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Localization of the cotyledon reserves of *Theobroma grandiflorum* (Willd. ex Spreng.) K. Schum., *T. subincanum* Mart., *T. bicolor* Bonpl. and their analogies with *T. cacao* L.

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ABSTRACT – (Localization of the cotyledon reserves of *Theobroma grandiflorum* (Willd. ex Spreng.) K. Schum., *T. subincanum* Mart., *T. bicolor* Bonpl. and their analogies with *T. cacao* L.). Cotyledon mesophyll cell morphology and lipid and protein synthesis of *T. grandiflorum*, *T. subincanum* and *T. bicolor* were analyzed and compared with *T. cacao*. These species possess foliar cotyledons folded around the hypocotyl radicle axis, typical of Sterculiaceae. Fruit size, morphology and weight are very distinct amongst the four species and so are the respective seeds. The main axis of the *T. grandiflorum* and *T. bicolor* seeds measured about 30 mm, while *T. subincanum* and *T. cacao* seeds measured 17 mm and 26 mm respectively. The seed weights of *T. grandiflorum*, *T. bicolor*, *T. subincanum* and *T. cacao* were 11.6 g, 9.4 g, 2.1 g and 3.0 g, respectively. The cotyledon mesophylls of the four species contained mainly polysaccharides and lipid-protein reserve cells. *Theobroma cacao*, *T. grandiflorum* and *T. subincanum* were composed of greater than 50% lipids. For the four species, lipid globules gradually accumulated adjacent to the cell wall, and these globules measured from 1 to 3 µm. TEM showed low-density proteins inside the central vacuole of the young mesophyll cells of *T. cacao*. The protein reserves of the mature cells were densely scattered amongst the lipid bodies, and a few starch granules occurred together with the cotyledon mesophyll of the four species. Polyphenolic cells were found throughout the mesophyll cells or aligned with the respective vascular bundles. Immature cells demonstrated the capacity to synthesize all these reserves, but gradually the pre-determined cells produced mainly lipid-protein reserves. Besides the unique characteristics of the *T. cacao* products, the lipid-protein synthesis capacities of *T. grandiflorum*, *T. subincanum* and *T. bicolor* suggest various possibilities for new industrialized food, pharmaceutical and cosmetic products.

Key words - comparative study, histology, reserves quantity, *Theobroma*

RESUMO – (Localização das reservas cotiledonares de *Theobroma grandiflorum* (Willd. ex Spreng.) K. Schum., *T. subincanum* Mart., *T. bicolor* Bonpl. e analogias com *T. cacao* L.). As células do mesofilo cotiledonar e respectivas sínteses lípide-protéicas de *Theobroma grandiflorum*, *T. subincanum* e *T. bicolor* são analisadas e comparadas ao *T. cacao*. As espécies mencionadas possuem os cotilédones foliáceos e redobrados em torno do eixo hipocótilo-radicular, típicos de Sterculiaceae; o tamanho dos frutos e também a morfologia, o peso e as respectivas sementes são distintos entre as quatro espécies. As sementes de *T. grandiflorum* e *T. bicolor* medem 30 mm no eixo principal, enquanto em *T. subincanum* e *T. cacao* medem 17 mm e 6 mm, respectivamente e nesta mesma ordem pesam 11,6 g, 9,4 g, 2,1 g e 3,0 g. Os mesófilos cotiledonares contêm células com reservas polissacárides e lípide-proteicas. *Theobroma cacao*, *T. grandiflorum* e *T. subincanum* contêm acima de 50% de lípideos. Nas quatro espécies, os glóbulos lipídicos acumulam-se gradualmente, adjacentes à parede celular e medem entre 1 a 3 µm. A microscopia eletrônica de transmissão mostrou a precipitação pouco densa das proteínas no vacúolo central das células jovens do mesofilo de *T. cacao*. Nas células maduras, a proteína de reserva está densamente entremeada por glóbulos lipídicos; poucos grãos de amido ocorrem no mesofilo cotiledonar destas quatro espécies. As células polifenólicas estão dispersas no mesofilo cotiledonar ou alinhadas próximas ao feixe vascular. As células imaturas demonstraram capacidade de síntese de todas as reservas, mas gradualmente as células predeterminadas, produzem principalmente reservas lípide-proteicas. Não obstante a característica única dos reconhecidos produtos de *T. cacao*, a capacidade de síntese lípide-proteica do *T. grandiflorum*, *T. subincanum* e *T. bicolor* sugerem novas possibilidades para a indústria de alimentos, produtos farmacêuticos e cosméticos.

Palavras-chave - estudo comparativo, histologia, reservas, *Theobroma*

Introduction

All *Theobroma* species present the same chromosome number: $2n = 2x = 20$ (Muñoz 1948). The botanical classification of *Theobroma* into sections was defined according to the vegetative and morphological aspects of the trees, fruits and seeds (Cuatrecasas 1964), and

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the comparative seed morphology of *T. grandiflorum* (Willd. ex Spreng.) K. Schum, *T. subincanum* Mart., *T. bicolor* Bonpl. as compared to *T. cacao* L. was still necessary. The genetic polymorphism has been studied by Figueira *et al.* (1994), Silva *et al.* (2001) and Silva & Figueira (2005). *Theobroma* contains 22 species classified into six sections (Cuatrecasas 1964), which have great importance as a gene reservoir for cacao improvement (Gilabert-Escrivá *et al.* 2002).

The full development of chocolate flavour requires fermentation and roasting of the *T. cacao* seeds (Voigt *et al.*, 1995). *Theobroma grandiflorum* seeds are also fermented and roasted to achieve the “cupulate” flavour (Venturieri & Aguiar 1988). *Theobroma cacao* seeds contain 53% fats (Pires *et al.* 1998) and 15% to 20% protein on a dry weight basis. The protein fraction is composed mainly of albumin (52% of total protein) and globulins (43%) (Biehl *et al.* 1982, Spencer & Hodge 1992, Voigt *et al.* 1993, 1995). Other *Theobroma* species contain higher fat and protein levels (Silva *et al.* 2001, Gilabert-Escrivá *et al.* 2002). Biehl *et al.* (1977, 1982) analysed the evolution of proteins from mature seeds in *T. cacao* during germination and fermentation. It has been proposed that during fermentation, *T. cacao* seed storage proteins are degraded by endo- and exo-proteases to oligopeptides, which react during roasting with other flavour precursors, such as sugars and polyphenols, to develop the full chocolate flavour (Voigt *et al.* 1995). It would be interesting to test the vicilins (Whitlock & Baum 1999) from other *Theobroma* species in comparative fermentation studies, to elucidate the role of vicilin in the development of chocolate flavour. Reisdorff *et al.* (2004) characterized the activities of proteolytic enzymes (aspartic endopeptidase and carboxypeptidase) and storage seed globulins from *T. grandiflorum* and *T. bicolor*, observing small differences in the metabolic and biochemical pathways as compared to *T. cacao*. These three species are still being timidly exploited for human purposes. The development of chocolate-like products from these species could potentially be achieved by adapting the fermentation procedures to the particular biochemical cotyledon features of *T. grandiflorum*, *T. subincanum* and *T. bicolor* seeds. In this study we present the first simultaneous analysis and comparison of these three species with *T. cacao* in terms of early reserve synthesis, and synthesis of cotyledon protein, lipid and polyphenols.

Material and methods

Theobroma grandiflorum, *T. subincanum*, *T. bicolor* and *T. cacao* seeds were obtained from mature fruits from

the germplasm collection of the “Comissão Executiva do Plano da Lavoura Cacaueira – Ceplac”, located in Marituba, Pará (1°12' S; 49°30' W), Brazil and also from Itabuna, Bahia (14°48' S; 39°18' W), Brazil.

The analysis employed two mature fruits and a third one beginning to mature and 12 seeds from each species. A further five large mature *T. cacao* fruits from Itabuna were also employed. The cotyledons were split into two halves. One half was employed for microscopic analysis and the other for chemical analysis.

The cotyledons were fixed (2% glutaraldehyde and 4% paraformaldehyde), included in glycol methacrylate, and prepared for the morphological and histochemical study using light microscopy (FM) (O'Brien & McCully 1981). Histological preparations for Scanning Electronic Microscopy (SEM) and Transmission Electronic Microscopy (TEM) were prepared according to Harris (1991).

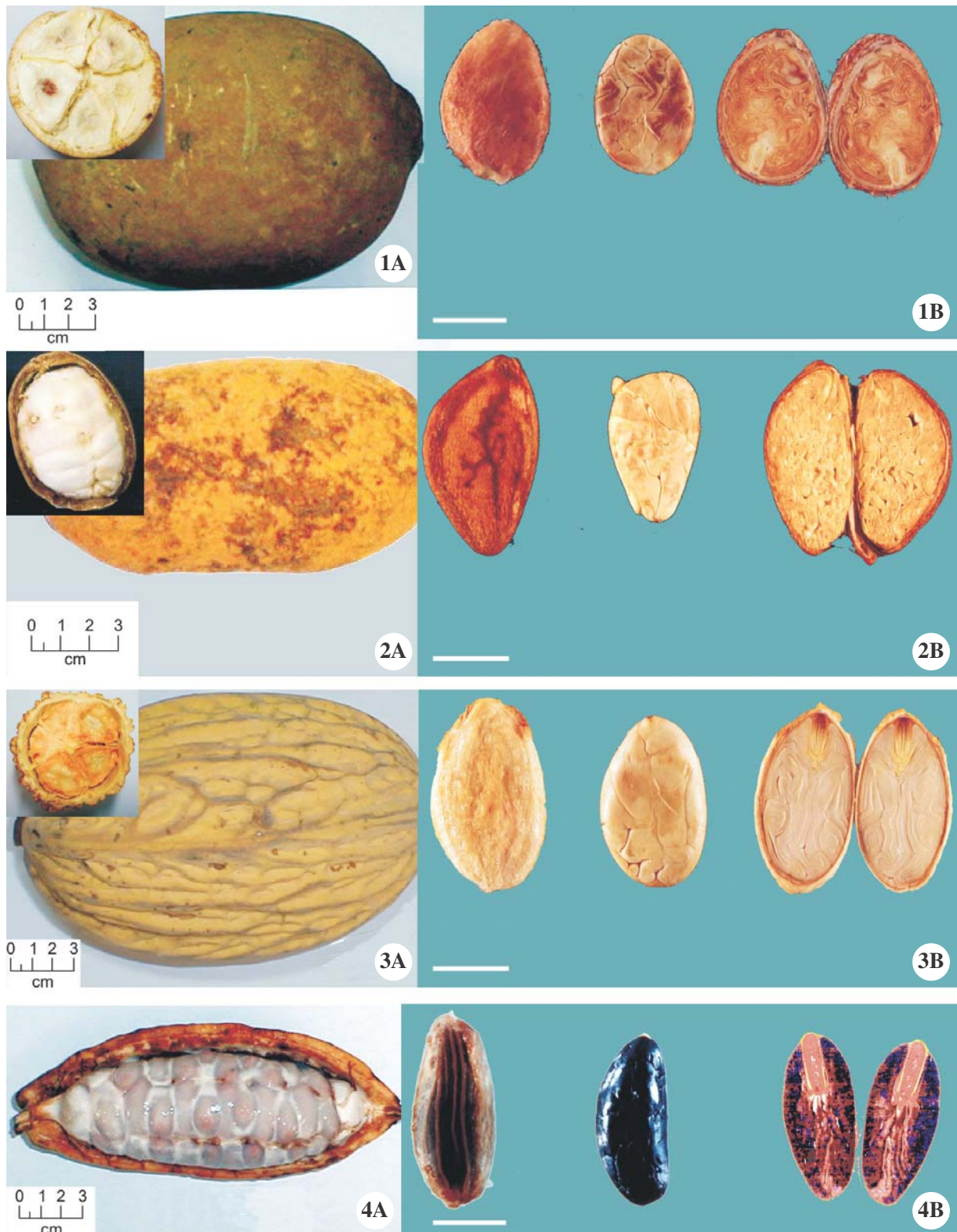
The total lipid contents were determined by the Soxhlet method (963.15 AOAC 1997) based on both ground and freeze-dried samples. The proteins were determined using the Kjeldahl method (970.22 AOAC 1997) using a conversion factor of 5.4. Polyphenols were determined by spectrophotometry (Marigo 1973).

Results and discussion

The seed genetics of this collection (CEPLAC) have been studied by Silva *et al.* (2001), who established the total seed protein profile and random amplified polymorphic DNA (RAPD). Silva & Figueira (2005) analysed the trypsin inhibitor gene of the same collection.

The *Theobroma grandiflorum*, *T. subincanum*, *T. bicolor* and *T. cacao* fruits were almost ellipsoids (figures 1A-4A) and displayed variation in seed size (figures 1B-4B). The cotyledon mesophylls of these species showed the following genus characteristics: a flattened mesophyll, which was always densely folded around the hypocotyl radicle axis and whitish mesophylls, or darker if anthocyanin synthesis was abundant (figures 1-4). The cotyledon foliar morphology was evident from the vascular bundles scattered around the entire mesophyll tissue (Barroso *et al.* 1999, Silva *et al.* 2001). Before germination, the thickness of the cotyledon mesophyll was 0.7 mm in *T. grandiflorum* and *T. subincanum* (figures 1B-2B), reaching 1.4 mm in *T. bicolor* and *T. cacao* (figures 3B-4B). The cotyledonary mesophylls of *T. bicolor* and *T. cacao* were thicker than the others and showed less folds. The name *T. bicolor* was well suited to this plant because its young cotyledons (figure 5A) sheltered a translucent, detachable tissue amongst their folds, which was homogeneous parenchyma containing no reserve substances (figure 5B).

The seeds of *T. grandiflorum* and *T. bicolor* were heavier in relation to the *T. subincanum* and *T. cacao*



Figures 1-4. Fruits and their seeds: 1. *Theobroma grandiflorum*. 2. *T. subincanum*. 3. *T. bicolor*. 4. *T. cacao*. The *Theobroma* genus displays significant fruit shape diversity. Variations in ellipsoid fruit shape (1A-4A) and respective seed sizes (1B-4B) are shown. *T. grandiflorum* (1B) and *T. subincanum* (2B) cotyledons are thinner and larger than in *T. bicolor* (3B). Bars = 10 mm (1B, 2B, 3B and 4B).

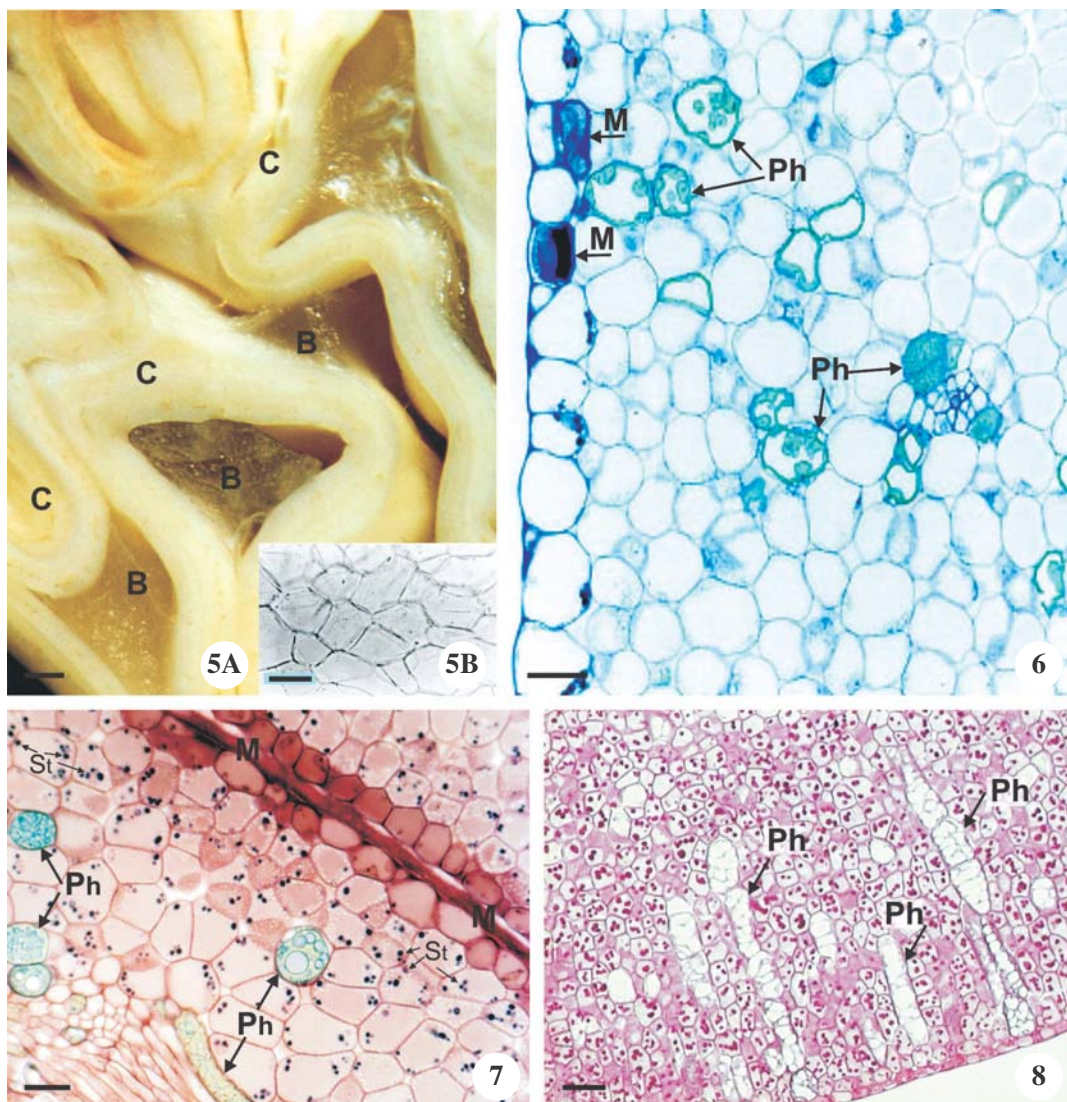
seeds (table 1). The seed weights of *T. cacao* were variable, due to the ellipsoidal shape of the fruit. However, as described by Silva & Figueira (2005), in fact the *T. cacao* varieties from CEPLAC displayed distinct seed shapes amongst the varieties.

Figures 5-8 display the mesophyll characteristics found using FM. The species studied initially synthesized mucilage, starch granules, lipid and protein bodies concomitantly (figures 5-7). Intense mucilage synthesis occurred in all the seed cells and also in other fruit tissues.

Table 1. Average size and weight of the seeds of *Theobroma subincanum*, *T. cacao*, *T. bicolor* and *T. grandiflorum*.

Species	Size* (mm)	Weight** (g)
<i>Theobroma subincanum</i>	17 x 11	2.1 ± 0.2
<i>T. cacao</i>	26 x 13	3.0 ± 0.2
<i>T. bicolor</i>	30 x 24	9.4 ± 1.1
<i>T. grandiflorum</i>	31 x 24	11.6 ± 2.8

* longitudinal and transversal axis; ** values: average ± standard deviation.



Figures 5-8. FM-Cotyledon mesophyll. 5A-5B. *Theobroma bicolor*. This fresh folded cotyledon (5A) is concealing a translucent tissue (B) characteristic of *T. bicolor*. This non-differentiated tissue is the original seed suspending-axis (5B). 6. *T. subincanum*. Toluidine Blue (pH4) staining mucilage and phenolic deposits. 7. *T. grandiflorum*. I/KI reaction staining of the mesophyll. Mucilage, small starch granules and the phenolic deposits. 8. *T. cacao*. PAS/Schiff stain. Starch granules and mesophyll cells are red. It is characteristic to *T. cacao* these rolls of phenolic cells toward the mesophyll border. (B = translucent tissue; C = cotyledon; M = mucilage; Ph = polyphenolic cells; St = starch granules). Bars = 10 mm (5A), 10 µm (5B), 50 µm (6, 7), 5 µm (8).

The synthesis of acid and neutral polysaccharides, which filled the epidermis and its mesophyll cells was demonstrated using Toluidine Blue for *T. subincanum*, (figure 6) or the Schiff PAS/Reactive stain for *T. cacao* (figure 8).

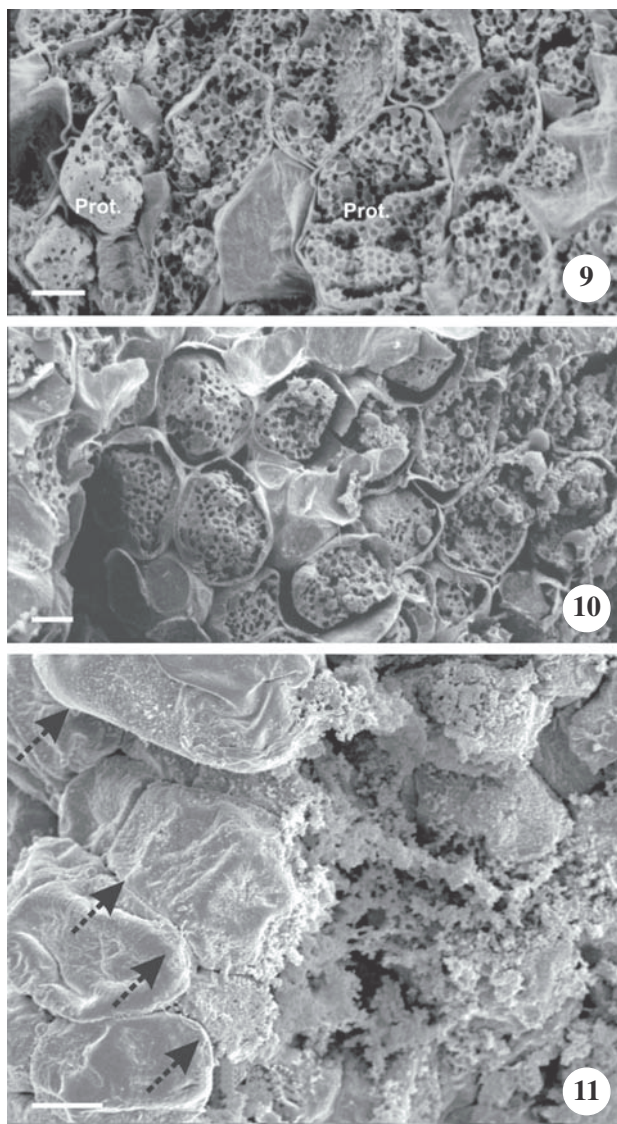
The *T. cacao* cotyledon showed a great amount of idioblast polyphenolic cells interspersed with the mesophyll cells. A peculiarity of the mature seeds of both *T. cacao* and *T. grandiflorum* (Martini 2004) was the presence of rows of polyphenolic cells displayed perpendicular to the cotyledon mesophyll edge (figure 8).

The cotyledon mesophylls of the four species presented protein, lipid and polyphenols (table 2) and the syntheses of these components followed a maturation gradient inside the fruit. Young mesophyll cells started accumulating protein reserves in their central area (figures 9-10) and also lipid droplets inside the cytoplasm, which accumulated adjacent to the cell walls (figure 11). These figures show intercellular spaces, which are characteristics of *T. cacao* and of other species from the same genus, described previously by Brooks & Guard (1952). We have also previously shown that *T. subincanum* displays large and a great number of intercellular spaces (Martini, 2004). In fresh sections of the *T. subincanum* cotyledon mesophyll cells were dissociable under gentle mechanical compression on a slide. We suggest that the weak union between the *T. subincanum* cells promoted natural mucilage storing spaces, a phenomenon already described to *T. speciosum* Willd. ex Spreng. (Martini *et al.* 2003). We have associated the same predominance of mucilage with smaller amounts of polyphenols in *T. bicolor* and furthermore we demonstrated the accumulation of great pools of mucilage in the middle of the mesophyll cells in *T. speciosum* and the poorer content of polyphenols (Martini *et al.* 2003).

When close to maturity the lipid and protein reserves prevailed over the cell structure components, as, for instance, the starch granules. The protein reserves in *T. cacao*, *T. bicolor* and *T. grandiflorum* were very expressive (table 2), which explains the incipient South

American industrialization of *T. bicolor* and *T. grandiflorum* in addition to that of *T. cacao*.

Lipids were definitely the main cell reserves in the four species (table 2). The lipid globules, with diameters ranging from 1 to 3 μm , were found in a spacial association with the protein reserves (figures 9-10, 12,13). When fresh mesophylls were submitted to temperatures above 28 $^{\circ}\text{C}$, the lipid globules of those live cells quickly coalesced. Figure 11 (left side) displays the characteristic



Figures 9-11. SEM-Lipid and protein reserves. 9. *T. cacao*. The opened mesophyll cells are exposing protein reserves; the sieve-like structures are due to protein deposition around lipid droplets. 10. *T. bicolor*, similar protein and lipid mass from figure 9. 11. *T. grandiflorum*. The four closed cells on left side (arrows) show the loose association of the mesophyll cell tissue. A mass of leaked proteins is seen on the right. (Prot = protein reserves). Bars = 10 μm (9, 11), 20 μm (10).

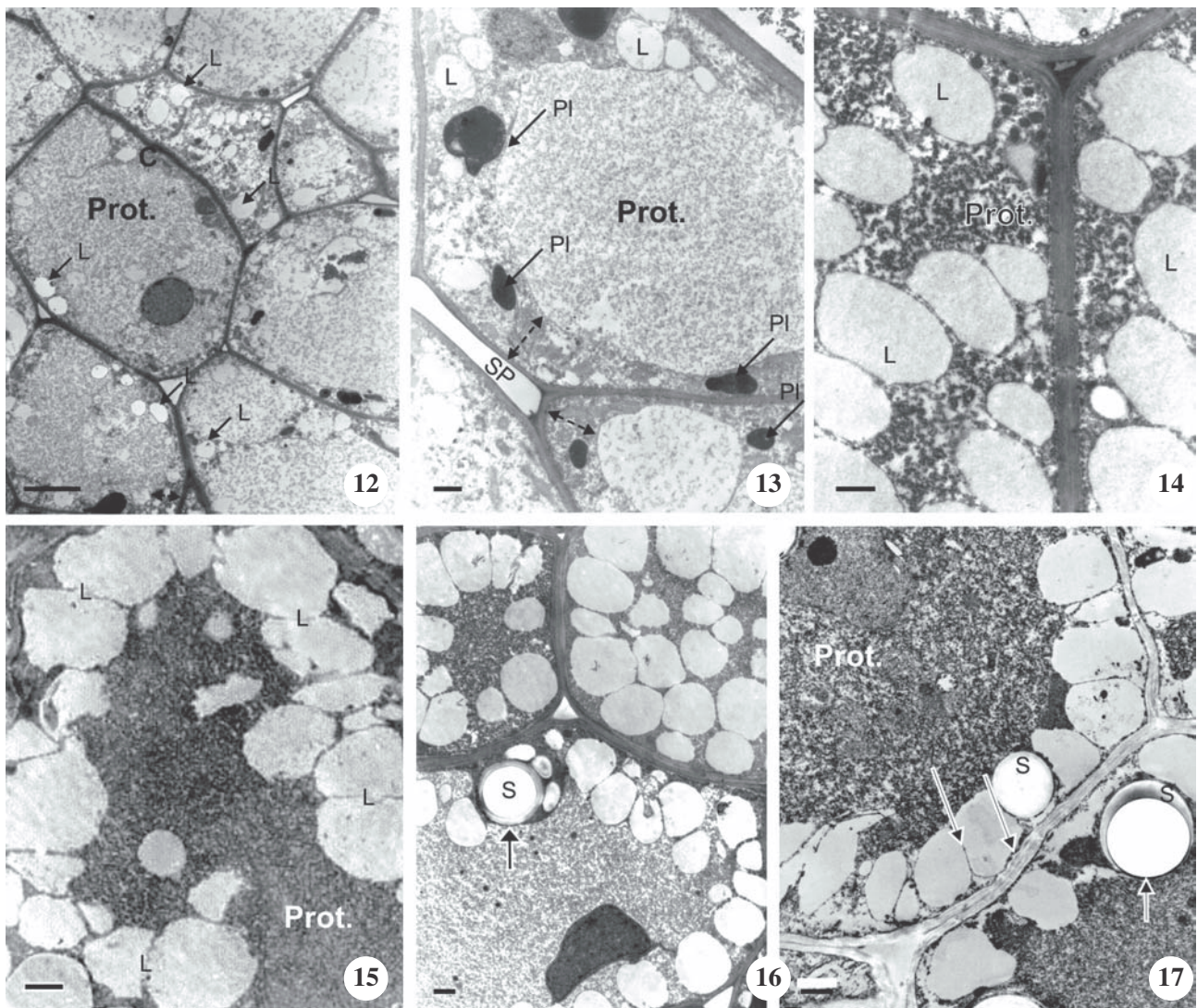
Table 2. Concentrations of the main seed reserves (dry basis, mg g^{-1}). Values of triplicates, average \pm standard deviation.

Sample	Protein	Lipid	Polyphenols
<i>Theobroma cacao</i>	143.5 \pm 1	492.4 \pm 6	79.9 \pm 6
<i>T. subincanum</i>	94.8 \pm 5	598.8 \pm 4	55.2 \pm 2
<i>T. bicolor</i>	190.7 \pm 2	308.6 \pm 8	5.0 \pm 1
<i>T. grandiflorum</i>	129.5 \pm 1	542.2 \pm 0	42.7 \pm 2

loose association of the *T. grandiflorum* mesophyll cells, as mentioned above for *T. speciosum*. During fotonic microscopy at 15 °C, the fresh cotyledons of *T. grandiflorum*, *T. bicolor* and *T. subincanum* preserved the lipid globules, which were evident from their fluorescence (Martini 2004). In fact, due to the interaction of the lipid globules with the protein reserves, it was not possible to define the oleosin membrane (figures 14-17). Although Guilloteau *et al.* (2003) reported the existence of two classes of oleosins in *T. cacao*, the rapid coalescence

amongst the lipid bodies, when exposed to temperatures above 28 °C (fresh histological sections), did not help to confirm the existence of the oleosin membrane enclosure.

Gilabert-Escrivá *et al.* (2002) determined the fatty acids and triacylglycerols of the same species from the same geographical location and demonstrated the predominance of oleic and stearic acids. These authors stressed the higher palmitic acid concentration in *T. cacao* as compared to the other three species.



Figures 12-17 TEM-Mesophyll of four *Theobroma* species. 12-13. *Theobroma cacao*, immature mesophyll. 12. The initial phase of reserve protein synthesis it is inside the large central vacuole and in the peripheral cytoplasm are visible appearance the first lipid bodies. 13. The plastid structure became enlarged due to polyphenols synthesis. Intercellular spaces are conspicuous. 14-17. Mature mesophyll. 14. *T. cacao*. Protein and lipid synthesis characterize this mesophyll. 15. *T. grandiflorum*. Lipid globules are coalescing faster than in the other three species. 16. *T. subincanum*. A plastid shelters the external halo of a starch granule. 17. *T. bicolor*. Mature cells have the protein reserves are surrounding the lipid globules (arrows) and two starch granules only contains the external part of the granule. (C = cytoplasm; L = lipid; PI = plastid; Prot = protein reserves; S = starch granule; SP = intercellular spaces). Bars = 4 µm (12), 1 µm (13-16), 2 µm (17).

The common cotyledon mesophyll feature of the four species was the large, densely folded mesophyll. In all these species lipid synthesis and storage prevailed over that of proteins.

The main purpose of the present study was to focus on the synthesis of the reserves from the first signals of cell deposition up to fruit maturity. Besides lipids, the younger mesophyll cells were shown to synthesize a wide variety of reserves, such as proteins, mucilage, starch and polyphenols. Despite the differences in quantity and important physicochemical distinctions between these reserves, mesophyll morphology was found to be somewhat similar, especially between *T. cacao* and *T. grandiflorum*.

Our analysis of fresh tissue and microscopic (FM, SEM and TEM) tissue samples did not provide evidence of oleosin membrane in those species.

The amount of polyphenols was relatively high in *T. cacao*, followed by *T. subincanum* and *T. grandiflorum*; however this is not a common feature in the other species.

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