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Seed structure and histochemistry in the palm *Euterpe edulis*

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The *Euterpe edulis* embryo consists of a prominent single cotyledon, a very short radicle-hypocotyl axis and an epicotyl. The epicotyl is obliquely angled with respect to the cotyledon; consequently it corresponds to one of the two categories recognized for palm seeds by DeMason (1988). Parenchyma, protoderm and procambium can be distinguished on the basis of position and shape of their cells, which are highly vacuolated with one central vacuole and the cytoplasm restricted to a thin parietal layer. Initial cells from both apical meristems are also vacuolated but they have small vacuoles distributed around the nuclei. Silica occurs in cell walls of some protodermal cells. Raphides, silica bodies and tannins all occur occasionally in vacuoles, especially in the basal cotyledon region. Most embryo cells lack storage reserves and exhibit an active state, with numerous mitochondria, RER cisternae and Golgi apparatus, indicating a strategy of continuous development without the interposition, at maturity, of a dry state. The endosperm consists of living cells with very large nuclei and thickened cell walls. Similar to the endosperm of other studied palm species, their cells exhibit a quiescent appearance with lipid, protein, minerals (in the cytoplasm) and mannans (in the cell walls) as the insoluble storage reserves. *Botanical Journal of the Linnean Society*, 2004, **145**, 445–453.

ADDITIONAL KEYWORDS: embryo – minerals – mitochondria – palmito – phytoferritin – protein bodies – recalcitrance – vacuole.

INTRODUCTION

Although the structural features of seed in only a few palm species have been reported (Meier, 1956, 1958; DeMason & Thomson, 1981; Alang, 1981; Meier & Reid, 1982; Alang, Moir & Jones 1988), it is possible to determine some common characteristics in the family. The two areas of storage reserves in a palm seed are the massive, hard endosperm and the cotyledon of the small embryo. The stored reserves in the endosperm are lipids, proteins and hemicelluloses. The lipids and proteins are present in the cytoplasm in the form of protein and lipid bodies. The hemicelluloses, mainly mannans, are in the form of thickened cell walls. In the embryo, the cotyledon is the area of stored reserves and contains lipid bodies and protein bodies, the last with enclosed globoid crystals. To date, detailed histochemical studies of the embryo axis have not been described in any palm species.

Studies on conservation of *Euterpe edulis* seeds, carried out by Bovi & Cardoso (1978), de Queiroz & Cavalcante (1986), Reis *et al*. (1999), Martins *et al*. (2000), Andrade (2001) and V. Panza *et al*. (unpubl. data), have shown that the mean moisture content of the whole seed is 48.5% (wet weight) and 85.3 and 48.2% (wet weight) for isolated embryo and endosperm, respectively, recognizing their recalcitrant behaviour.

In this paper we study the structural and ultrastructural characteristics of the mature seed of *Euterpe edulis* using light and transmission electron microscopy (LM and TEM). We also determined the nature of the main food reserves using histochemical methods and Energy Dispersive X-ray (EDX) analysis. In order to find some differences between recalcitrant

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vs. orthodox palm species, we compared *E. edulis* with *Phoenix dactylifera* L. and *Washingtonia filifera* (Lindl.) Wendl., both orthodox. Finally, we concluded that the embryo tissue structure indicates a strategy of continuous development without the interposition, at maturity, of a dry state. This is part of a comprehensive study on seed conservation in *E. edulis.*

MATERIAL AND METHODS

Mature fruits of *Euterpe edulis* Martius were harvested from trees growing in the Parque Nacional Iguazú, Provincia de Misiones Argentina, during the month of August of three consecutive years, from 1999 to 2001, and shipped by expedited post to the Bank of Germplasm of INTA, Castelar, Buenos Aires, where the studies were conducted.

For histochemical and ultrastructural studies, ten seeds were analysed from each year. Cotyledon and axis were processed for TEM separately. Sections (1 mm thick) of both were fixed overnight in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, at 4 °C. For TEM, tissues were postfixed in 1% OsO_4 in water for 2 h, then dehydrated in a graded ethanol-propylene oxide series and embedded in Spurr's resin (Hawes, 1994). Sections were mounted on grids, stained in uranyl acetate followed by lead citrate, and examined in a Zeiss EM109T transmission electron microscope. For histochemical studies, semithin sections embedded in wax (Harris, Spence & Oparka, 1994), were stained with toluidine blue O (Sigma T 3260 CI 52040), fast green FCF (Sigma F 7252 CI 42053) and anilino-8-naphthalene sulphonic acid (Sigma A 8142 CI 82-76-8) for detecting proteins (Feder & O'Brien, 1968; Yiu, Altosaar & Fulcher, 1983). The presence of starch and lipids was determined on fresh sections with iodine potassium iodide (Sigma L 6146) and Sudan black B (Sigma S 2380 CI 26150), respectively (Harris *et al.*, 1994). Tannins were identified in tissues postfixed in 1% OsO_4, as dense deposits in vacuoles; tannins were also visible as blue deposits with 1.0% ferric chloride in 0.1 M HCl (Harris *et al.*, 1994). The thickened cell walls of the endosperm stained with the PAS reaction and fluoresced after application of calcofluor white (DeMason, 1986).

For EDX analysis, fresh sections of mature seeds (five seeds from each year) were analysed. The analysis was carried out using a Philips XL 30 ESEM. Globoid crystals, raphides, silica bodies and vacuoles were analysed for 60 s at an accelerating voltage of 20 kV and a count rate of between 1500 and 2000 cp.

RESULTS

The main area of food reserves in *E. edulis* seeds is the massive endosperm (Fig. 1). The embryo consists of a large single cotyledon, a short radicle-hypocotyl axis and an epicotyl (Figs 2–6). The axis is curved, with the epicotyl obliquely angled with respect to the cotyledon (Figs 5, 6). The shoot apical meristem (SAM) and root apical meristem are well differentiated (Fig. 6). Two or three leaf primordia cover the SAM (Fig. 5). A small slit is evident in the base of the cotyledon under which the SAM is located (Fig. 5). In the cotyledon, two regions can be differentiated, i.e. apical and basal regions (Fig. 2). During germination, the basal region emerges as a germinative button prior to the emergence of the radicle. The apical region develops, during germination, in a haustorium.

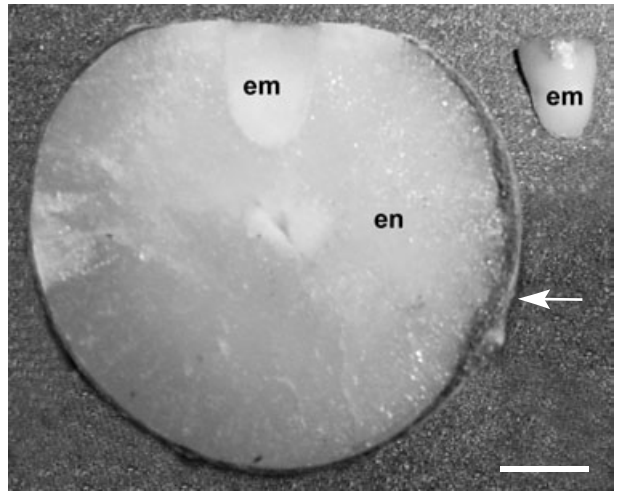
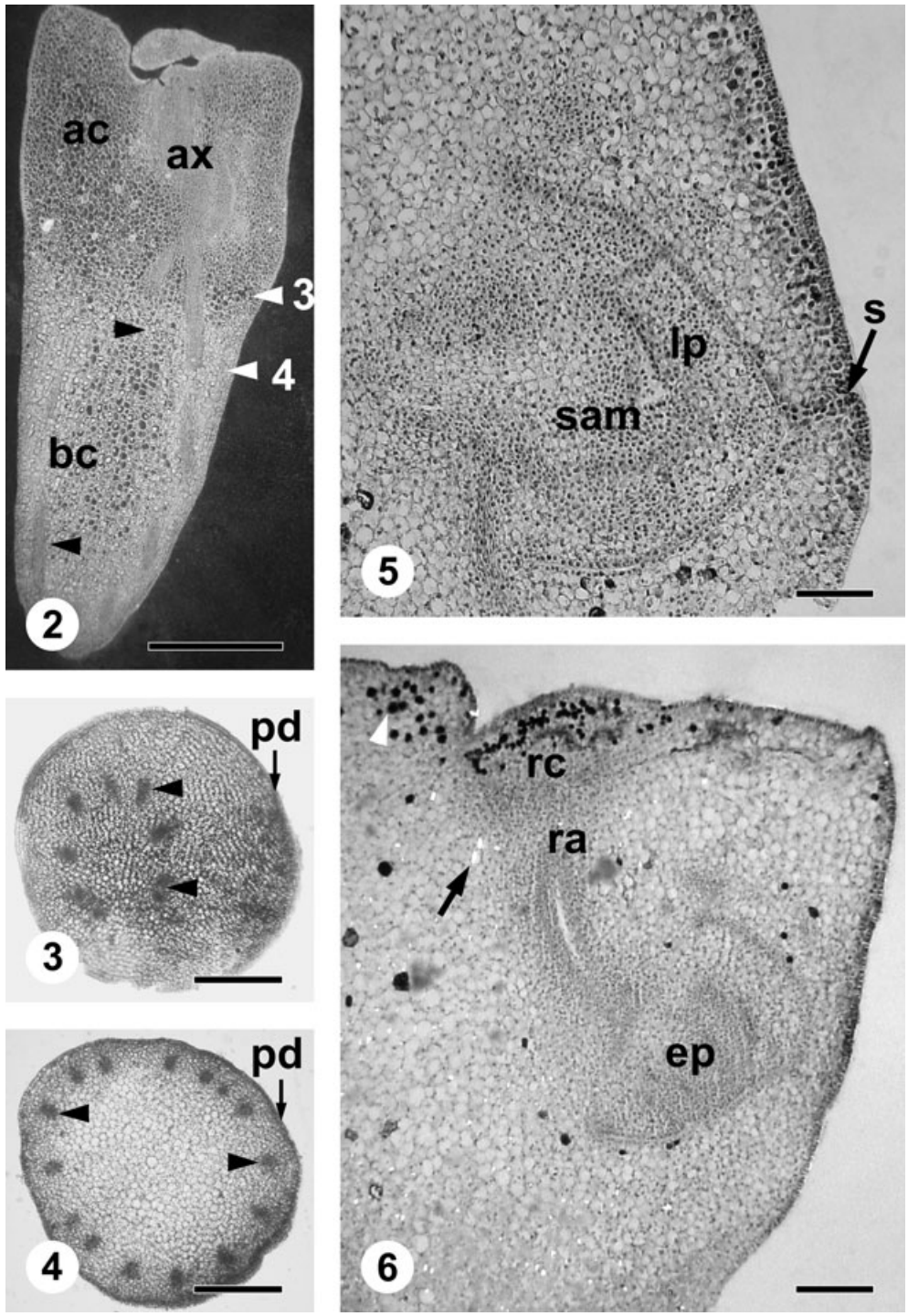


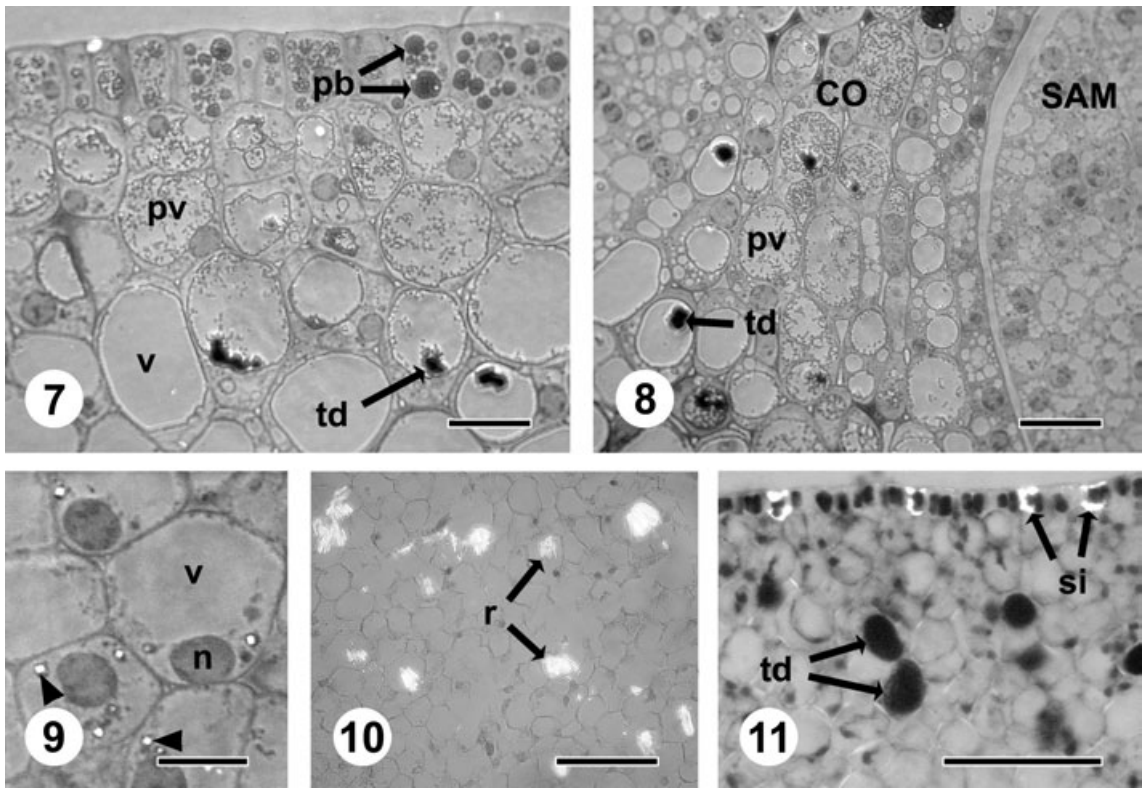
Figure 1. Micrograph of a median longitudinal section of a *Euterpe edulis* seed showing the embryo (em) the endosperm (en) and the seed coat (arrow). In the right upper angle, an embryo, separated from the seed, is shown. Scale bar = 2 mm.

EMBRYO

In the cotyledon, protodermis, parenchyma and procambium are clearly distinguished by the shapes and positions of their cells (Figs 3, 4). The protodermal cells are tabular in outline; parenchyma cells are isodiametric, and procambial cells are narrow and elongate in the long axis of the embryo. Protein bodies occur in the protodermal and subdermal cells of the cotyledon basal region (Fig. 7) and also in the small region of the cotyledon facing the SAM (Fig. 8). Protein bodies include globoid crystals but not protein crystalloids. Cells from the parenchyma and procambium, as well the protoderm, are highly vacuolated, with one central vacuole and the cytoplasm restricted to a thin parietal layer (Figs 7–11). Raphides, silica bodies or tannins occur frequently in vacuoles (Figs 10–12). Silica is also found as deposits in the cell walls of protodermis and parenchyma (Fig. 11).



Figures 2–6. *Euterpe edulis* embryo (LM). Fig. 2. Longitudinal section of an embryo showing the apical and basal regions of the cotyledon (ac and bc, respectively), axis (ax) and procambial strands (black arrow head). Micrograph was obtained using dark field microscopy. White arrow heads indicate approximate levels of Figs 3 and 4. Scale bar = 1 mm. Figs 3, 4. TS apical regions of cotyledon with procambial strands (arrow heads) and protodermis (pd). Scale bars = 500 μm. Fig. 5. Shoot apical meristem (sam) with two leaf primordia (lp). Note the slit (s) facing the shoot apical meristem. Scale bar = 100 μm. Fig. 6. Median LS cotyledon basal region showing the epycotyl (ep), radicle (ra) and root cap (rc). Note tannins (white arrow head) and raphides (arrow). Scale bar = 200 μm.



Figures 7–11. *Euterpe edulis* embryo tissues (LM). Fig. 7. Protodermis and three subjacent parenchyma layers from the basal region of cotyledon, with protein bodies (pb), protein vacuoles (pv), fluid-filled vacuoles (v) and tannin deposits (td). Scale bar = 20 μm. Fig. 8. Note shoot apical meristem (SAM) and that part of cotyledon tissues (CO) facing the SAM, and protein vacuoles (pv) and tannin deposits (td). Scale bar = 20 μm. Fig. 9. Parenchyma of the cotyledon with proplastids containing protein crystalloids (arrow head) as seen using phase microscopy; n, nucleus; v, vacuole. Scale bar = 20 μm. Fig. 10. From the basal region of cotyledon, raphides (r) as seen using polarized light. Scale bar = 100 μm. Fig. 11. Tannin (td) and silica deposits in cell walls (s), as seen using phase microscopy. Scale bar = 100 μm.

Raphides and silica occur especially in the basal region of the cotyledon. Protein crystalloids and starch grains are detected frequently in plastids (Figs 9, 13, 14).

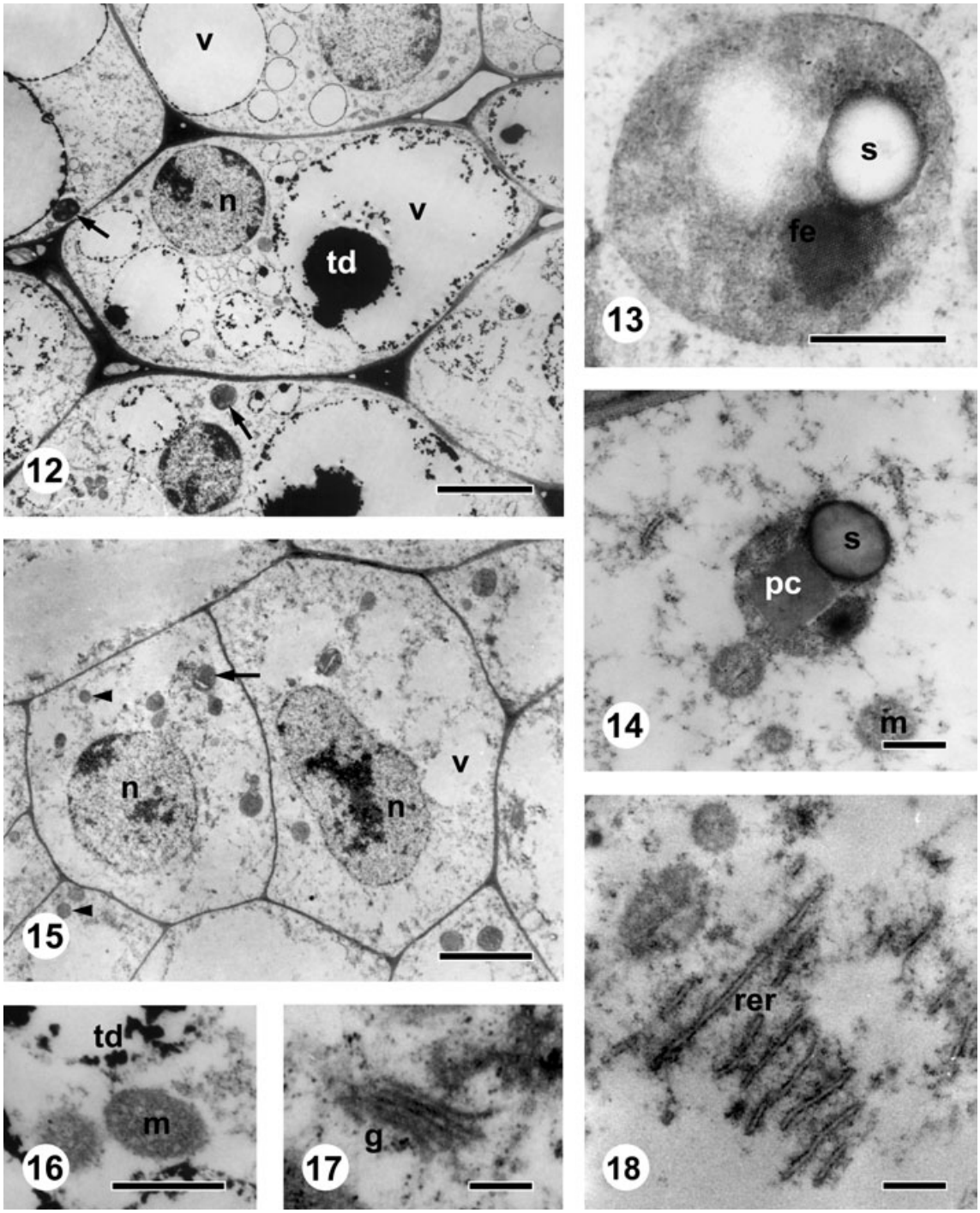
The vascular system is laid out as a system of procambial strands, connecting the axis to the apical region of cotyledon (Figs 2–4). Two procambial strands diverge from beneath the epicotyl. Each one ramifies eight to ten times to produce 16–20 procambial strands at the distal end of the cotyledon.

In the axis, parenchyma cells are notoriously small compared with those of the cotyledon. They are also vacuolated with a central vacuole. In the initial cells of both root and shoot meristems, the nucleus is central with small vacuoles distributed around it (Fig. 15). Small plastids and some lipid bodies are present. Plastids contain small starch grains and clusters of electron dense particles of phytoferritin (Fig. 13). Numerous mitochondria with well defined cristae, cisternae of rough endoplasmic reticulum (RER) and Golgi apparatus are frequently observed (Figs 16–18).

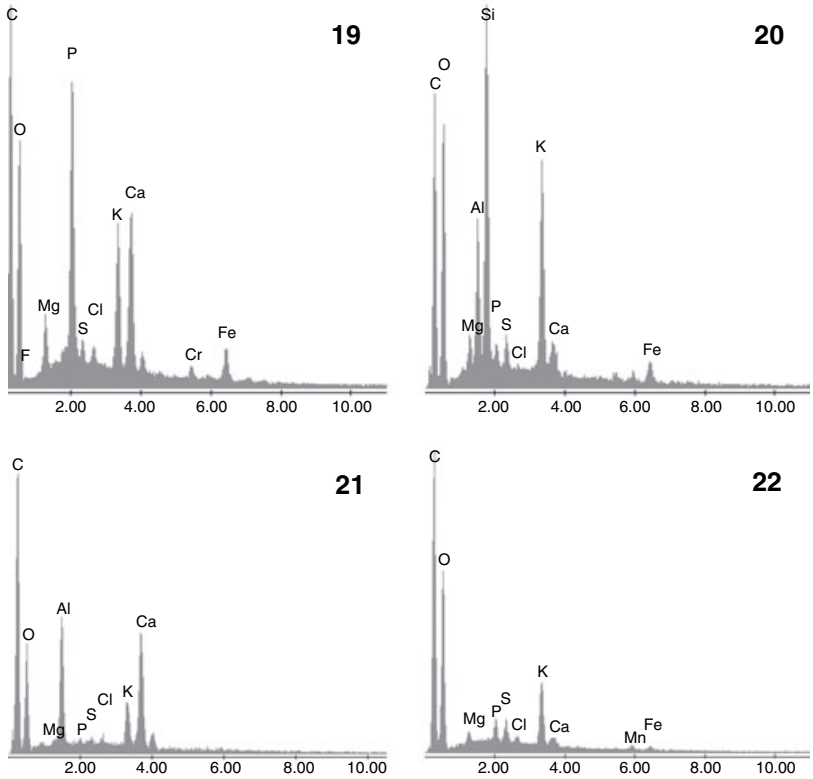
All mineral elements found in the embryo tissues, i.e. globoids, deposits of silica and raphides, were detected by EDX analysis (Figs 19–22). Globoids contain P, Mg, K and frequently also, Ca and Fe. EDX analysis of vacuoles always revealed a high K content (Fig. 22).

ENDOSPERM

The endosperm consists of living cells with thickened cell walls interconnected by primary pit fields (Figs 23–26). Cell walls are the site of mannan storage. Mannan organization is highly crystalline as demonstrated by its strong birefringence upon polarized light. Endosperm tissue is very hard, with resulting difficulty in obtaining sections from endosperm of mature seeds. The cells store lipid and proteins in the form of lipid and protein bodies (Figs 23, 24). The appearance of the protein bodies is not uniform throughout; the outermost cells, just inside the seed coat, contain several small protein bodies (Figs 23,



Figures 12–18. *Euterpe edulis* embryo tissues (TEM). Fig. 12. Cells from the embryo axis with several vacuoles (v) containing tannin deposits (td), some plastids (arrow) and a large nucleus. Scale bar = 10 μm. Fig. 13. Detail of a starch plastid from a cell containing a starch grain (s) and a cluster of phytoferritin particles (fe). Scale bar = 1 μm. Fig. 14. Detail of a starch plastid containing a protein crystalloid. Scale bar = 1 μm. Fig. 15. Cells from the shoot apical meristem showing small vacuoles, mitochondria (arrow head) and some plastids (arrow). Scale bar = 10 μm. Fig. 16 Details of mitochondria (m), active in appearance, with well defined cristae. Scale bar = 1 μm. Fig. 17. Detail of a Golgi apparatus (g). Scale bar = 1 μm. Fig. 18. Several cisternae of rough endoplasmic reticulum (rer) can be observed. Scale bar = 1 μm.



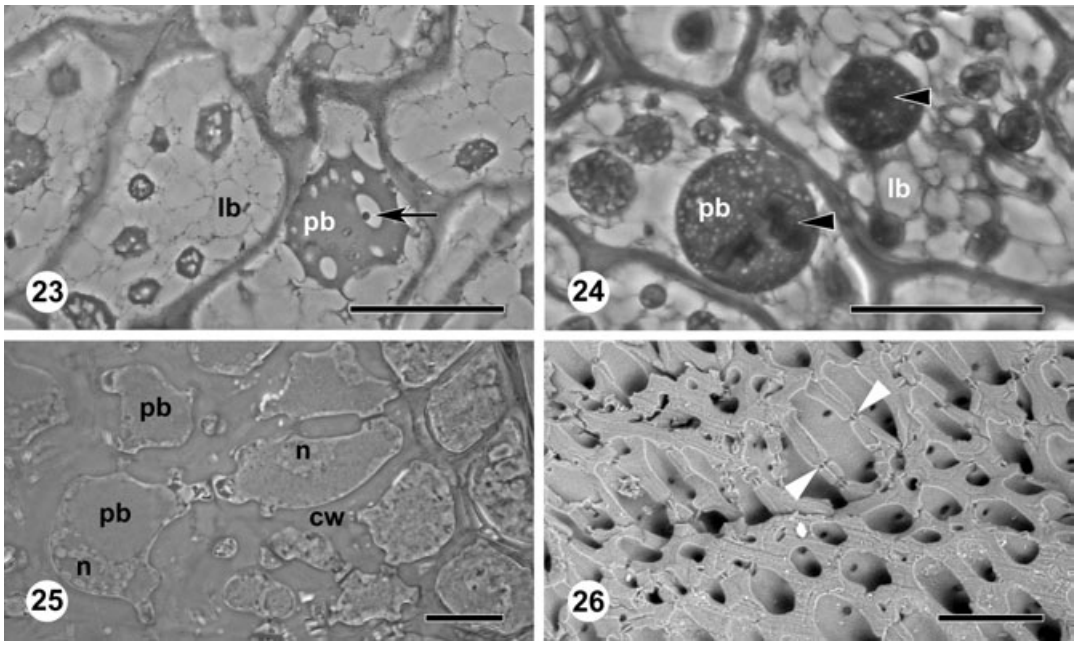
Figures 19–22. EDX analysis of minerals in the *Euterpe edulis* embryo tissues. In all cases, material was placed on aluminium stubs. Fig. 19. Spectrum of a globoid crystal from the protodermis of the cotyledon (basal region). Fig. 20. Spectrum of a silica deposit from a vacuole. Fig. 21. Spectrum of a raphide-crystal. Fig. 22. Spectrum of the vacuolar content from a cell of the embryo axis.

24). More deep-seatedly, the cells only contain a small number of lightly staining protein bodies resembling vacuoles in their structure (Fig. 25). The protein matrix of the protein bodies contains protein crystalloids and crystal globoids (Figs 23, 24). One notably large nucleus per cell is always observed (Fig. 25). The cytoplasm is reduced to the interstices between protein bodies, lipid bodies and nucleus (Figs 23, 24). No evidence of reserve mobilization is observed in the endosperm at this stage.

DISCUSSION

Studies on embryos of a number of palm genera, i.e. *Archontophoenix*, *Phoenix*, *Sabal*, *Cocos*, *Elaeis* and *Washingtonia*, indicate that they consist of a large single cotyledon, a very short radicle-hypocotyl axis and an epicotyl (Sach, 1862; Lloyd, 1910; Biradar, 1968; Biradar & Mahabale, 1968; Haccius & Philip, 1979; Alang, 1982; DeMason & Thomson, 1981; DeMason, 1988). Even though those studies revealed very similar palm embryo organography, differences could be established on the orientation of the embryo axis. In this way, DeMason (1988) distinguished palm embryos into two categories: (1) those with epicotyls parallel to the cotyledon (*Phoenix*); and (2) those with the epicotyls obliquely angled with respect to the cotyledon (*Archontophoenix*, *Cocos*, *Elaeis* and *Washingtonia*). We found that the embryo of *E. edulis* corresponds to the second category.

There has been very little work on the characteristics of palm embryo tissues. The only structural studies of the embryo have been made on the cotyledon tissue of three palm species: *Elaeis guineensis* (Alang, 1982), *Phoenix dactylifera* (DeMason & Thomson, 1981) and *Washingtonia filifera* (DeMason, 1988). In those studies, protodermis, parenchyma and procambium were distinguished in the cotyledon. The three tissues store proteins and lipids in the form of protein and lipid bodies, but not starch. As in the above species, in the cotyledon of *E. edulis*, three tissues are recognized, but some conspicuous differences could be determined at cellular and subcellular levels. Cells from most tissues lack insoluble storage reserves and are highly vacuolated with one central vacuole and the cytoplasm restricted to a thin parietal layer. The presence of vacuoles had not yet been reported in mature embryos of any palm species.



Figures 23–26. Sections of endosperm from a mature seed of *Euterpe edulis.* Scale bars = 100 μm. Figs 23, 24. Peripheral endosperm layer; both are sections parallel to surface. Both figures in phase contrast microscopy. Fig. 23. The tissue was stained with fast green FCF. Lipid bodies (lb) and protein bodies (pb) can be seen. The arrow indicates a globoid crystal. Fig. 24. Endosperm tissue stained with toluidine blue with protein crystalloids in the protein bodies (pb) (arrow head). Note lipid bodies (lb). Fig. 25. Peripheral radial section using phase contrast microscopy. Note the thick cell walls (cw). Nuclei (n) and protein bodies resembling vacuoles (pb) are observed in the inner layers. Fig. 26. ESEM of the central endosperm tissue showing the very thick cell wall with primary pit fields (arrow head).

According to Iljin (1957) plant cells which tolerate desiccation must withstand the mechanical stresses associated with volume reduction. This may be partly achieved by reduction of the volume of fluid-filled vacuoles by shrinkage, breaking up of one or a few large vacuoles into many considerably smaller ones, or by their becoming filled with insoluble reserve material. Even though embryo development has not been studied in either *W. filifera* or *P. dactylifera*, it is possible to infer that during development, large vacuoles divide into many, each becoming filled with protein reserves. Because of the presence of fluid-filled vacuoles and the absence of insoluble reserves, the mature *E. edulis* embryo seems similar to the embryo of *Avicennia marina*, the most recalcitrant species currently known (Farrant, Pammenter & Berjak, 1992). In addition, the presence of numerous mitochondria, RER cisternae and Golgi indicate that they are in a very active state. Such features are associated with a strategy of continuous development, almost without the interposition, at maturity, of a dry state, and differentiate *E. edulis* from the seeds of the palm species with orthodox behaviour, i.e. *P. dactylifera* and *W. filifera* (Sento, 1972; Krigman, 1974; Carpenter & Ream, 1976; Dickie, Balik & Linnington, 1992).

In studies on *E. edulis* seeds carried out by Andrade (2001), Bovi & Cardoso (1978), Martins, Nakagawa & Alves Bovi (2000), Queiroz & Cavalcante (1986), Reis *et al*. (1999) and V. Panza *et al*. (unpubl. data), the mean moisture content of the whole seed was recorded as 48.5% (wet weight), and 85.3 and 48.2% (wet weight) for isolated embryo and endosperm, respectively, recognizing their recalcitrant behaviour. Here, we present the structure of their embryo tissues and infer that such a high water content is accounted for by the water of vacuoles.

As in *A. marina*, the data are consistent with the hypothesis that germination of *E. edulis* seed is initiated at or around shedding and, as the germination-associated changes continue in storage, the seed became increasingly desiccation sensitive. The *E. edulis* embryo should thus be viewed as a developing seedling.

Alang (1982) states that starch grains occurred in cells of the embryonic axis in *Elaeis guineensis*. Starch grains were absent in the cotyledon tissues of *E. guineensis*, *W. filifera* and *P. dactylifera*. In the *E. edulis* embryo, plastids of parenchyma cells frequently exhibited small starch grains, together with protein crystalloids and clusters of electron dense

particles of phytoferritin. Protein crystalloids have been reported in *W. filifera* (DeMason, 1988), but were absent in *P. dactylifera* embryos (DeMason & Thomson, 1981). Until now, phytoferritin deposits have not been detected in embryo tissues of other palm species.

The presence of tannins had been reported by Haccius & Philip (1979) in *Cocos nucifera* L., one of the palm species recognized as recalcitrant. (Chin, 1989; Assy-Bah & Engelmann, 1992; Engelmann *et al*., 1995a,b). Other ergastic substances were not observed anywhere in the embryos of the other palm species studied. In *E. edulis*, tannins, raphides and silica frequently occurred in the embryo tissues.

The cotyledon of two palm species, *P. dactylifera* and *W. filifera*, exhibited electron-opaque granules identified by EDX analysis as polyphosphate deposits (DeMason & Stillman, 1986). Such granules were not identified in embryo tissues of the species in this study.

The endosperm in *E. edulis* consists of living cells storing carbohydrate in the form of thickened cell walls, and lipids and proteins in the form of lipid and protein bodies, respectively, in the cytoplasm. Determination of the causes why the endosperm, but not embryo, accumulates storage reserves, remains the subject of further investigations.

Three layers were differentiated in the thickened cell walls of the endosperm, middle lamella, and outer and inner walls, all stained differentially with toluidine blue. In this respect, toluidine stainibility was similar to that of *W. filifera* and *P. dactylifera* (DeMason, 1986). Calcofluor white was used in order to demonstrate that cell walls are composed mainly by K(1–4) linkage polysaccharides in a microfibrillar arrangement, similar to that of *Washingtonia* and *Phoenix*. All cells were similar in composition. The only evidence of differentiation was the fact that protein bodies are not uniform throughout. The outermost cells, i.e. just the outer layer, contain several small protein bodies; more internally, cells only contain one or two lightly staining protein bodies similar to vacuoles in their aspect. The protein matrix contains protein crystalloids and crystal globoids. Protein crystalloids inclusions were present in the protein bodies of the outer endosperm cells. The same reserves are stored in the endosperm of *P. dactylifera* (DeMason, Sexton & Reid, 1983) and *W. filifera* (DeMason, 1986).

In the endosperm of both *W. filifera* (DeMason *et al.*, 1983) and *P. dactylifera* (DeMason, 1986), nuclei occupy only a small fraction of the cell volume. In *E. edulis,* they were notably large.

Overall, there is no evidence of storage mobilization and the endosperm appears to be in an inactive state.

CONCLUSIONS

The histochemical and ultrastructural characteristics of *E. edulis* embryo tissues described here clearly differentiate this recalcitrant species from the two orthodox species, *P. dactylifera* and *W. filifera*. The embryo tissue features of *E. edulis*, i.e. fresh tissues, highly vacuolated, almost lacking storage reserves, with abundant endomembranes, producing ergastic substances, are associated with this strongly recalcitrant seed behaviour.

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