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Seed and seedling anatomy in *Euterpe oleracea* Mart. during the germination process

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Abstract

The aim of the study was to investigate the seed and seedling anatomy, as well as to identify the reserve degradation pathway during the germination process in *Euterpe oleracea* seeds. Longitudinal and transversal sections of endosperm and embryo seeds, as well as transversal sections of different parts of the seedling were analyzed. The seeds are ruminated with the endosperm formed by cells of several sizes and forms, as well as by presenting very large walls and pits. The embryo is formed by a hypocotyl-radicle axis, procambium and parenchyma with several idioblasts composed of raphides. The seed endosperm has the hypocotyl base and haustorium as sites of starch accumulation. The results obtained by scanning and transmission electron microscopy demonstrate that the endosperm cells present several pits with plasmodesma connections and the haustorium can regulate the endosperm degradation process. Seedling sections between the endosperm and haustorium indicate that the testa projections can have the function of transport during the degradation process of the endosperm reserves.

***Key words*:** *Euterpe oleracea* Mart., seed, endosperm, reserve, degradation.

Introduction

The species *Euterpe oleracea* Mart. is considered the most important of the genus *Euterpe*, being found in the entire Amazon region, but mainly in the states of Pará, Amazonas, Maranhão and Amapá [10]. This palm presents great economic importance, where the food industry uses the fruit to produce sub-products like energetic drinks, ice creams, juices and yogurts. In addition, the leaves are used as decorations in houses and palm hearts can be found in salads and diet bases [21].

Euterpe oleracea fruits are drupes with a green or lilac pericarp, when immature and mature, respectively, as well as presenting a fibrosis mesocarp, composed by one seed with a spherical format [10, 16]. The morphological characteristic constant in these palm fruits are abundant endosperms and small embryos [1, 19].

The seed morphology in *Euterpe oleracea* was investigated by Costa *et al*. [10], which indicated that the endosperm is ruminated, solid and constituted by large cell walls and several pits. Studies conducted by Dassanayake and Sivakachchan [11] on *Borassus flabellifer* L. suggested that the endosperm is formed by two regions, one extreme and another central, in which it presents different characteristics, as disposition and cell size.

Morpho-anatomy studies with other palm seeds were undertaken aiming at responding linked with embryogenesis and germination [2,15,22], as well as some experiments explaining the degradation process and chemical composition of the endosperm of palm seeds [20]; however, the anatomy aspects are little explained in *Euterpe oleracea*.

The aim of the study was to investigate the seed and seedling anatomy, as well as identify the reserve degradation pathway during the germination process in *Euterpe oleracea* seeds.

Materials and Methods

Plant material and growth conditions: *Euterpe oleracea* seeds were immersed in containers with vermiculite as substrate and placed in growth chambers under a photoperiod of 12 hours of light, photosynthetic active radiation (PAR) of 60 mol/m²/s and temperature 30 ± 3°C.

Evaluation period and section localizations: On days 0, 10, 25, 50, 75 and 100 after the experiment implementation, the germination and development stages were evaluated and photographed, where we considered germinated those seeds presenting protrusion of the hypocotyl-radicle axis. The transversal sections were carried out in the seed endosperm, haustorium, hypocotyl base and eophyl at a distance of 1 cm from the distal part, using only seedlings 50 days old (Fig. 1). Furthermore, the biological material was stained with toluidine blue at 1% and Lugol's solution containing iodine, potassium iodine and ultra pure water in the proportion 1:3:300 (v/v) for 10 min.

Cell capture: The endosperm and haustorium of the seedlings were obtained on the 50th day after experiment implantation, in which they were incubated in liquid nitrogen and softly macerated

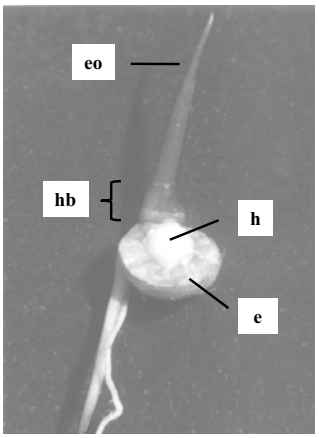

Figure 1. *Euterpe oleracea* seedling. The transversal section presents the haustorium (h), endosperm (e) hypocotyl base (hb) and eophyl (eo).

in water. Subsequently, the separation of some cells was carried out through a nylon membrane with 60 μm of diameter and placed into a 1 ml microtube. The cells that remained on the membrane were stained with toluidine blue for 10 min. These samples were used to make the slides, for subsequent visualization under light microscopy.

Transmission electron microscopy: The samples of the dry seed endosperm were removed and fixed in Karnovsky solution for 24 hours [17]. Subsequently, the samples were washed 3 times in cacodylate buffer, for 10 min each time and afterwards, the materials were fixed in osmium tetroxide solution at 2% (v/v) for 6 hours. Immediately after the fixation process, the samples were dried with acetone solutions at 25, 50, 75, 90 and 100% (v/v) for 10 min in each solution, as well as to finalize the process the samples were immersed in pure acetone 3 times. Following, the materials were submitted to Spurr resin and acetone under concentrations of 30, 70 and 100% (v/v). Consecutively, the blocks containing biological material and resin were sliced with an ultramicrotome, plus the cuts were submitted successively to uranile acetate 3% (v/v) and lead acetate 3% (v/v) for 3 minutes each. These cuts were viewed in a transmission electron microscope (Zeiss, EM-109).

Scanning electron microscopy: The endosperm and haustorium samples were removed from the seedlings on the 50th day after experiment implementation, in which they were immersed in fixative solution with a 7.2 pH for 24 hours [17]. Afterwards, the samples were transferred to a solution with osmium tetroxide at 1% (v/v) for 1 hour and subsequently dehydrated with acetone in 6 concentrations (30%, 50%, 70%, 90%, 100% only 1 time and 100% by 3 times), as well as immediately conduced to critic point system (BAL-TEC, model CPD 030). Additionally, the samples were covered with gold through a gold evaporator (BAL-TEC, SCD 050) and viewed in a scanning electron microscope (LEO EVO, 40XVP).

Results

Embryo constitution and organization: The embryo in longitudinal (Fig. 2A) and transversal (Fig. 2B) sections is constituted by a hypocotyl-radicle axis, procambium that expands in 6 lines and subsequently, opposite to the hypocotyl-radicle axis, forms approximately 30 lines of procambium in the haustorium. Fig. 2C shows that the pits of adjacent endosperm cells do not present plasmodesma connections. Despite there being a reduction in the wall thickness in regions with pits, there is a very considerable cell portion occurring between the lamella media and cytoplasm content of adjacent cells (Fig. 2D).

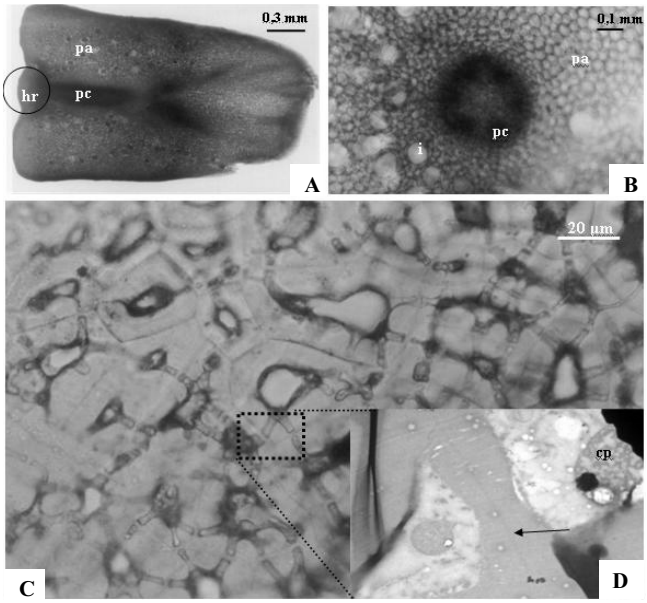

Figure 2. Longitudinal section of the *Euterpe oleracea* embryo (A), where it is shows the hypocotyl-radicle axis (hr), procambium (pc) and parenchyma (pa). Transversal section of the embryo in the hypocotyl region (B) showing the procambium (pc), parenchyma (pa) and idioblasts (i). Transversal section of the endosperm, in which the rectangle presents several pits (C). The detail of two pits between adjacent cells (D), showing the protein body (cp), arrow indicates the lamella media.

Endosperm cell structure: The endosperm cells are long, with different forms and a very large cell wall, presenting several pits, covering a large part of the total cell volume (Fig. 3A). The isolated cells of the endosperm present the cytoplasm content in dark color and cell walls, rich in pits, in white, confirming that these have no plasmodesmata (Fig. 3B), since the cytoplasm contents probably are lost to the external environment during the maceration and isolation processes. In addition, the form of the cytoplasm cells in the endosperm is characterized as extended and containing several pits.

Seed germination: Evaluating the germination and development phases proves that the seed presents a conical and small embryo, as well as a solid and large endosperm (Fig. 4). On the 10th day after implementing the experiment, the seeds were considered germinated due to the protrusion of the hypocotyl-radicle axis. In addition, on the 20th day the rootless emission and initial development of the haustorium into the endosperm occurred. On the 50th day, the emission of secondary roots with the presence of two sheaths and one more developed haustorium was observed, where the haustorium occupied more than 50% of the endosperm. On the 75th day, the eophyl broke the second sheath and the

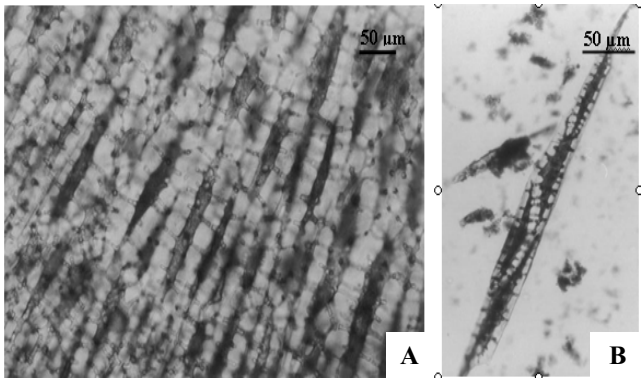

Figure 3. Longitudinal section of the endosperm in *Euterpe oleracea* seed (A). Isolated cells of the endosperm (B).

haustorium continued to develop with more than 60% of the endosperm volume, as well, in this stage, the testa projections forming lines that outline the endosperm extremity to the proximities of the center is clearly viewed. On the 100th day, the first leaf emerged which partially consumed the endosperm, in which the haustorium now occupied all of the area that was used previously by the endosperm. Apparently, this moment marks the independency of the seedling in relation to seed endosperm.

Testa rumination: The seed testa is ruminated shown by the projection into the endosperm's interior, as well as forming cell lines. In the seed endosperm these lines originate from the extremity to the centers, being visible in the seedling at 75 days old, when the haustorium is very developed (Fig. 4).

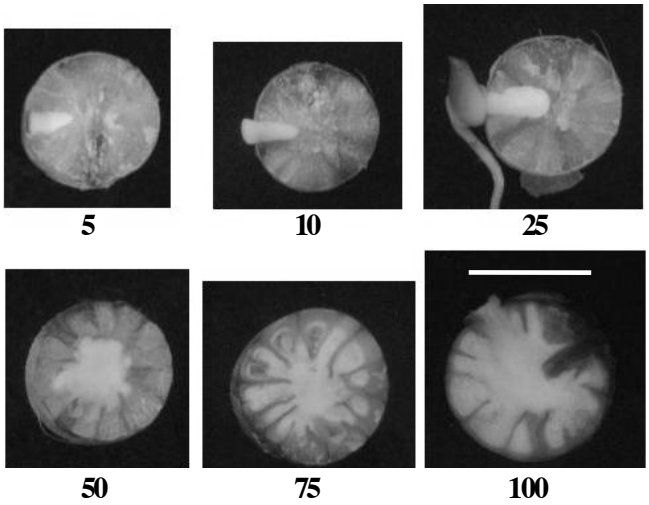

Figure 4. Development phases of the haustorium in *Euterpe oleracea* seed in the 0, 10th, 25th, 50th, 75th and 100th day after implementing the experiment. The arrow is equal to 1 cm.

Haustorium and endosperm cells: The isolated cells of the haustorium (Fig. 5A) and endosperm (Fig. 5B) were intensely stained, evidencing large amounts of starch. The haustorium cell walls were very slim and starch grains were seen in the interior. The endosperm cells were irregular, with thick walls and an intensely stained cytoplasm. In addition, the starch accumulated in the haustorium cells is coming from starch degradation and walls of endospermic cells, where it probably kept the embryo axis growing.

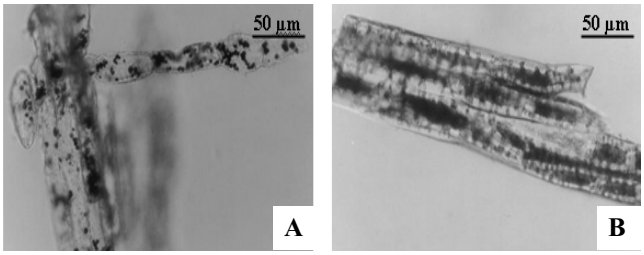

Figure 5. Isolated cells of the haustorium (A) and endosperm (B) of a seedling aged 50 days after experimental implementation.

Hypocotyl base: The central region of the hypocotyl base of the seedlings is also a site of high starch accumulation as shown in Fig. 6.

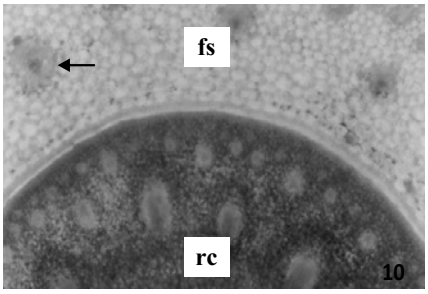

Figure 6. Transversal section of the hypocotyl base, where the central region (rc) and first sheath (fs) with several vascular lines (arrow) are presented.

Scanning electron photo-microscopy of the endosperm: A part of the seed endosperm is presented in Fig. 7A, where the haustorium was removed showing the haustorium cavity, in addition to Fig.7B which presents the removed haustorium. Furthermore, Fig. 7C presents in detail the rest of the endosperm cells, with several regions with intact endosperm cells and other partially degraded endosperm cells. Therefore, the structures observed confirm that the haustorium controls endosperm degradation and this process occurs from the center to the extremity. Besides this, the endosperm region connected with the haustorium was partially degraded, where its central portion was totally consumed and taken up by the haustorium.

Endosperm degradation: In the transversal section of the seed endosperm on the 50th day after implementing the experiment, four endosperm regions under different degradation stages were observed, one degraded totally, one degraded partially, one under an initial degradation process and another not affected (Fig. 8).

Reserve mobilization in the endosperm and haustorium: Fig. 9A presents the endosperm portion partially degraded between the testa projection, where endosperm degradation can occur, initially, in endosperm cells found in the central endosperm portions covered by the testa projections, as well as continuing until reaching the projections, which are not degraded. Fig. 9B presents the haustorium portion after endosperm degradation; showing the externally developed vascular system and promoting direct contact with testa projections. The degradation of endosperm cells and distribution of the haustorium vascular tissue is

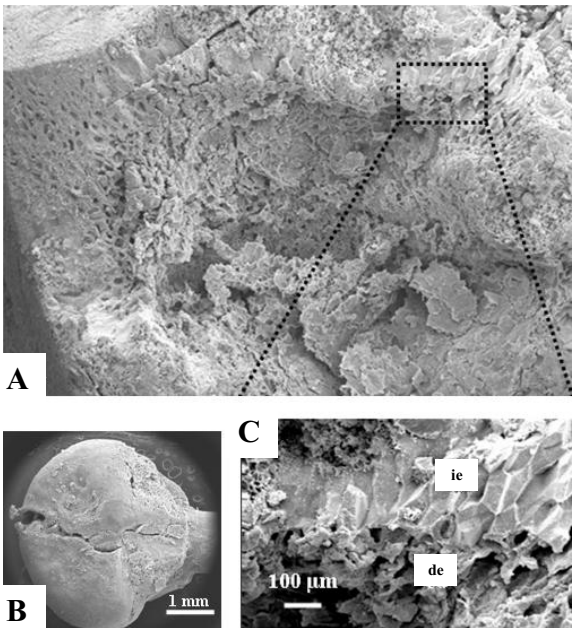

Figure 7. Scanning electron photo-microscopy of part of the endosperm. Cavity formed after the haustorium to be removed (A). Isolated haustorium (B). Detail of the region in transition between degraded and intact areas (C), where a degraded endosperm (de) and intact endosperm (ie) are presented.

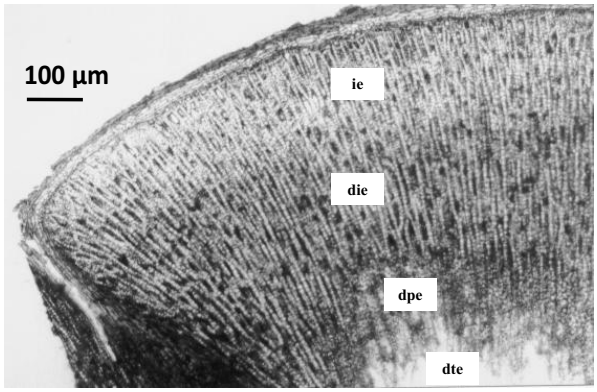

Figure 8. Transversal section of the endosperm in a seedling, presenting a totally degraded endosperm (tde), partially degraded endosperm (dpe), initially degraded endosperm (die) and intact endosperm (ie).

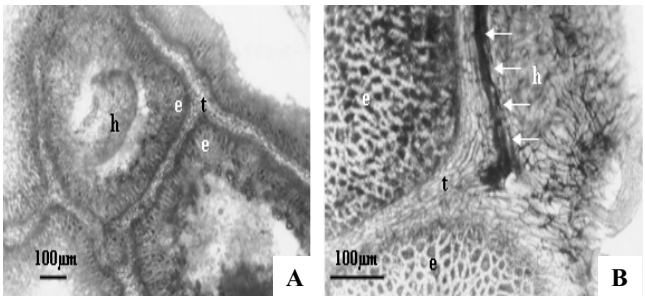

Figure 9. Transversal sections of the endosperm (A) and haustorium (B) portions, where the testa (t), haustorium (h) and the endosperm (e) are presented. The arrows indicate the vascular system.

influenced by the projections, as well as having direct contact with the endosperm cells. These results suggested that the testa projections might present some function in the transport of substances coming from the endosperm degradation to the conductive tissue of the haustorium.

Discussion

The non-existence of plasmodesma connections between the endosperm cells in *Euterpe oleracea* seeds induces the degradation process of reserves contained in walls and consequently this occurs gradually in haustorium proximities in development. With the wall degradation in pit regions, the enzymes responsible for mannan degradation (mannanase and mannosidase) might change for other cell layers and consequently begin degradation. Therefore, this process probably occurs from the center to the extremity and is controlled by the haustorium. The hypothesis of plasmodesma connections occurrence is not consistent since it provokes generalized degradation, in which the process is initiated in a more internal layer and conducted to more external layers, consequently promoting wall breakdown. In addition, the haustorium does not have the capacity to assimilate the sugar liberated from the endosperm wall degradation. The *E. oleracea* embryo presents structures similar to *Phoenix dactylifera* embryo [12], as well as having a reserve parenchyma rich in idioblasts and raphides. Moreover, a study conducted by Volk *et al*. [23] proves the degradation of calcium oxalate crystals in conditions of calcium deficiency.

The endosperm cell wall of the studied species is probably constituted by mannan, like in palms investigated by Meier and Reid [18], in which it is a polysaccharide wall formed by an extensive linear group of mannose in connections β-1,4, partially without ramifications. In seeds, the mannan promotes mechanical resistance and consequent higher embryo protection, besides, it acts like a carbohydrate reserve for seedling nutrition during the development [7]. Similar results on endosperm cells were found by Chandra Sekhar and DeMason [8], which proves that the endosperm cell walls of *Phoenix dactylifera* seed can reach approximately 65% of the total cell volume and present a great number of the protein bodies with reduced cytoplasm content and few mitochondria and plastids.

The anatomic study of the seedlings presented the main sites of starch accumulation as the endosperm and haustorium, where they fill almost 50% of the endosperm volume, besides the hypocotyl base. De Paula [14], studying the *Euterpe oleracea* anatomy, describes these tegument projections being 8 to 13 cell layers wide and having fine walls rich in reserve substance. Bayer and Appel [3] confirm that one seed can be ruminated due to the existence of endosperm or irregular tegument, in which the irregular tegument can occur in the same palms, where the tegument is irregularly developed within the endosperm. However, it has not been established that the endosperm rumination and/or testa have the same function during seed development or germination. Boesewinkel and Bouman [6] suggested that endosperm rumination could facilitate the entrance of water, oxygen and/or nutrients coming from the testa due to an increase in the surface contact between the testa and the endosperm.

The monocotyledonous seeds are normally albuminous and the germination is hypogeal. However, species from the Arecaceae family (palms) present abnormal behavior during germination, in which the distal portion of the cotyledon remains in the seed and

due to expansion, this structure works as a haustorium, absorbing the endosperm reserves and occupying this site. This process is followed by the production of endo-ß-mannanase and ß-mannosidase [5], these are enzymes that degrade the mannan.

In agreement with Chandra Sekhar and DeMason [9], the *Phoenix dactylifera* haustorium has two functions, primarily to secrete hydrolytic exoenzymes into the endosperm and secondly, to absorb products coming from endosperm degradation. This probable excretion of hydrolytic enzymes to the endosperm through the embryo is compatible with the observation that *P. dactylifera* embryo cells have more than half of their cell volume covered by protein bodies [8]. This characterizes an amino acids reserve that can be used during enzyme synthesis to be excreted to the endosperm; other investigations will be conducted to elucidate this process.

The polysaccharide hydrolysis of endosperm walls in *Phoenix dactylifera* occurs due to exoenzyme liberation by the haustorium, where mannan is also transformed to mannose, when it is absorbed by the haustorium and transported to the embryonic axis, where it is converted to sucrose [4]. Furthermore, these researchers report that the larger part of mannose is accumulated in the secondary wall. However, results obtained by Chandra Sekhar and DeMason [9] described that the wall of the *P. dactylifera* endosperm cell is primary. During our study, we did not define if the endosperm cell was primary or secondary. Moreover, in lettuce seeds, the mannan mobilization of the endosperm cell walls is promoted by the mannanase synthesis from the endosperm.

Results obtained by DeMason *et al*. [13] studying *Phoenix dactylifera* germination revealed that the haustorium cannot be responsible for the production of the ß-mannanase and ß-mannosidase enzymes, which might be active in the endosperm cells by haustorium signalization. In addition, these researchers also suggested that the content of each endosperm cell is initially mobilized, followed by cell mobilization, then the cell wall digestion of each endosperm cell occurs from the cytoplasm to the middle lamella. The endosperm cell walls in *P. dactylifera* seeds degrade from the center to the ends, an important evidence of the pit functions in endosperm cells of *Euterpe oleracea*. It is probable that pits facilitate the entrance of endo-ß-mannanase coming from the haustorium because a wall presenting pits is thicker than a wall which does not contain them. After entering the cell, the endo-ß-mannanase begins the cell wall degradation process from the center to the ends through wall degradation of the thinner pit regions.

The irregular form of the walls can be important during the degradation of wall polysaccharides during germination, due to an increase in the surface contact between endo-ß-mannanase and the main constitute in the endosperm cell, mannan. Studies conducted by DeMason and Thomson [12] on *Phoenix dactylifera* proved that this species has only one cotyledon, originating from the hypocotyl-radicle axis. The cotyledon presents a protodermis, reserve parenchyma and procambium. The procambium system is formed by one line in the hypocotyl that expands within the ring with 7 to 8 lines, continuing the division process until forming 49 or more lines separated at the end of the cotyledon.

The existence of a continuous pro-vascular system in embryo of *Euterpe* gender is common, using rootless and hypocotyl until to reach the cotyledon, however, this system presents no connections with the seed testa. The vascular system can remain meristematic, as a procambium in mature embryo or change into vascular elements during some development stages. For the embryos that depend on reserves present in external tissues to germinate, the cotyledon is the organ that has the absorption function for these reserves. In addition, the cotyledon of several monocotyledons suffers modifications, originating the haustorium, a process common in palms. The haustorium vascularization in *Euterpe oleracea* and the tendency of this tissue to develop in the testa projection proximities, they are characteristics that suggest that the seed presents a very efficient system for degrading endosperm reserves.

The sugar transport coming from the endosperm degradation to the haustorium is little explained in palms. Results obtained by Zambou and Spyropoulos [24] with *Trigonella foenum-graecum* L. legume indicate that D-mannose is partially transported by a transporter that is specific and not by the system of co-transport sugar/H^+, in which this transporter undertakes an important role during the activation process of the regulation in *T. foenum-graecum* cotyledon during the seedling development.

Conclusions

This study over seed and seedling anatomy of *Euterpe oleracea* reveals that the embryo is formed by a hypocotyl-radicle axis, procambium and parenchyma with several idioblasts composed of raphides. The seed endosperm has the hypocotyl base and haustorium as sites of starch accumulation. Scanning and transmission electron microscopy demonstrate that the endosperm cells present several pits with plasmodesma connections and the haustorium can regulate the endosperm degradation process. Other results found suggesting that seedling sections between the endosperm and haustorium indicate that the testa projections can have the function of transport during the degradation process of the endosperm reserves.

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