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Haustorium–endosperm relationships and the integration between developmental pathways during reserve mobilization in *Butia capitata* (Arecaceae) seeds

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• **Background and Aims** Palm seeds are interesting models for studying seed reserve mobilization at the tissue level due to the abundance and complexity of reserves stored in their living endosperm cells and the development of a highly specialized haustorium. We studied structural and physiological aspects of the initial phases of reserve mobilization in seeds of a neotropical palm, *Butia capitata*, and sought to characterize the interactions between the different developmental pathways of the haustorium and endosperm.
• **Methods** Morphological and histochemical evaluations of the haustorium, the endosperm adjacent to the embryo, and the peripheral endosperm of dry, imbibed, dormant seeds and seeds geminating for 2, 5 and 10 d were performed. Biochemical analyses included determinations of endo-β-mannanase activity, hormonal profiling (20 hormones belonging to eight classes) and H_2O_2 quantification in various tissues.
• **Key Results** The mobilization of haustorium reserves was associated with germination and involved distinct hormonal alterations in the endosperm related to H_2O_2 production. The mobilization of endosperm reserves occurred as a post-germination event controlled by the seedling and involved major structural changes in the haustorium, including growth (which increased contact with, and pressure on, the endosperm) and the formation of an aerenchyma (thus facilitating O_2 diffusion). The flow of O_2 to the endosperm and changes in endogenous contents of H_2O_2 and hormones (cytokinins, auxins, brassinosteroids and ethylene) induced the establishment of an endosperm digestion zone and the translocation of reserves to the haustorium.
• **Conclusions** The haustorium–endosperm relationship during reserve mobilization plays a pivotal role in signal integration between growth and degradation pathways in germinating seeds of *Butia capitata*.

Key words: *Butia capitata*, endosperm, germination, H_2O_2, haustorium, hormonal profile, post-germination development, reserve mobilization, seedling.

INTRODUCTION

Palm seeds commonly store large quantities of endosperm reserves, many of which have economic value (Alang *et al.*, 1988; DebMandal and Mandal, 2011; Pires *et al.*, 2013). The mobilization of these reserve compounds after germination is a complex process involving the development of a highly specialized haustorium from the cotyledonary blade (DeMason, 1983, 1988) and the formation of a narrow digestion zone within the endosperm in which cells are gradually degraded (Oliveira *et al.*, 2013; Mazzottini-dos-Santos *et al.*, 2017). Although there have been previous studies of reserve mobilization in palm seeds (DeMason, 1985, 1986; Alang *et al.*, 1988; Panza *et al.*, 2009; Bicalho *et al.*, 2015; Mazzottini-dos-Santos *et al.*, 2017), they only considered a small number of species (the Arecaceae family has >2600 species; Baker and Dransfield, 2016).

Seeds commonly store carbohydrates in their cell walls (mannan, glucomannan and galactomannan), and their mobilization occurs through the actions of hydrolase enzymes (Buckeridge, 2010). Reserve mobilization control has been well studied, especially in lettuce, tomato and legume seeds (Buckeridge *et al.*, 2000; Buckeridge, 2010; Rodríguez-Gacio *et al.*, 2012; Bewley *et al.*, 2013). Palm seeds store abundant mannan reserves in their endosperm cells (DeMason, 1988; Mazzottini-dos-Santos *et al.*, 2017). Although it is recognized that the mobilization patterns of these reserves in palm seeds follows a general pattern found (for example) in endospermic legumes (Alang *et al.*, 1988; DeMason, 1988; Buckeridge, 2010; Bewley *et al.*, 2013), there are still many aspects to be elucidated from a biochemical

point of view, particularly in terms of the hormonal regulation of this mobilization at the tissue level.

The balance between gibberellins (GAs) and abscisic acid (ABA) released by the embryo into the aleurone layer of cereal seeds controls the activation of enzymes that will degrade endosperm reserves (Weiss and Oris, 2007; Tan-Wilson and Wilson, 2012; Bewley *et al.*, 2013). There are also reports of the participation of cytokinins (CKs), brassinosteroids (BRs) and auxins in these processes (Holdsworth *et al.*, 2008; Bewley *et al.*, 2013). In Arecaceae, however, there is evidence that digestive enzymes (particularly endo-β-mannanase) are stored in living endosperm cells, and that they can be activated by signals emitted by the embryos (Sekhar and DeMason, 1990; Mazzottini-dos-Santos *et al.*, 2017). Hormone profile evaluations are still scarce for palm seeds (Bicalho *et al.*, 2015; Ribeiro *et al.*, 2015; Dias *et al.*, 2017), however, and a better understanding of their enzyme interactions may contribute to characterizing the diversity of seed reserve mobilization patterns.

Albuminous seeds show distinct stages of embryonic and endospermic reserve mobilization associated with germination and post-germination development (Bewley *et al.*, 2013). It is known that embryonic reserve mobilization begins with seed hydration, and it represents one of the first events associated with the activation of germinative metabolism (Müntz *et al.*, 2001; Nonogaki *et al.*, 2010; Bewley *et al.*, 2013; Baskin and Baskin, 2014; Nonogaki, 2014; Bellieny-Rabelo *et al.*, 2016). Endosperm reserve mobilization in palm seeds, which in most cases is dependent on the conclusion of germination (Domínguez and Cejudo, 2014; Yan *et al.*, 2014), is still poorly understood. The presence of stomata and the formation of aerenchyma in the haustorium of the seedlings of some palm species (Ribeiro *et al.*, 2012; Neves *et al.*, 2013; Oliveira *et al.*, 2013) suggest that increased oxygen pressure may be an important inducer of reserve mobilization (Ribeiro *et al.*, 2015). The dynamics between reactive oxygen species (ROS) and the enzymes involved in reserve metabolism is viewed as crucial for controlling germination and the mobilization of seed reserves (Bailly, 2004; Bailly *et al.*, 2008; El-Maarouf-Bouteau and Bailly, 2008). Considering that there have been no studies related to these themes in Arecaceae, evaluations of the roles of ROS in activating reserve mobilization would contribute to furthering our understanding of post-germination development in this family.

The present study examined the morphological, anatomical and physiological aspects of the initial phases of reserve mobilization in the seeds of *Butia capitata*, an endemic palm of the Cerrado (neotropical savannah) biome having significant ecological and economic importance (Lorenzi *et al.*, 2010; Magalhães *et al.*, 2013), to address the following questions: (1) what are the differences between embryonic and endosperm reserve mobilization? (2) what changes are associated with the activation of endosperm reserve mobilization? and (3) what are the interactions between the different developmental pathways of the haustorium and the endosperm?

MATERIALS AND METHODS

Fruit collections and preliminary evaluations

Mature fruits of *Butia capitata* (identified by their yellow exocarp and easy abscission from the bunch) were collected from 20 plants in a natural population growing in the municipality of Bonito de Minas in the northern region of the Minas Gerais State, Brazil (15º25′59.8″ S, 44º41′31.7″ W). After the fruits were depulped (in a low-rotation blender), the pyrenes (seeds enveloped by the endocarp) were dehydrated naturally in a shaded and ventilated location for 15 d (to release the seeds from the endocarp). Four replicates of ten seeds each were removed from the pyrenes (with the aid of a manual vice) to determine their water content by the difference between fresh mass and dry mass after dehydration at 105 °C for 24 h (Ribeiro *et al.*, 2012). The other pyrenes were kept in a ventilated shed until they were used in additional experiments.

Sowing and sampling

Seeds of *B. capitata* were disinfected in 6 % sodium hypochlorite for 15 min, rinsed three times in distilled water, and subsequently hydrated by immersion in water for 5 d under laboratory conditions (with daily water replacement) (Carvalho *et al.*, 2015). As the seeds of *B. capitata* show pronounced dormancy, a dormancy-breaking treatment was performed on half of the seeds (consisting of removing the operculum under semi-aseptic conditions, taking

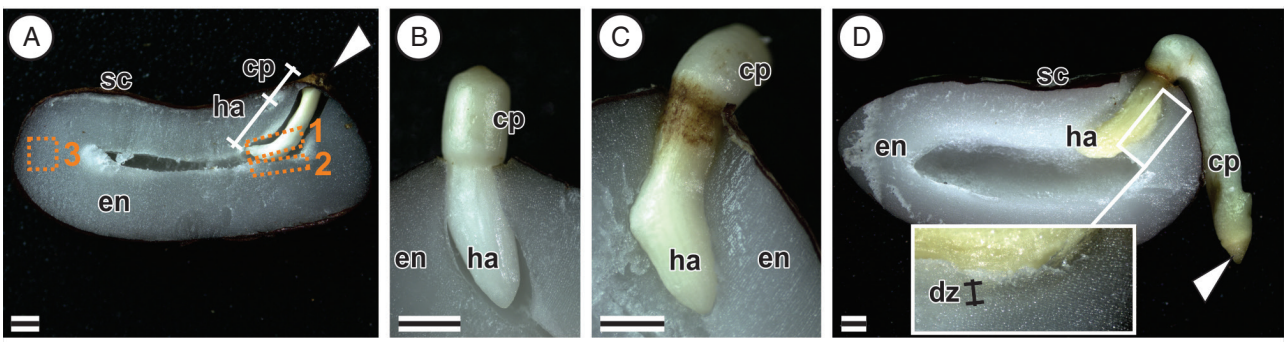

FIG. 1. Morphology of *Butia capitata* seeds. Longitudinally sectioned seeds. (A) Dry seed, showing the location of the operculum (arrowhead), regions of the embryo, and the regions from which samples were obtained for evaluation: haustorium (1), adjacent endosperm (2) and peripheral endosperm (3). (B, C) Details of the micropylar region of seeds with the operculum removed, 2 d (B) and 5 d (C) after sowing, showing the protrusion of the cotyledonary petiole. (D) Germinated seed 10 d after sowing, showing the newly emerged root (arrowhead) and endosperm digestion zone (enlarged detail below with the thickness of the digestion zone indicated by the I-shaped lines). cp, cotyledonary petiole; dz, digestion zone; en, endosperm; ha, haustorium; sc, seed coat. Scale bars = 1 mm.

care to guarantee embryo integrity) (Oliveira *et al.*, 2013). Seeds with their opercula intact (control treatment) and seeds without the operculum were sown into moist vermiculite (at 80 % of its water retention capacity) at 25 °C, in the absence of light. Samples of haustorium tissue (removed from the distal cotyledon region), the endosperm adjacent to the embryo/seedling (1 mm thickness) and peripheral endosperm tissue (distal region of the seed) were removed from dry seeds, imbibed seeds and seeds cultured with and without the operculum, after 2, 5 and 10 d, following Dias *et al.* (2017) (Fig. 1A). These structures were used for morphological and micromorphometric evaluations, histochemical tests and evaluations of their hormone profiles, H_2O_2 contents and endo-β-mannanase activities.

Biometric evaluations

Photographs of the haustoria were obtained from five replicates of ten seeds held under the conditions described above, and the lengths and diameters of these structures were measured using an image analysis program (Image-Pro Plus™, Media Cybernetics, Rockville, MD, USA); the water contents and dry masses of the haustoria were also determined.

Histochemical and micromorphometric evaluations

Samples of the haustorium and adjacent endosperm were fixed in Karnovsky's solution (Karnovsky, 1965), dehydrated in an ethanol series, and embedded in 2-hydroxyethyl-methacrylate (Leica Microsystems, Heidelberg, Germany) following Paiva *et al.* (2011). Longitudinal sections (5 µm thick) were obtained with the aid of a rotating microtome (Atago, Tokyo, Japan), stained with toluidine blue (0.1 %, pH 4.7) (O'Brien *et al.*, 1964, modified) and mounted in acrylic resin. The sections were then stained with xylidine ponceau (Vidal, 1970) for protein identification; periodic acid–Schiff reagent (PAS) (Feder and O'Brien, 1968) for neutral polysaccharide identification; and Sudan black in glycerine (10 %) (Johansen, 1940, modified) to identify lipids. The slides were mounted in water and photographed with the aid of a photomicroscope (AxioVision LE, Zeiss, Jena, Germany).

The lengths and widths of five cells were measured in the central region of the haustorium, 1 mm from the distal end. These cells are components of the ground meristem (in the case of the control treatment), but were differentiated into parenchyma in the case of germinated seeds. Measurements were performed on 20 structures per treatment using photomicrographs, with the aid of an image analysis program (Image-Pro Plus, Media Cybernetics, Rockville, MD, USA).

Quantification of H_2O_2

To quantify H_2O_2 we used the protocol described by Alexieva *et al.* (2001), which is based on the specific reaction between H_2O_2 and KI. The tissue samples were macerated in liquid nitrogen and four replicates (35 mg each) were homogenized in 0.1 % trichloroacetic acid and centrifuged at 10 000 *g* for 15 min at 4 °C; 500 µL of the supernatant was then added to 500 µL of sodium phosphate buffer (100 mM, pH 7.5) and 2 mL of KI (1 M) at 4 °C, and then kept in an ice bath in the dark for 60 min, followed by holding in a water bath at room temperature for 10 min. The samples were analysed in a spectrophotometer (UV-1800, Shimadzu, Tokyo, Japan) at 390 nm; the blank consisted of 0.1 % trichloroacetic acid, 100 mM sodium phosphate buffer (pH 7.5) and 1 M KI. The H_2O_2 concentrations in the samples were calculated based on a standard curve constructed using known concentrations of H_2O_2 subjected to the same procedures as those described here. The results were expressed in micromoles of H_2O_2 per gram of fresh mass.

Hormone quantification

Quantifications of hormones belonging to eight classes – ABA; GAs (GA_1, GA_4, GA_9, GA_{19}, GA_{20}, GA_{24}); CKs [isopentenyladenosine (IPA), 2-isopentenyladenine (2-iP), zeatin (Z), zeatin riboside (ZR), dihydrozeatin (DHZ), dihydrozeatin riboside (DHZR)]; BRs [brassinolide (BL), castasterone (CS), cathasterone (CT)]; indole 3-acetic acid (IAA); jasmonic acid (JA); salicylic acid (SA); and the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) – were performed according to Müller and Munné-Bosch (2011). Four replicates of 80 mg of the plant tissue samples were frozen in liquid nitrogen and pulverized in a cryogenic mill (MM400, Retsch, Haan, Germany). The solvent (methanol and 1 % glacial acetic acid) and the internal standards specific for each hormone (d_6-ABA, d_2-GA_1, d_2-GA_4, d_2-GA_9, d_2-GA_{19}, d_2-GA_{20}, d_2-GA_{24}, d_6-IPA, d_6-2iP, d_5-Z, d_5-ZR, d_4-ACC, d_3-BL, d_3-CS, d_3-CT, d_5-IAA, d_5-JA, d_4-SA; with d_5-Z and d_5-ZR being used as standards for DHZ and DHZR, respectively) were added to the samples, which were then homogenized in a vortex mixer and exposed to an ultrasonic bath. Samples were then centrifuged at 9500 *g* for 15 min at 4 °C and the supernatant was removed. The solvent was again added to the precipitate and the extraction procedure was repeated three times. The supernatant was filtered through a 0.22 µm PTFE membrane and analysed by ultrahigh-performance liquid chromatography coupled to electrospray ionization tandem spectrometry (Waters, Milford, MA, USA) using a HALO™ C18 column (2.1 × 75 mm, 2.7 mm) (Advanced Materials Technology, Inc. Wilmington, USA) with 0.05 % glacial acetic acid (solvent A) and acetonitrile with 0.05 % glacial acetic acid (solvent B). A standard curve of pure hormones was used for the quantifications, and the composite/standard ratio was calculated with the aid of the Analyst® program (Applied Biosystems, Foster City, CA, USA). The results are expressed as nanograms per gram of fresh mass.

Endo-β-mannanase activity

The enzymatic activity of endo-β-mannanase was determined according to Pinho *et al.* (2014), based on reactions between enzyme hydrolysis products with galactomannan locust bean gum (LBG) and *p*-hydroxybenzoic acid hydrazide (PHBAH). The samples (four replicates of 5 mg of tissue) were macerated in liquid nitrogen, and 1 M sodium acetate buffer (pH 4.7) was added to each powder, followed by vortex homogenization and centrifugation at 16 000 g for 45 min at 4 °C. The supernatant was then added to the enzymatic substrate (0.25 % LBG in 0.1

M sodium acetate buffer, pH 4.7), and held for 3 h at 40 °C; 5 % (w/v) *p*-hydroxybenzoic acid prepared in 0.5 M HCl was then added and stabilized with 0.5 M NaOH and the system was kept in a water bath at 95 °C for 5 min. The reactions were stopped by incubation at room temperature for 10 min, and the products of the reactions between the reducing sugars released by enzymatic action and PHBAH were evaluated at 398 nm (Cary 60 UV/Visible spectrophotometer, Agilent, Australia). A sample extract with the addition of only PHBAH was used as the blank. A standard curve was constructed by replacing the seed extracts with an endo-β-mannanase standard (Megazyme, Ireland). The results were expressed in terms of micromolar equivalent of reducing sugar per minute per gram of fresh sample mass.

Statistical analyses

Data were submitted to ANOVA and compared by the Tukey test (at the 5 % level of probability). At each growth period (2, 5 and 10 d) the control treatment was compared with seeds without their operculum using Student's *t*-test for independent samples (5 % probability).

RESULTS

Biometric evaluations

Butia capitata seeds have a thin seed coat, an abundant endosperm, and a falciform embryo located in the micropylar region adjacent to the operculum; the latter structure is formed by the opercular seed coat and micropylar endosperm, and it restricts germination (Fig. 1A). The embryo is composed of two regions: the cotyledonary petiole (which includes the embryonic axis), which is cylindrical, has a yellowish colour and is situated in a more external position; the haustorium is curved, has a whitish colour and is located internally. The embryos of dry seeds (water content of 4.9 %) partially occupy the seminal cavity and their contact with the endosperm is restricted. Immersion increases the water contents of the seeds and the haustoria to 29.5 and 60 %, respectively, causing the embryo to swell and expand and have greater contact with the endosperm (Figs 1A and 2A–C). Seeds maintained intact after imbibition (control treatments, i.e. seeds that remained dormant) did not germinate during the evaluation period and showed no changes in haustorium size.

Forty percent of the seeds with the operculum removed showed protrusion of the cotyledonary petiole (Fig. 1B) 2 d after sowing. These seeds will be referred to here as germinated seeds. Germination proceeded in 82 and 90 % of the seeds 5 and 10 d after sowing, respectively. Germinating seeds showed increased haustorium lengths, but the increase was not enough for the entire structure to come into contact with the endosperm (Figs 1B and 2B). Five days after sowing, germinated seeds showed increases in water content and the length of the haustorium; the surface of the haustorium was then in total contact with the endosperm (Figs 1C and 2A, B). Ten days after sowing, reserve mobilization could be confirmed by the formation of a digestion zone (having a soft consistency and a bright, translucent coloration) in the endosperm adjacent to the haustorium (Fig. 1D). Access to the endospermic reserves was associated with increases in the dimensions and dry mass of the haustorium (Fig. 2B–D), accentuated growth of the cotyledonary petiole and root emergence (Fig. 1D).

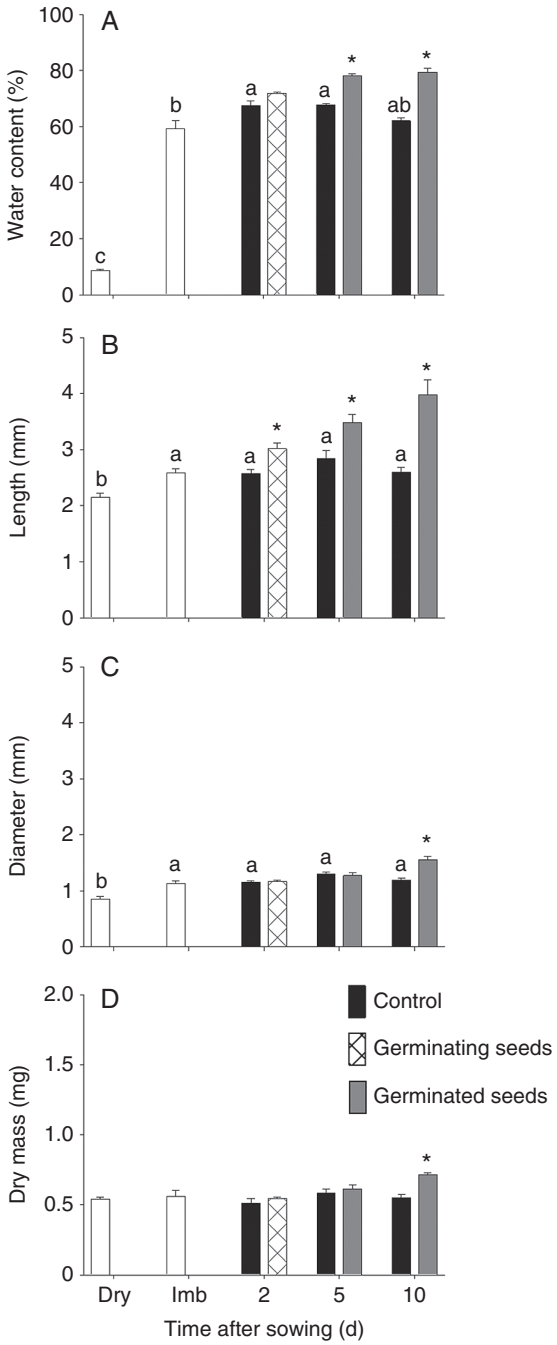

FIG. 2. Biometric evaluations of the haustorium of *Butia capitata* seeds. Water content (A), length (B), diameter (C) and dry mass (D) of the haustorium of dry (Dry) and imbibed (Imb) seeds, and seeds with intact operculum (control treatment, i.e. seeds that remained dormant) and without their operculum (germinating and germinated) 2, 5 and 10 d after sowing. Different lowercase letters indicate statistically significant differences in relation to seeds with an intact operculum (Tukey test, 5 % probability). Asterisks indicate differences between cultivated seeds of the control treatment and seeds without their operculum at each evaluation time (Student's *t*-test, 5 % probability). $n = 5$ replicates of ten seeds. Bars represent standard error of the mean.

Histochemical and micromorphometric evaluations

In dry seeds, both the embryonic ground meristem cells and the endosperm cells were filled with abundant protein, carbohydrate and lipid reserves. Proteins, which were predominant, were stored in protein bodies of varying sizes; lipids were stored in small, peripherally disposed lipid bodies (Fig. 3 and Supplementary Data Fig. S1). Carbohydrates were present in the cell walls and in peripherally disposed starch grains in the embryonic cells; starch reserves in the endosperm cells were present in the thick cell walls and large vacuoles; the cells also accumulated proteins. Adjacent to the haustorium were layers of collapsed endosperm cells, indicative of reserve mobilization in the final stage of embryogenesis.

Partial mobilization of the embryo protein and starch reserves occurred during imbibition, associated with the vacuolation of the cells (Fig. 3 and Supplementary Data Fig. S1). The carbohydrate reserves present in the glycoprotein vacuoles were mobilized in a narrow strip of endosperm adjacent to the haustorium, and the protein bodies merged to form large vacuoles. Two days after sowing, the mobilization of protein reserves in the haustorium intensified in the control treatment seeds, and there were discrete depositions of starch (Fig. 3 and Supplementary Data Fig. S2). There was also evidence of protein mobilization in the adjacent endosperm, with slight enlargements of the vacuolated cells. Lipid reserves remained intact, and there were no increases in the sizes of the collapsed cells. No significant changes were observed in relation to the previous phase 5 d after sowing (Fig. 3 and Supplementary Data Fig. S3), but the lengths of the cells of the fundamental meristem increased in relation to dry seeds (Fig. 4A). The mobilization of protein reserves in the germinating seeds advanced in the haustorium

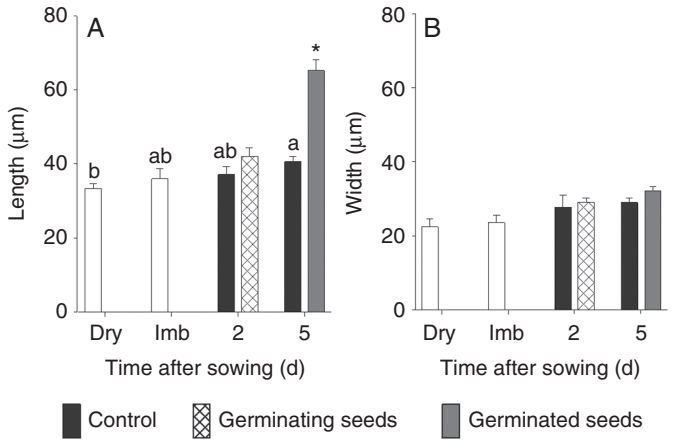

FIG. 4. Micromorphometric evaluations of the haustorium of *Butia capitata* seeds. Length (A) and width (B) of the cells of the ground meristem/parenchyma in the central median region of the haustorium of dry (Dry) seeds, imbibed seeds (Imb) and seeds with intact operculum (control treatment) and without the operculum (germinating and germinated) 2 and 5 d after sowing. Different lowercase letters indicate statistically significant differences in relation to seeds with intact operculum (Tukey test, 5 % probability). Asterisks show differences between cultured seeds of the control treatment and seeds without the operculum at each evaluation time (Student's *t*-test, 5 % probability). $n = 5$ replicates (cells) of 20 seeds. Bars represent standard error of the mean.

cells, associated with vacuolation and starch deposition (Fig. 3 and Supplementary Data Fig. S2). The size range of vacuolated cells had expanded in the adjacent endosperm, and mobilization of most of the lipid reserves was also evident. The accumulation of remnants of collapsed cell walls in the region of

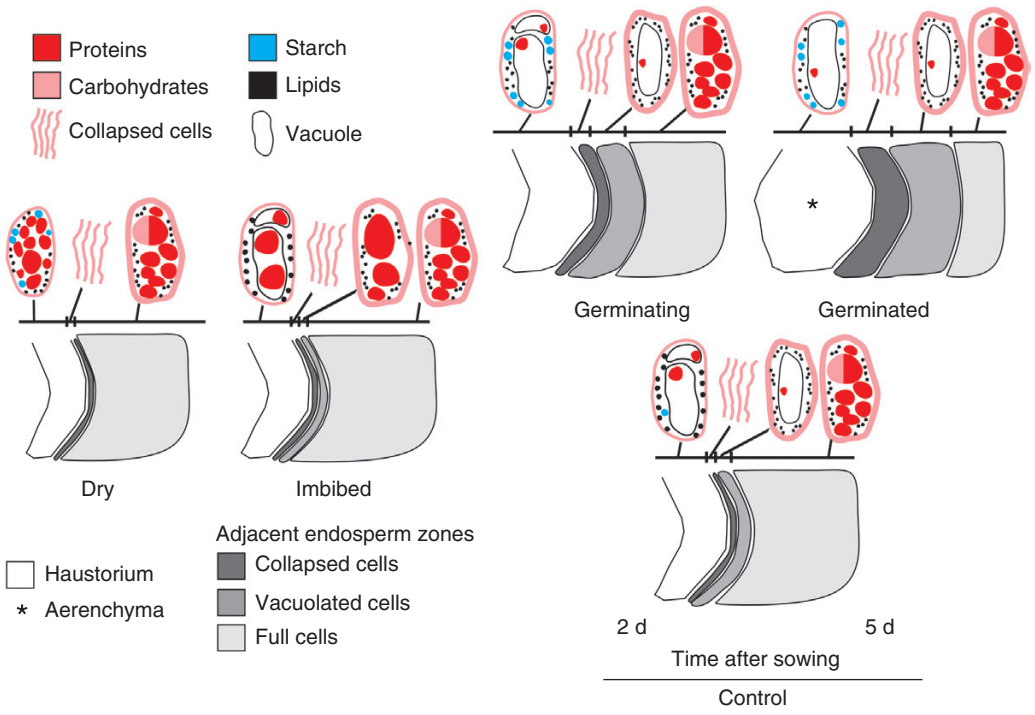

FIG. 3. Schematic representation of the cytological changes in the haustorium and endosperm, and the establishment of the endosperm digestion zone in *Butia capitata* seeds, based on histochemical evaluations (presented in detail in Supplementary Data Figs S1–3).

contact with the haustoria showed that final cell wall mobilization occurred after protoplast degradation. The collapsed cells in the control, imbibed, germinating and germinated seeds were identical, indicating that the cells observed near the haustorium in early germination stages later became degraded.

There were significant elongations of the haustorium cells in germinated seeds (Fig. 4A), although cell width did not show any significant variations (Fig. 4B). Cell growth in the haustorium was associated with the consumption of most of the protein reserves and part of the lipid reserves, continuing starch deposition and the formation of an aerenchyma of schizogenous origin (Fig. 3 and Supplementary Data Fig. S3). This pattern of reserve mobilization also occurred in the adjacent endosperm, with the enlargement of bands of cells showing different degrees of alteration of their reserve contents.

Quantification of H_2O_2

We detected H_2O_2 in the haustorium and the adjacent and peripheral endosperms of dry seeds (Fig. 5A–C). Concentrations of H_2O_2 were not altered significantly after imbibition and sowing in the control treatment seeds. Five days after sowing, the haustorium of germinating seeds showed increases in their H_2O_2 content; similar increases occurred in the adjacent and peripheral endosperms of germinated seeds.

Hormonal profile

There were reductions in the ABA contents of all three tissue structures after sowing in the control treatment seeds and in the haustoria of germinated seeds (Figs 6 and 7A–C). Increases in the concentrations of the main active GAs (GA_{at} = [GA_1]+[GA_4]) and reductions in the concentrations of the GA_4 precursors (GA_9, GA_{20}, GA_{24}) were observed in germinating and germinated seeds (Figs 6 and 7D). Concentrations of GA_{at} in the adjacent endosperm increased with imbibition (Fig. 7E), but no significant changes were observed in the peripheral endosperm (Fig. 7F). The GA_{at}/ABA ratios increased significantly in the haustoria of germinated seeds (Fig. 7G), a phenomenon related to cellular expansion (Fig. 4A) and to increases in the length of this structure (Fig. 2B). By the end of the evaluation period, the ABA contents in the haustoria of germinated seeds were 9.6 times lower (and the GA_{at}/ABA ratios 42.8 times higher) in relation to control treatment seeds (Fig. 7A, G). No significant changes were observed, however, in the GA_{at}/ABA ratio in the endosperm (Fig. 7H–I).

Increases in CK concentrations were responsible for most of the changes observed in the haustoria during the evaluation period (Fig. 6). Contents of Z and DHZ increased notably in both the control treatment and in germinating and germinated seeds. The CK concentrations in the haustorium of germinated seeds decreased, however, 5 d after sowing (Fig. 7J). There were no changes in CK concentrations in the endosperms with imbibition, but increases in the Z contents of germinating seeds were observed (Fig. 6). A predominance of CK concentration reductions was observed in the adjacent endosperm of germinated seeds in relation to control treatment seeds. The CK concentrations were not altered in the endosperm, except for a reduction observed in the peripheral endosperm of germinated seeds 5 d after sowing (Fig. 7K, L).

The BR concentrations increased in the haustorium and adjacent endosperm of control treatment seeds, but decreased in germinated seeds (Fig. 7M–O). The CS contents increased in the haustoria of control treatment and germinating seeds (Fig. 6). There were increases in the concentrations of CS and CT in the adjacent endosperms of control treatment seeds and of CT in germinating and germinated seeds.

Increases in the IAA contents in the haustoria were observed in all of the evaluations carried out, as well as in the endosperms of the germinated seeds 5 d after sowing (Fig. 6). Contents of ACC (the precursor of ethylene) increased in the adjacent endosperm of germinating seeds, but became reduced in the three

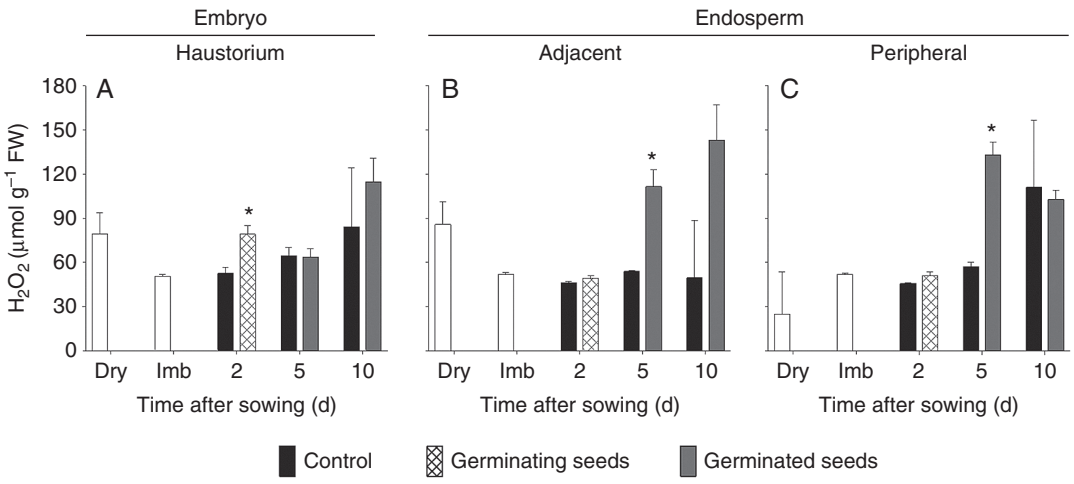

FIG. 5. Concentration of H_2O_2 in the haustorium and adjacent and peripheral endosperms of *Butia capitata* dry seeds (Dry), imbibed seeds (Imb) and seeds with an intact operculum (control treatment) and without the operculum (germinating and germinated) 2, 5 and 10 d after sowing. Different lowercase letters indicate statistically significant differences in relation to seeds with intact operculum (Tukey test, 5 % probability). Asterisks show differences between cultivated seeds of the control treatment and seeds without operculum at each evaluation time (Student's *t*-test, 5 % probability). n = 4 replicates of 35 mg. Bars represent standard error of the mean. FW, fresh weight.

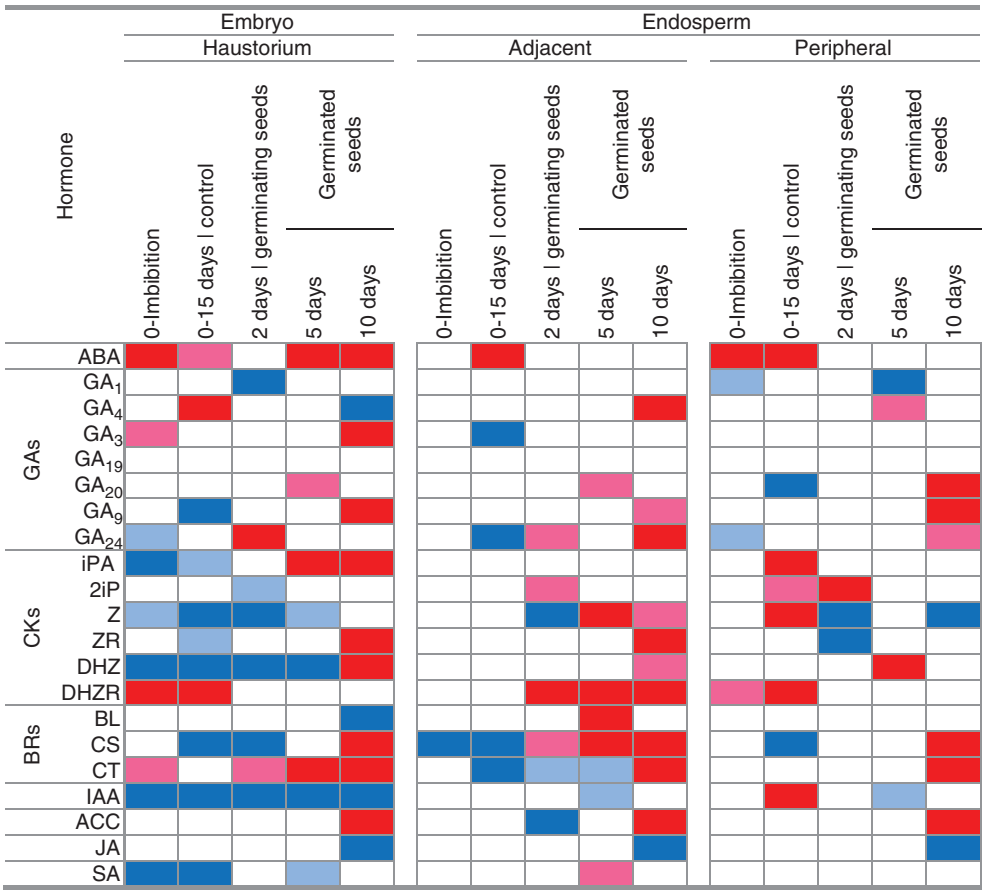

FIG. 6. Summary of hormone content changes in *Butia capitata* seed structures. Blue and red colours indicate positive and negative hormonal variations, respectively. Dark and light tones indicate significant differences at the 5 and 10 % probability levels (Student's *t*-test), respectively. Comparisons were made with each structure (haustorium, adjacent endosperm and peripheral endosperm) between dry and imbibed seeds (0–imbibition), dry seeds, and seeds at the end of the evaluation period in the control treatment (0–15 d), and between seeds with intact opercula (control) and without opercula (germinating and germinated) 2, 5 and 10 d after sowing. Averages are available as Supplementary Data Tables S1–3.

tissue structures analysed in germinated seeds 10 d after sowing. Unlike most hormones, JA increased in concentration in germinated seeds 10 d after sowing. Contents of SA increased in the haustorium of imbibed seeds, germinated seeds and in control seeds during the evaluation periods, but became reduced in the adjacent endosperm of germinated seeds.

In summary, imbibition, dormant seed culture and germination were related to increases in the GA/ABA ratios and CK, IAA and CS contents of the haustoria (Fig. 6, Supplementary Data Tables S1–3). Reserve mobilization activation during germination was associated with increases in the contents of Z, IAA and ACC in the adjacent endosperm. There were decreases in the hormone levels of all structures after establishment of the endosperm digestion zone in germinated seeds.

Endo-β-mannanase activity

Endo-β-mannanase was detected in the haustorium of dry seeds, principally in the adjacent and peripheral endosperms (Fig. 8A–C). After imbibition and sowing, there were no changes in the activities of this enzyme in the seed structures evaluated in the control treatment seeds. There were increases in enzymatic activity in the haustorium and in the adjacent endosperm of germinated seeds 10 d after sowing, but reductions in their peripheral endosperm.

DISCUSSION

The mobilization of seed reserves in the neotropical palm *B. capitata* involves interactions of distinct events in the embryo/seedling and endosperm in two developmental stages: germinating and post-germination. These processes are particularly complex as a consequence of the diversity of stored compounds, the participation of the highly specialized haustorium and the integration of two antagonistic developmental pathways: growth of the haustorium and reserve degradation and mobilization in the endosperm. A schematic summary of the main events associated with reserve mobilization in *B. capitata* seeds is presented in Figure 9.

A hard endocarp that surrounds the seeds and a seed coat rich in phenolic compounds are commonly found in the diaspores of palm trees, and they may hinder oxygen diffusion (Hussey, 1958; Debeaujon *et al.*, 2000; Benech-Arnold *et al.*, 2006; Carvalho *et al.*, 2015). With the displacement of the

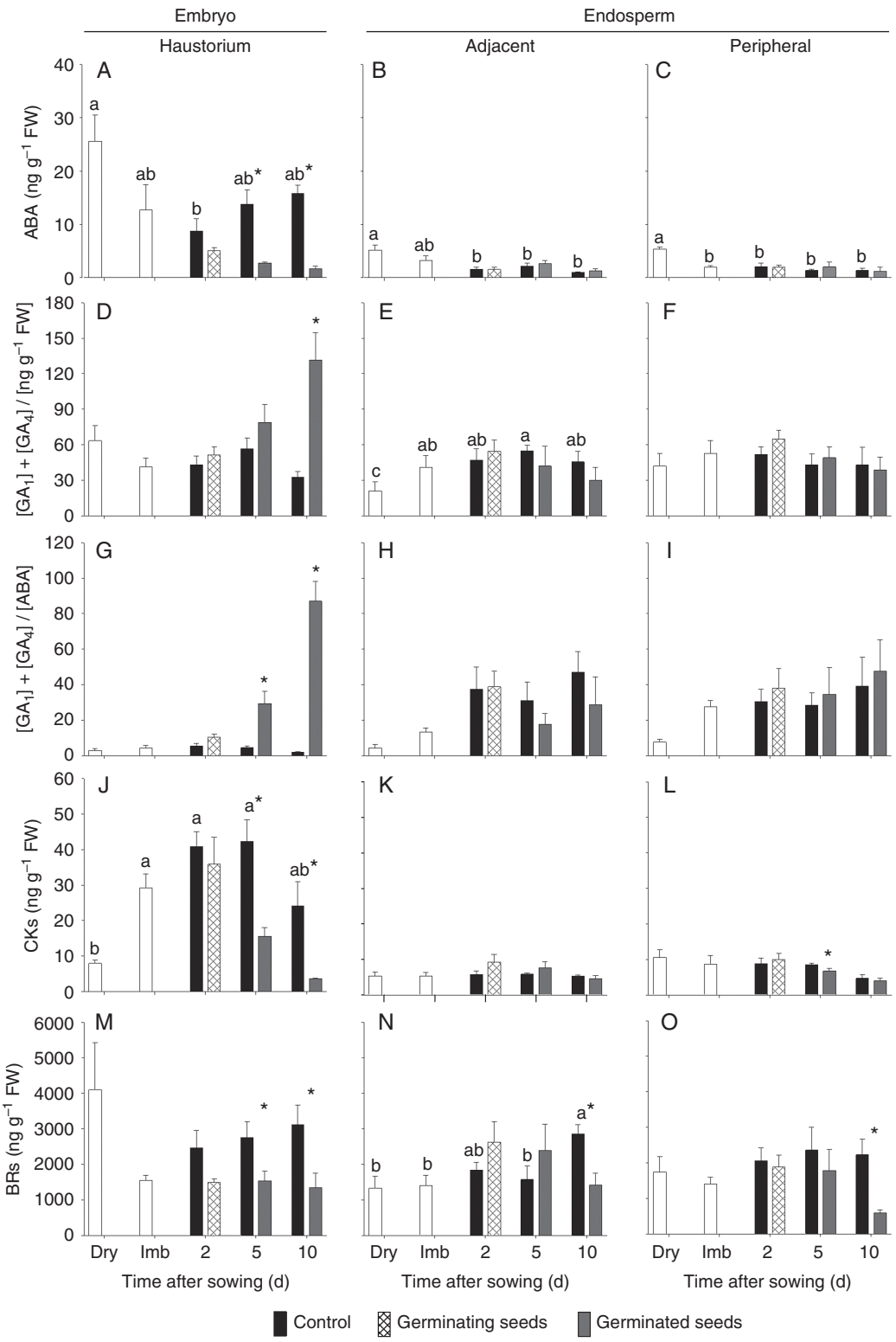

FIG. 7. Concentrations of ABA and main active GAs ([GA_1]+[GA_4]), main active GA/ABA ratios and concentrations of CKs ([iPA]+[2-iP]+[Z]+[ZR]+[DHZ]+[DHZR]) and BRs ([BL]+[CS]+[CT]) in the haustorium and the adjacent and peripheral endosperm of *Butia capitata* dry seeds (Dry), imbibed seeds (Imb), and seeds with intact operculum (control treatment) and without operculum (germinating and germinated) 2, 5 and 10 d after sowing. Different lowercase letters indicate statistically significant differences in relation to seeds with intact operculum (Tukey test, 5 % probability). Asterisks show differences between cultured seeds of the control treatment and seeds without operculum at each evaluation time (Student's *t*-test, 5 % probability). $n = 4$ replicates of 80 mg. Bars represent standard error of the mean. FW, fresh weight.

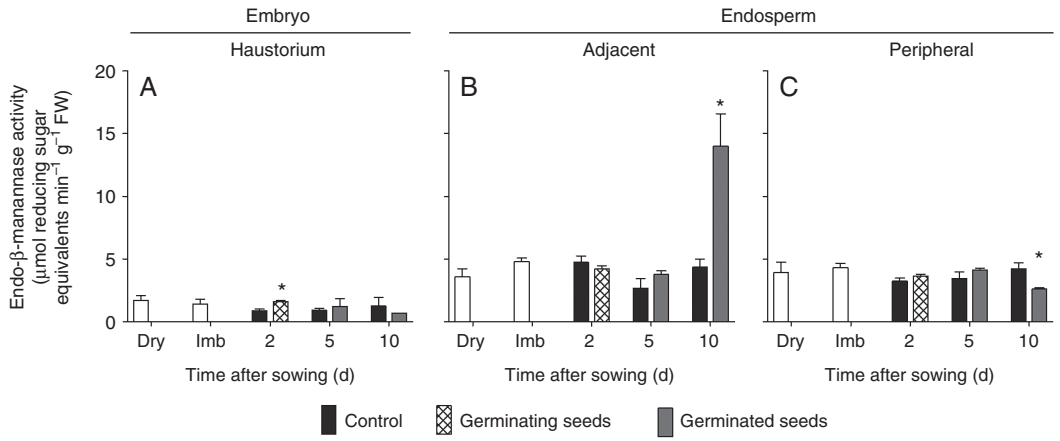

FIG. 8. Endo-β-mannanase activity in the haustorium and adjacent and peripheral endosperms of *Butia capitata* dry seeds (Dry), imbibed seeds (Imb) and seeds with intact operculum (control treatment) and without operculum (germinating and germinated) 2, 5 and 10 d after sowing. Different lowercase letters indicate statistically significant differences in relation to seeds with intact operculum (Tukey test, 5 % probability). Asterisks show differences between cultured seeds of the control treatment and seeds without operculum at each evaluation time (Student's *t*-test, 5 % probability). $n = 4$ replicates of 5 mg. Bars represent standard error of the mean. FW, fresh weight.

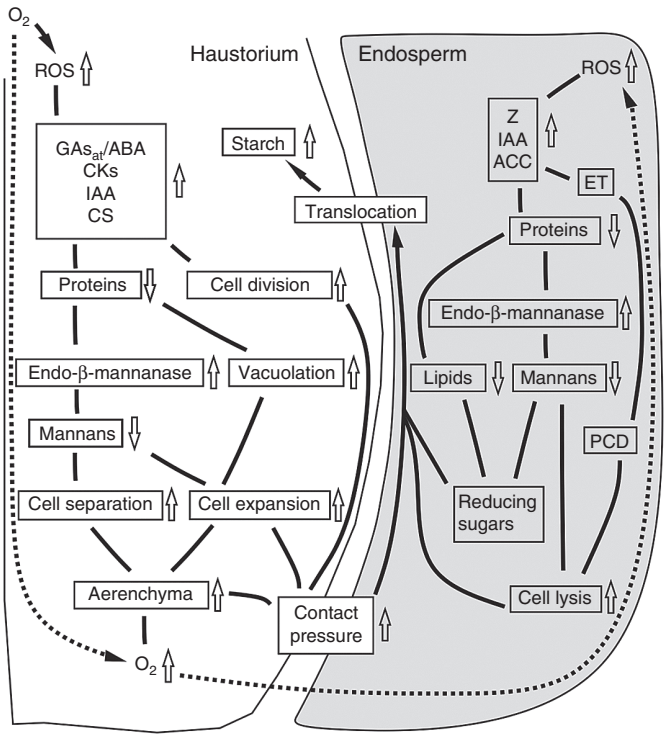

FIG. 9. Schematic representation of the main events related to reserve mobilization and the establishment of the endosperm digestion zone in *Butia capitata* seeds. Arrows pointing to the upper and lower sides (to the right side of the text boxes) represent, respectively, increases and decreases in the content of the compound, enzymatic activity or cytological changes. Dashed lines indicate the flow of O_2 into the haustorium, and from there to the endosperm. ET, ethylene; PCD, programmed cell death.

operculum at the conclusion of germination, the cotyledonary petiole comes into contact with the atmosphere (Fig. 1B), which allows direct O_2 penetration into the seed (Fig. 9). The existence of stomata in the cotyledonary petiole adjacent to the operculum and the development of large cell spaces resulting from the imbibition of embryos in the palm species *Acrocomia aculeata* and *Mauritia flexuosa* have been pointed out as evidence of the importance of O_2 diffusion in germination and reserve mobilization (Ribeiro *et al.*, 2012; Silva *et al.*, 2014). In the present work, ROS formation was observed in the haustorium of germinating seeds in the form of increases in H_2O_2 concentrations.

Alterations in the hormonal profiles of the haustoria of germinating and germinated seeds promoted a series of cytological changes that contributed to the establishment of an endosperm digestion zone (Fig. 9). The elevations of GA_{at}/ABA ratios and of CK, IAA and CS contents in haustoria typically occur as a result of ROS signalling (El-Maarouf-Bouteau and Bailly, 2008), while adjustments to CK/IAA ratios are related to cell cycle control and promoting cell division (Weyers and Paterson, 2001). Increasing GA_{at}/ABA ratios induce cellular expansion and separation through cell wall weakening due to the activity of hydrolytic enzymes (such as endo-β-mannanase), which occurs in association with vacuolation (Bewley *et al.*, 2013; Gómez-Maqueo and Gamboa-deBuen, 2016). Associations of those events in the haustoria of *B. capitata* promote the formation of aerenchyma, which facilitates O_2 diffusion. Aerenchyma formation, together with cell division, also contributes to the growth of the haustorium and increases in the contact surface area with (and pressure on) the endosperm, an aspect considered crucial in reserve mobilization and translocation in the seeds of *A. aculeata* (Mazzottini-dos-Santos *et al.*, 2017).

The formation of the aerenchyma in the haustorium favours O_2 diffusion to the adjacent endosperm, and increases in H_2O_2 concentrations were observed in this tissue in germinated seeds (Fig. 9). Reactive oxygen species have been shown to be important signalling agents for the mobilization of seminal reserves in several studies (El-Maarouf-Bouteau and Bailly, 2008; Gomes *et al.*, 2014; Verma *et al.*, 2015; Wojtyla *et al.*, 2016). Increases in the concentrations of the hormones Z, CT, IAA and ACC (an ethylene precursor), as observed here in the endosperm, are considered to be related to ROS signalling (Palma and Kermode, 2003; Bewley *et al.*, 2013). Auxins

are known to induce carbohydrate mobilization in legume seeds (Brandão *et al.*, 2009), and CKs likewise increase proteolytic activities and carbohydrate and lipid mobilization (Bewley *et al.*, 2013). There is also evidence that ethylene induces galactomannan mobilization (Tonini *et al.*, 2010) and the accumulation of ROS (which signal in the programmed cell death pathway) (Bailly, 2004; Gadjev *et al.*, 2008; Gomes *et al.*, 2014; Wojtyla *et al.*, 2016). We observed that these processes resulted in the mobilization of cell wall carbohydrates, extravasation of cellular contents, and the extension of the range of collapsed cells in the adjacent endosperm of germinated *B. capitata* seeds.

The enzyme endo-β-mannanase plays a central role in the establishment of the endosperm digestion zone because it is involved in the mobilization of abundant cell wall mannan reserves (Fig. 9); it has been shown to be involved in the mobilization of endosperm reserves in the palms *Phoenix dactylifera* (DeMason *et al.*, 1985), *Elaeis guineensis* (Alang *et al.*, 1988) and *A. aculeata* (Mazzottini-dos-Santos *et al.*, 2017). There is evidence that endo-β-mannanase is not synthesized in the haustorium, but is present in an inactive form in the endospermic protein bodies of that organ, and is activated through haustorium signalling (Mazzottini-dos-Santos *et al.*, 2017). Embryonic signalling, possibly through GA and CK, is involved in overcoming the ABA-imposed blockage of endo-β-mannanase activity in the endosperm of lettuce seeds (Bewley, 1997; Bewley *et al.*, 2013). The results obtained in the present work are in agreement with this proposal, since endo-β-mannanase activity was much lower in the haustorium than in the endosperm, and endosperm enzyme activity increased after the mobilization of these protein bodies.

Significant seedling growth was seen after the establishment of the digestion zone (Fig. 1D) and was associated with the onset of lipid degradation in the endosperm and with the accumulation of transient starch in the haustorium. The greatest negative hormonal changes were noted in seed structures during this period, and were expressed to a greater degree in the adjacent endosperm. The results presented here show that endospermic reserve mobilization is related to increases in the levels of several hormones (possibly associated with hormonal signalling), and that once this process is initiated hormone levels generally become reduced. It is important to note that increases in the levels of some hormones (GA_1, IAA, Z) and H_2O_2 in the peripheral endosperm may represent signalling for processes that favour subsequent mobilization in peripheral seed regions.

The results obtained in the present work indicated that seminal reserve mobilization in *B. capitata* involves the integration of growth and degradation pathways in the haustorium and adjacent endosperm, respectively. The mobilization of haustorium reserves is associated with germination and involves hormonal changes (especially the elevation of GA/ABA ratios) distinct from those seen in the endosperm. The mobilization of endospermic reserves is a post-germination event controlled by the seedling that involves structural changes in the haustorium, including its growth (which increases contact and pressure) and the formation of an aerenchyma, which facilitates O_2 diffusion. The establishment of a digestive zone in the adjacent endosperm is related to H_2O_2 production and Z, CT, IAA and ethylene hormones in degradation pathway processes that result in reserve translocation and the deposition of transient starch in the haustorium.

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/aob and consist of the following. Figures S1–3: images of longitudinal sections through the median and internal regions of the haustorium and adjacent endosperm of dry, imbibed, germinating and germinated seeds of *Butia capitata* submitted to histochemical tests for the identification of proteins, carbohydrates and lipids. Tables S1–3: tables presenting average values of endogenous hormones in the haustorium, adjacent endosperm and peripheral endosperm of dry, imbibed, germinating and germinated seeds of *Butia capitata*

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