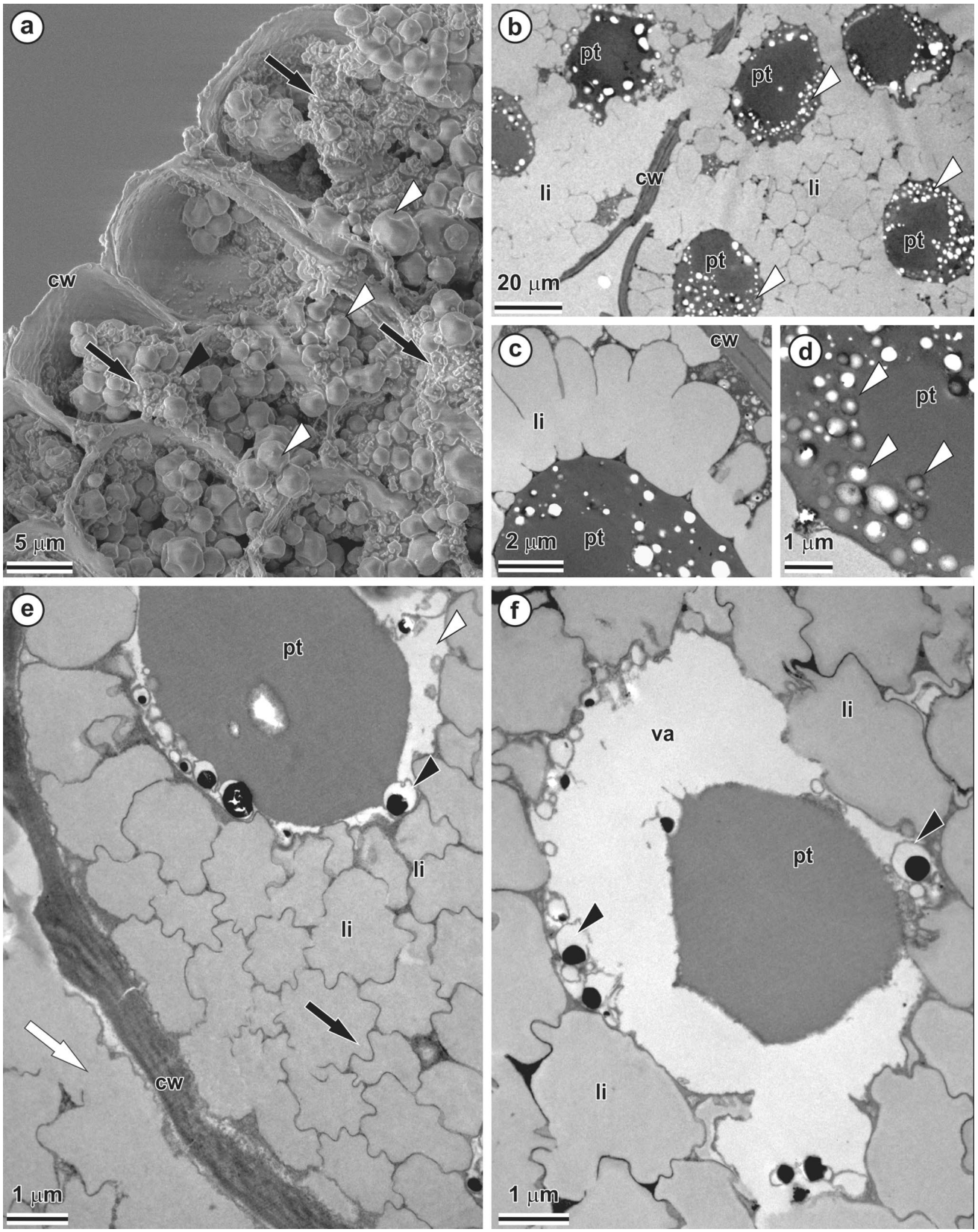
A number of anatomical characteristics indicate that *C. brasiliense* seeds have features that favor rapid seedling development soon after overcoming dormancy. The embryo of this species is of the hypocotyledonary type, and during its development the endosperm reserves are almost completely incorporated by the embryo (Barradas 1973; Lima et al. 2007)—which facilitates access to reserves during germination and early seedling development (Linkies et al. 2010). Our results indicate that the mobilization of protein reserves (which begins with imbibition) is more directly related to germination, while lipid mobilization (which takes place later) is more complex (Bewley et al. 2013) and more important to seedling nutrition. These aspects, as well as transient starch formation, indicate that the mobilization of seminal reserves in *C. brasiliense* follows the most common pattern observed in non-endospermic seeds (Werker 1997; Tan-Wilson and Wilson 2012; Bewley et al. 2013).

C. brasiliense embryos have differentiated vascular systems, which is rare (Werker 1997). Vascular bundles with functional aspects and organized in a eustelic pattern, connect the plumule and root pole to the hypocotyl, facilitating reserve transport to growth regions. We also observed vascular cambium activity in the hypocotyls of newly germinated seeds, indicating that early secondary growth constitutes a strategy to expand the translocation capacity of the seminal reserves and facilitate the rapid seedling establishment. We believe that this is the first report of secondary growth in newly germinated seeds.

Seeds have significant morphogenetic capacities, with the potential for the initial development of stem systems as an alternative to root development. On the other hand, although the radicle is not differentiated, a vigorous root system can develop rapidly after germination. The hypocotyl has the capacity of early emission of adventitious roots if the principal root is mechanically damaged or attacked by fungi, as was also observed by Barradas (1973). The rapid development of a vigorous root system is typical of Cerrado species seedlings (Hoffmann 2000) as it facilitates access to deeper soil layers with higher moisture contents (Silveira et al. 2013). The formation of a thick strip on the anticlinal walls of the epidermal cells of epicotyls and cotyledons in newly germinated seeds is also noteworthy. The presence of cell wall thickenings (of the Casparian strip type) associated with vascular tissue in the endoderm has been reported in the leaves of several species of angiosperms (Lersten 1997). We are not aware, however, of reports of this type of structure in the epidermis of seedling plumules during early development. Considering that these structures are often associated with water flow control (Metcalfe and Chalk 1989), these cellular thickenings may have a specialized role in preventing dehydration in *C. brasiliense* seedlings, which should be investigated in future studies.

Ecological aspects of germination control

The ontogenesis of the fruit of *C. brasiliense* was described in detail by Barradas (1973), who found that the oleaginous pulp that coats the endocarp represents the inner portion of the mesocarp. Our field observations confirm that after fruit abscission the exocarp and the outer and inner mesocarps are consumed by animals (that disperse the pyrenes) as well as by insects and possibly by soil microorganisms. The hard and aculeate endocarp protects the embryo (which is resistant to dehydration, according to Mendes 2015) against predation, thus favoring seed bank establishment. The sophisticated germination control mechanisms in *C. brasiliense* represent adaptations to the climatic conditions of the Cerrado with its marked seasonality and irregular rainfall. The rainy season occurs between the months of September and April, and the dry season between the months May and September (corresponding to Austral winter) (Carvalho et al. 1994; Sano et al. 2008). Most *C. brasiliense* diaspores are dispersed in the second half of the rainy season (December–March). The partial restriction of water absorption provided by the endocarp serves as a germination control mechanism, since a prolonged period in moist soil is required to promote seed hydration, as reported for *Attalea vitrivir* (Arecaceae) (Neves et al. 2013). Periods of drought are a common occurrence even during the rainy season, and the seeds may not be able to access water available in the soil long enough to complete hydration,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◂**Fig. 7** Hypocotyl cells of *Caryocar brasiliense* in the region of the cotyledonary node by SEM (**a**) and TEM (**b–f**) imagery. Dry seeds (**a–d**). Newly germinated seeds (**e–f**). **a** Cells of the protoderm and ground meristem with numerous lipid bodies (*arrows*) and protein bodies (*arrowheads*). **b** Ground meristem cell, indicating globular inclusions within the protein bodies (*arrowhead*). **c** Detail of the arrangement of lipid and protein bodies. **d** Detail of globular inclusions (possibly phytin globoids) in protein bodies (*arrowheads*). **e** Ground meristem cell in an early stage of reserve mobilization, showing loosened cell walls, lipid bodies with change in the original form (*black arrow*), coalescence of lipid bodies (*white arrow*), small vacuole with residues from the mobilization of the globoid content (*black arrowhead*), and a large vacuole in formation (*white arrowhead*). **f** Detail of large vacuole formed by protein mobilization, and small vacuoles formed by inclusions mobilization arrowheads. *cw* cell wall, *li* lipid bodies, *pt* protein bodies, *va* vacuole

delaying germination until the next rainy season. We found, however, that about 20% of the seeds still enveloped in the endocarp in control treatment germinated within 80 days of sowing, suggesting that some seeds have low-intensity dormancies that can quickly be overcome in the post-ripening period. This may constitute a strategy of the species for germination in very rainy years in the same season as their dispersal, to take advantage of unusually long periods with wet soils.

The pronounced physiological dormancy of many *C. brasiliense* seeds favors delayed germination under otherwise favorable conditions, as reported for several other species (Koornneef et al. 2002; Finch-Savage and Leubner-Metzger 2006; Miransari and Smith 2014). By remaining in the seed bank during the dry winter (which has a higher temperature range) the seeds undergo changes in the hormonal balance, favoring overcoming dormancy and germination at the beginning of the next rainy season following dispersal, as reported by Mendes (2015).

This is the first detailed report of the role of diaspore components in controlling germination in the

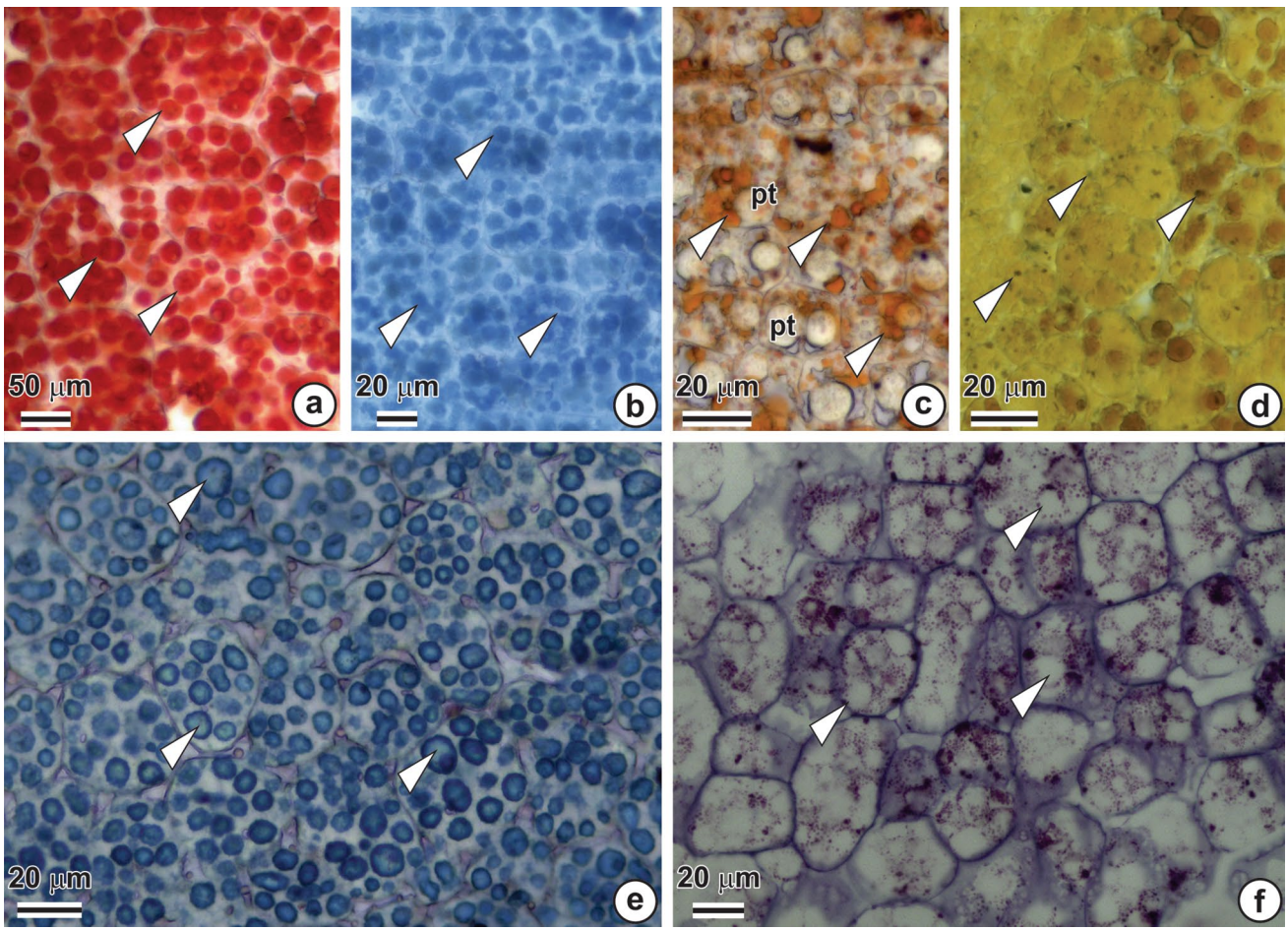
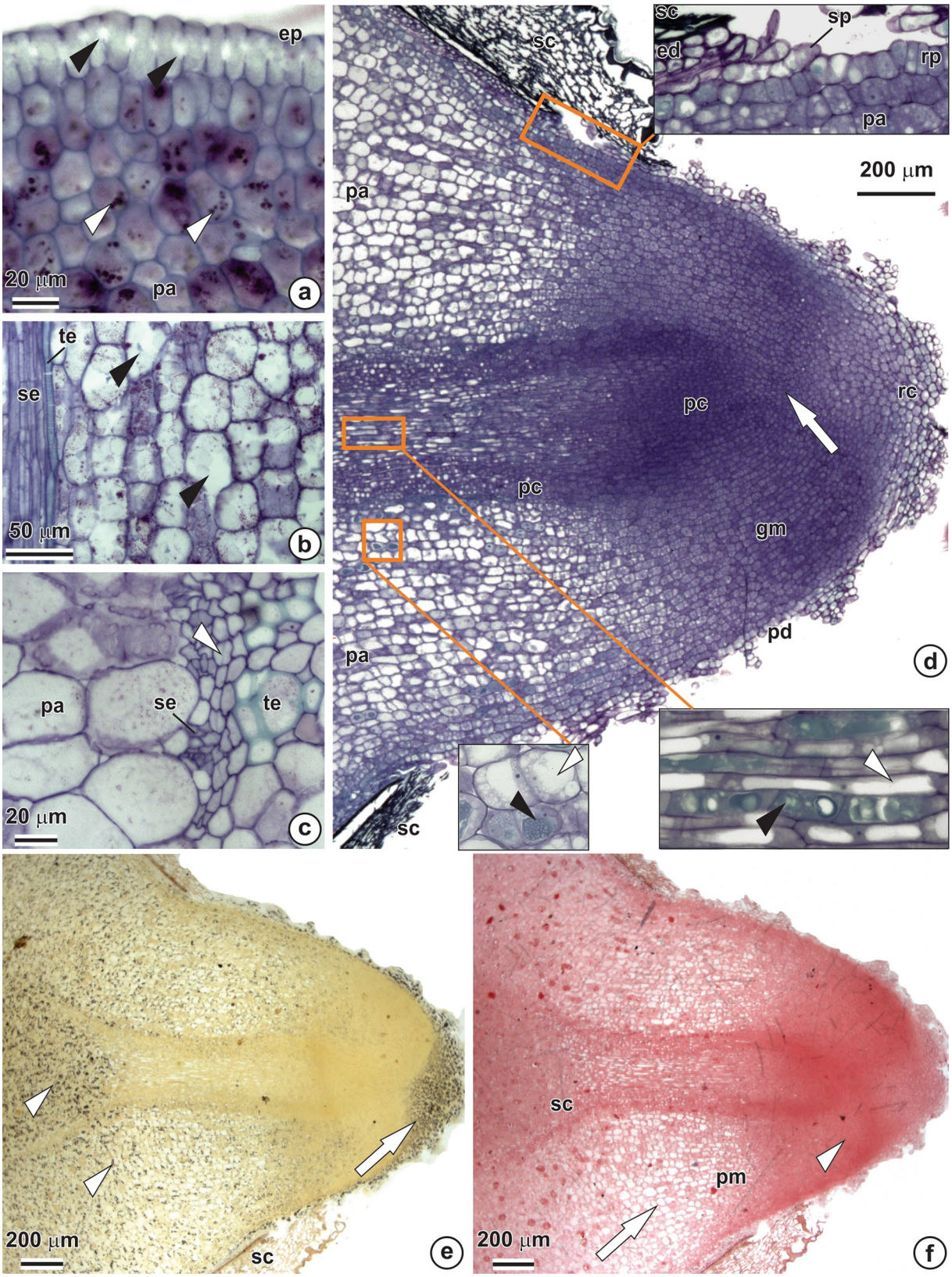


Fig. 8 Anatomical sections of the ground meristem of *Caryocar brasiliense* seeds in the region of the cotyledonary node, subjected to histochemical tests. Dry seeds (**a–d**). Seed, 1 day after sowing (**e**). Newly germinated seed (**f**). **a, b** Protein bodies (*arrowheads*) stained red with Xylidine Ponceau (**a**) and stained blue with bromophenol blue (**b**). **c** Lipids (*arrowheads*) stained orange with Sudan IV. **d** Small starch grains (*arrowheads*), associated with the protein bodies, stained purple with lugol. Partially consumed protein reserves stained blue with bromophenol blue (**e**) and stained purple with toluidine blue (**f**); *arrowheads* indicate vacuoles arising from reserve mobilization. *pt* protein bodies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◂**Fig. 9** Anatomical sections of newly germinated seeds of *Caryocar brasiliense*. Longitudinal sections (**b, d–f**). Cross-sections (**a, c**). Sections stained with toluidine blue (**a–d**). **a** Epicotyl, highlighting strips in the epidermis (*black arrowheads*) and reserves in the parenchymal cells (*white arrowheads*). **b** Epicotyl, highlighting vacuoles formed by reserve mobilization from nearby cells to the vascular bundles (*arrowheads*). **c** Dorsal peripheral region of the hypocotyl, highlighting cells in division at cambial region (*arrowhead*). **d** Radicular pole, showing the protrusion of the root and highlighting the radicular promeristem (*arrow*); *top corner* detail of seed coat, remnants of the endosperm, embryo, and root protoderm; *bottom right* elongated procambium cells with large vacuoles (*white arrowhead*) and accumulations of phenolic compounds (*black arrowhead*); *bottom left* parenchyma cells with large vacuoles (*white arrowhead*) and accumulations of phenolic compounds (*black arrowhead*). **e** Starch accumulation, stained purple with lugol, in the parenchymal cells of the hypocotyl (*white arrowheads*) and the root cap (*arrow*). **f** Accumulations of proteins stained red with Xylidine Ponceau in the root apex region and root cap (*arrowhead*) and vacuolated parenchyma cells of the hypocotyl without protein reserves (*arrow*). *ep* epidermis, *gm* ground meristem, *ed* endosperm, *pa* parenchyma, *pc* procambium, *pd* protoderm, *pm* root promeristem, *rc* root cap, *rp* root protoderm, *sc* seed coat, *se* sieve elements, *sp* embryo protoderm, *te* tracheary elements

Caryocaraceae family. Our results indicate that the germination of *C. brasiliense* is controlled by complex interactions between pyrene components. The rigid and aculeate endocarp protects the seeds and slows water absorption, thus establishing a germination control mechanism. The physiological dormancy of the seeds is determined by low potential for embryo growth associated with the mechanical resistance provided by the endocarp. The mobilization of seminal reserves and cell expansion mediated by gibberellins are possibly involved in the rupture of the endocarp that allows the completion of germination. The abundant reserves available and the high degree of differentiation of the embryo then contribute to rapid seedling establishment. The reproductive success of *C. brasiliense* in the Cerrado environment is, therefore, favored by the morpho-anatomical and physiological characteristics of the pyrene.

Author contribution statement PSNL and LMR conceived and designed the research. AMSS and MSA collected and prepared the plant material and conducted experiments. PSNL analyzed the physiological and biometric data. AMSS prepared histological slides. LMR and MOMS analyzed the anatomic and ultrastructural data. AMSS wrote the initial text. PSNL and LMR wrote the final text. All authors read and approved the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest related to the present study.

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