Biochemical, histochemical and ultrastructural characterization of *Centrolobium robustum* (Fabaceae) seeds

Javier Alberto Pinzón-Torres[1], Vanessa Rebouças dos Santos[2], Marlene Aparecida Schiavinato[1,4] and Sara Maldonado[3]

Received: 29.04.2008; accepted: 05.03.2009

ABSTRACT - (Biochemical, histochemical and ultrastructural characterization of *Centrolobium robustum* (Fabaceae) seeds). *Centrolobium robustum* seed coat is made up of an external layer of macrosclereids and an internal layer of osteosclereids, followed by a layer of dead cells. The endosperm is closely united to the seed coat and is made up of up to three layers of living cells rich in lipid and protein bodies. All the embryonic cells that form the different tissues of the cotyledons and embryonic axis, including the apical meristematic tissues, are storage tissues since they accumulate mainly lipids and proteins, in the form of lipid and protein bodies, water-soluble polysaccharides, and starch. In the seed coat, lignin, simple phenols and hydrolysable tannins were chemically detected.
Key words: Leguminosae, lipids storage, protein storage, seed ultrastructure

RESUMO - (Caracterização bioquímica, histoquímica e ultraestrutura das sementes de *Centrolobium robustum* (Fabaceae)). O tegumento que recobre o embrião da semente de *Centrolobium robustum* está constituído por uma camada externa de macroesclereídeos e uma camada interna de osteoesclereídeos, seguida por células mortas. O endosperma é constituído por até três camadas de células vivas ricas em corpos lipídicos e protéicos. Todas as células embrionárias que compõem os diferentes tecidos dos cotilédones e do eixo embrionário, incluindo os ápices meristemáticos, armazenam principalmente lipídeos e proteínas na forma de corpos lipídicos e protéicos, polissacarídeos solúveis e amido. No tegumento, foram detectados lignina, fenóis simples e taninos hidrolisáveis.
Palavras-chave: Leguminosae, reserva de lipídeos, reserva de proteínas, ultraestrutura de semente

Introduction

Centrolobium Mart. *ex* Benth. comprises six tropical species: *C. robustum* (Vell.) Mart. *ex* Benth., *C. microchaete* (Mart. *ex* Benth.) H. Lima, *C. tomentosum* Guill. *ex* Benth., *C. paraense* Tul., *C. sclerophyllum* H. Lima, and *C. minus* Presl. These species are distributed from Panama to the Southeast of Brazil and are known locally as guayacan hobo, balaustre (Colombia, Venezuela), amarillo (Peru), amarillo guayaquil (Panama, Ecuador), morosimo (Paraguay), and ararauba, ararauva, araribá, lei nova (Brazil) (Vidal 1978, Rojas 2005).

During the mid-period of the dry season, when the availability of food becomes notoriously scarce, seeds of different species become a source of food and are widely consumed by the fauna. Justiniano & Frederickse (1998) report observations in Bolivian forests of certain vertebrates breaking open the fruits of *Centrolobium microchaete* to consume the seeds; these include *Pyrrhura moline* (parrot gris), *Cebus apella* (monkey) and *Sciurus bolivianensis* (squirrel).

The species *Centrolobium robustum* are tall leafy trees that can reach up to 35 m in height, occupying the dominant and co-dominant levels in the tree stratum. According to their successional status they may be classified as heliophyte pioneer species or as early secondary species (Lorenzi 1992).

This species is found in "cerrados" or "capoeirões", gallery forests, humid and semi-humid forests, semi-deciduous forests of the coast and mountain-maritime

1. Universidade Estadual de Campinas, Instituto de Biologia, Departamento de Fisiologia Vegetal, Caixa Postal 6109, 13083-970 Campinas, SP, Brasil
2. Instituto de Botânica, Seção de Ornamentais, Caixa Postal 3005, 01061-970 São Paulo, SP, Brasil
3. Universidad de Buenos Aires, Ciudad Universitária, Departamento de Biodiversidad y Biología Experimental, C1428EGA, Buenos Aires, Argentina
4. Corresponding author: mschiavi@unicamp.br

areas and in regions with medium to good soil fertility (Vidal 1978, Lorenzi 1992).

The fruit is a samara of up to 25 cm in length, surrounded by a paranuclear coriaceus wing and a seminiferous nucleus globular in form, fibrous in structure and covered with numerous spikes (Vidal 1978, Justiniano & Frederickse 1998). The fruit contains from one to three seeds separated by transversal septa and located in a single locule, forming monospermic chambers (Vidal 1978) surrounded by fibrous-woody mesocarp tissue. The seed measures from 12 to 23 mm in length and from 9 to 10 mm in width, and has the consistency of a nut. It is surrounded by a paper-like tegument, reddish-brown to dark brown in colour (Vidal 1978). The embryo is constituted by a large hypocotyl-radicle axis with a poorly differentiated plumule, and two fleshy, plane-convex and asymmetrical cotyledons (Oliveira 1999).

The aim of the present study was to investigate *Centrolobium robustum* seed structure and ultrastructure and the spatial localization of food reserves. In order to produce a complete study of the seeds, we have used different but complementary methods. Along with a structural investigation using light microscopy, scanning electron microscopy and transmission electron microscopy biochemical studies for determination of lignin, phenols, tannins, protein and lipids composition were also carried out. To our knowledge, this is the first study on *Centrolobium robustum* seed. No studies have been made of the seeds of other species of the genus, particularly with regards to the nature of their storage compounds and nutritional value. This study was done as a prelude to physiological studies on seed structure behaviour.

Materials and methods

Plant material - The fruits of *C. robustum* were collected on the campus of the Universidade Estadual de Campinas, SP, Brazil, between August and September of 2005, and the seeds were carefully manually removed from the fruit. Immediately after collection, seeds had 5.2% (±0.44) water content and showed 100% germination. Water content (percentage wet weight basis) of the whole seed and the isolated embryos were determined on three replications of five seeds by the ISTA (2005) oven methods (103 ºC ± 1 for 17 h). In these conditions, seeds were considered mature. For histochemical and ultrastutural analysis, samples were fixed, immediately following collection. Other seeds were kept in a freezer, at -20 ºC, until use for biochemical analysis.

Histochemistry - For histochemical and ultrastructural studies, 10 seeds were analyzed. Cotyledons and seed coat were studied separately. 1.0 mm-thick sections of these structures were fixed overnight in 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.2 at 4 ºC, dehydrated through a graded ethanol series, embedded in LRWhite resin and examined under an Olympus microscope (model BX40F-3 - Exposure control unit PM20 - Olympus Optical Co. Ltd. Japan - Tokyo). Both fresh unfixed tissues and tissues that had been embedded in resins were used for light microscopy. Semi-thin sections were stained with toluidine blue O (Sigma T 3260 CI 52040) (Feder & O'Brien 1968) and 8-anilino-1-naphthalenesulfonic acid (Sigma A 3125), and epifluorescence (excitation filter: 395 nm; emission filter: 500 nm) (Feder & O'Brien 1968, Yiu *et al*. 1983) used for detecting proteins.

Starch and hemicelluloses were stained using the periodic acid Schiff (PAS) reaction preceded by aldehyde blockage with dimedone (Sigma D 3504), and hemicelluloses identified by fluorescence after application of calcofluor white (M2R, Polyscience) and by epifluorescence (excitation filter 365-380 nm; emission filter: 435-475 nm) (DeMason 1986). The presence of lipids was detected by epifluorescence (excitation filter: 520-560 nm; emission filter 510 nm) after reaction with Nile Blue A (Sigma N5632).
Preparation of tissue for light microscopy (LM) and transmission electron microscopy (TEM) - Excised embryos were cut to obtain separate samples of axis, cotyledons, endosperm and seed coat and endosperm. Samples were immediately fixed for 2 h at 4 °C, using 2.5 % glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, and washed three times with the same buffer. Then, samples were post-fixed in OsO_4 (1.0% in the buffer) for 2 h, dehydrated through a graded ethanol-propylene oxide series and embedded in Spurr's resin, according to Harris *et al*. (1994) and Kuo (2007). Semithin sections (1 μm thick) and ultrathin sections for LM and TEM, respectively, were obtained with an ultramicrotome (Reichert-Jung, Vienna, Austria) with a glass knife in buffer. Sections were mounted on grids, stained with uranyl acetate, followed by lead citrate, and examined under a TEM (JEOL 1200EX II - JEOL Ltd, Akishima, Japan).
Preparation of tissue for scanning electron microscopy (SEM) - Sections of seeds cut longitudinally or transversely were fixed as described for TEM and dehydrated through a graduated ethanol series and exchanged in isopentyl acetate. Then they were

subjected to critical-point drying, followed by mounting on aluminium stubs with double-sided adhesive, sputter coated with gold and examined under a SEM (JEOL JSM 5880LV - Japan) (Coimbra & Salema 1994).

Biochemical analyses - For determination of reserve compounds, the seed coat was separated from the embryo. Samples of 1.0 g of each tissue were homogenized with 25 mL of methanol-chloroform-water (MCW, 12:5:3 v/v/v) (Bieleski & Turner 1966). After centrifugation and phase separation following further addition of chloroform (1 vol.) and water (1.5 vol) to the extract supernatant (4 vol), total soluble sugars were determined in the aqueous phase by the phenol-sulphuric acid method (Handel 1968). The residue obtained after centrifugation of the initial extract was divided into two equal parts. One part was homogenized with 0.1 N NaOH to determine total soluble protein content (Bradford 1976), and the other part homogenized with 10% trichloro-acetic acid to extract water-soluble polysaccharides, followed by 30% perchloric acid for starch extraction. Both these components were determined by the anthrone method described by Graham & Smydzuk (1965).

Free lipid content was determined according to Gemmrich (1977) and lignin, after acid hydrolysis, according to Hatfield *et al*. (1994). Total tannin content was estimated by difference between total phenols (Singleton *et al*. 1999) and simple phenols. The latter determined with the Folin-Ciocalteu reagent, after precipitation of the tannins with polyvinilpolypyrrolidone (PVPP). The levels of condensed tannins were determined on aliquots of the aqueous extract obtained in the previous step, using the Stiasny reaction (Wissing 1955):

$(Abs_{(550\ nm)} \times 78.26 \times \text{dilution factor}) / (TE)$

Where:

$Abs_{(550\ nm)}$ = Absorbance reading at 550 nm; 78.26 = the Stiasny number; dilution factor = number of times the crude extract was diluted; TE = level of total extractives, calculated as follows:

[(Initial mass - Final mass) / Final mass] × 100

The data transformed in percentage represents the level of condensed polyphenols in the extract.

Results

Seed coat - At the mature seed stage, the seed coat of *C. robustum* was observed to be composed of two sclereid layers, the outer, constituted by macrosclereids, and an internal layer of osteosclereids (figure 1A, B). The remaining tissues of the outer integument and all the cellular layers derived from the inner integument do not persist in the mature seed; only some parenchymatous layers of dead cells remain. Cellulose and hemicellulose of the cell walls were detected through metachromatic staining with PAS and by epifluorescence with Calcofluor (excitation filter 365-380 nm; emission filter: 435-475 nm) (figure 1B).

SEM revealed that the surface of the *C. robustum* seed coat was rough, undulated, with shallow depressions (figure 1C). Stomata were observed on the external epidermis of the outer integument of the *C. robustum* seed (figure 1D).

Endosperm - An endosperm closely linked to the seed coat was observed. Endosperm was constituted by one to two layers of living cells with relatively thick walls and the remains of dead and flattened cells (figure 2A). The intense fuchsia staining of the PAS reagent detected the presence of hemicellulose in the endosperm cell walls. Histochemical tests and TEM observations revealed that the living cells of the endosperm contained a nucleus and that the cytoplasm was rich in lipid bodies and protein bodies (figure 2B).

Embryo - All cells that make up the different tissues of the embryo of *C. robustum*, including the apical meristematic tissues, accumulated mainly lipids and proteins in the form of lipid and protein bodies (figure 2B, C). As with the endosperm, protein bodies stained blue with toluidine blue and fluoresced with the ANS fluorochrome (figure 2D). The lipid bodies fluoresced yellow-green with the fluorochrome Nile Blue (data not shown). On staining the cotyledon cells with Calcofluor White, their fine walls fluoresced blue, indicating the presence of cellulose and hemicellulose (figure 2E). They also reacted with PAS, staining fuchsia.

Lipid bodies were observed surrounding the protein bodies. The lipid bodies of the cotyledonary cells seeds varied in size from 1.5 to 25 μm. It was observed that the protein bodies of cotyledonary cells consisted of a proteinaceous matrix enclosing globoid crystals of different sizes (figure 3A, B). Cytoplasm was very reduced and nuclei were relatively large and lobed (figure 3A-D). De-differentiated organellas, *i.e*. mitocondria and proplastids were difficult to identify because the poorly defined cristae in mitochondria (figure 3C), and absences of grana in proplastids.

Biochemical analysis - Lignin was present in the seed coat, at 445.6 g kg^{-1} fresh mass, corresponding to 64.7% of the embryo value (figure 4A). Simple phenols were

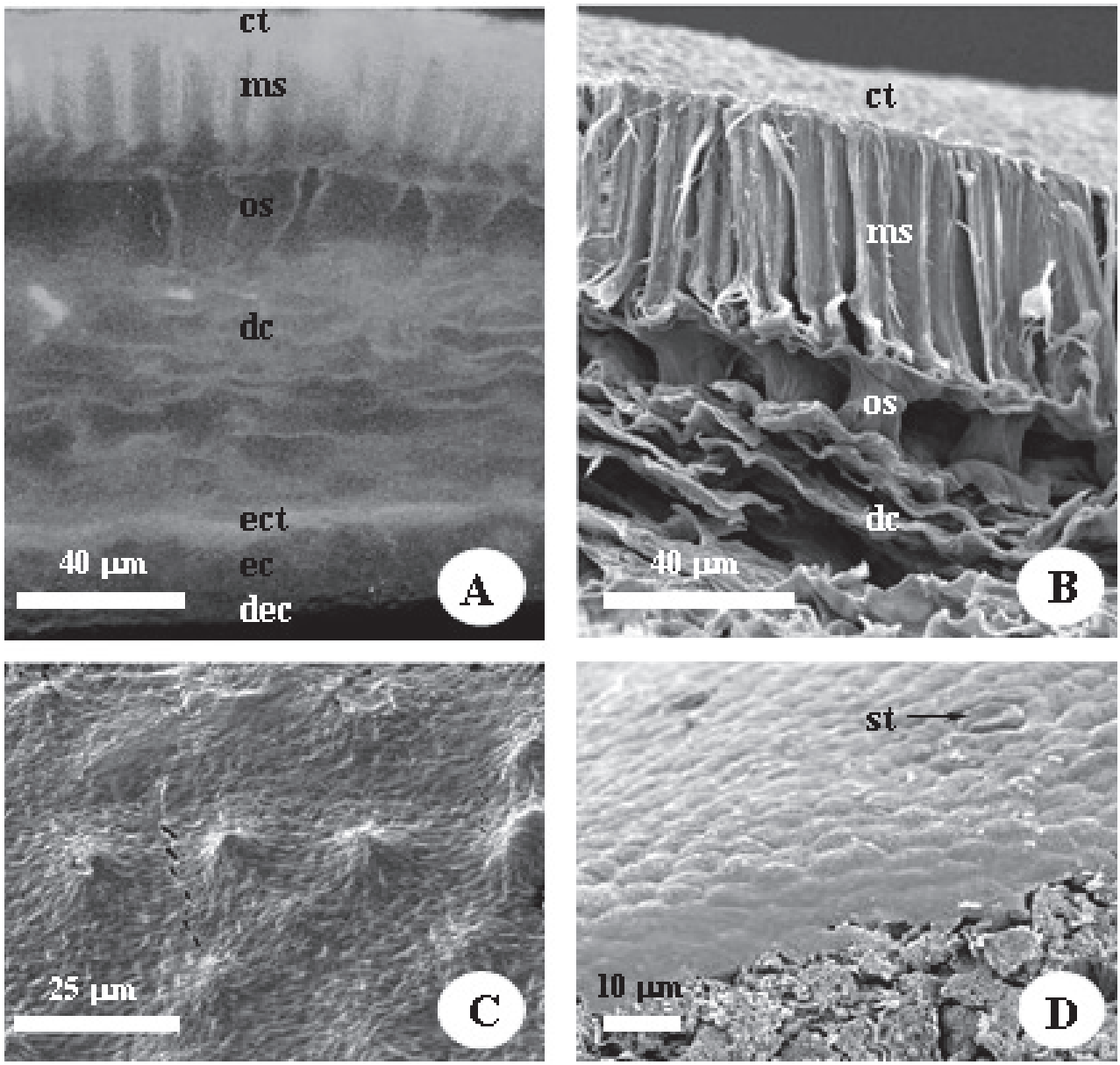

Figure 1. Transverse section of *Centrolobium robustum* seed coat, using Calcofluor White as fluorochromes and observed by epifluorescent microscopy (excitation filter 365-380 nm; emission filter: 435-475 nm). It can be seen that all cell wall were fluorescent (A). SEM (Scanning electron microscopy) of a transverse section of the seed coat (B). SEM of the seed coat surface. It can be observed that surface is rugose-foveate, with undulations, shallow depressions and small cavities (C). SEM of external epidermis of the outer integument, showing presence of stomata (D). ct = cuticle of the seed coat; dc = dead cells, remains of the internal layers to the outer integument and of the inner integument; dec = digested endosperm cells; ec = living endosperm cells; ect = endosperm cuticle; ms = macroesclereids; oc = osteosclereids; st = stomata.

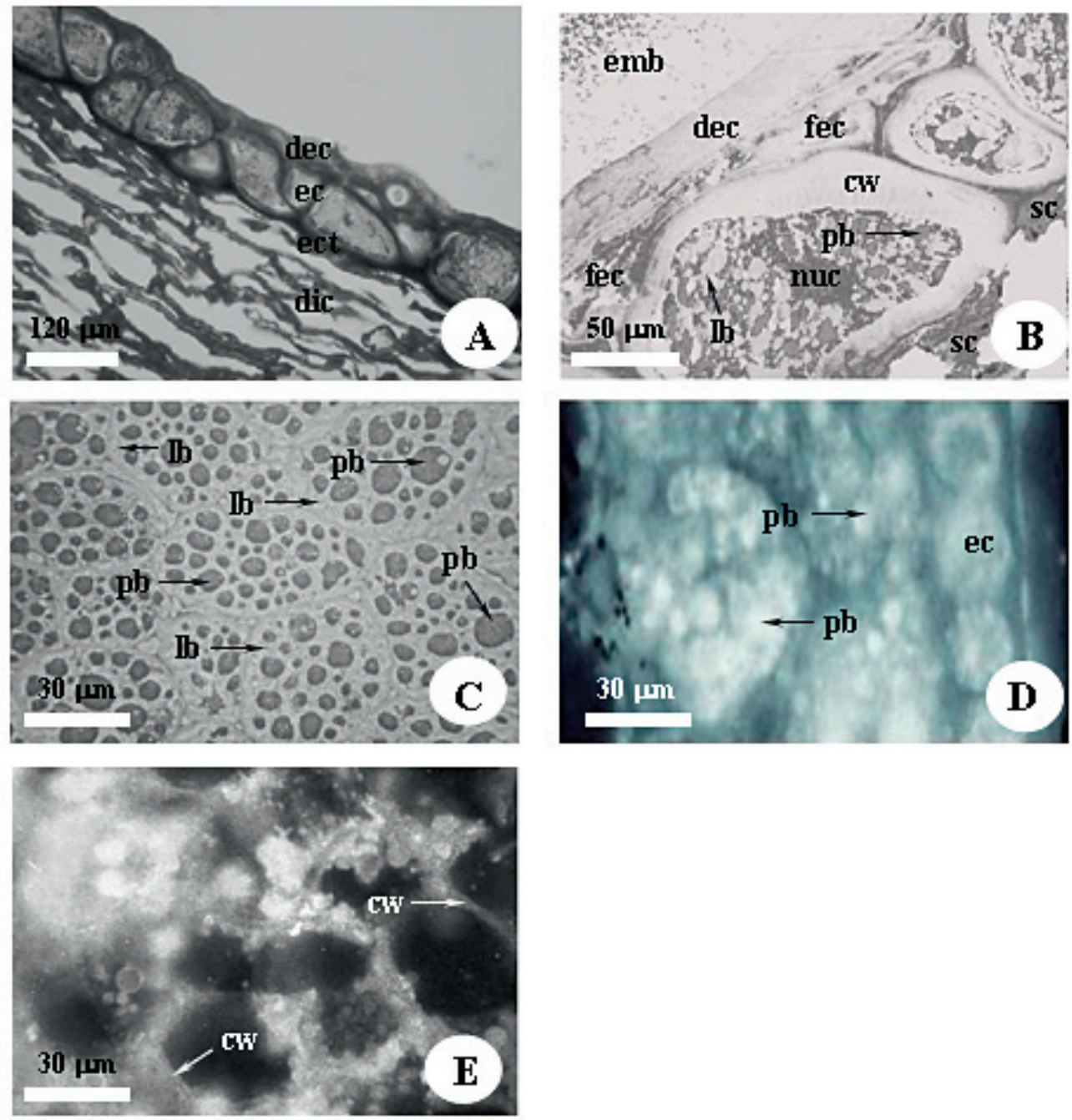

Figure 2. Micrographs of the *Centrolobium robustum* endosperm and inner integument. Thick cell walls of the endosperm reacted with PAS (A). Micrograph showing details of the content of the living endosperm layer and underlying collapsed layers, stained metachromatically with toluidine blue. In cells of the endosperm living layer can be seen (B). Micrograph of a cotyledonary section, stained with toluidine. Protein bodies and lipid bodies can be seen (C). Epifluorescence of protein bodies using ANS (excitation filter: 395 nm; emission filter: 500 nm)(D). Epifluorescence of cell walls (arrows) of the embryo using Calcofluor White (excitation filter 365-380 nm; emission filter: 435-475 nm) (E). cw = cell wall; dec = digested endosperm cells; dic = dead cells of the inner integument; ec = living endosperm cells; ect = endosperm cuticle; emb = embryo; fec = layer of flattened endosperm cells; lb = lipid bodies; nuc = nucleus; pb = protein bodies; sc = seed coat.

found to be present in the seed coat, at 114.4 g kg^{-1} fresh mass, corresponding to 95.2% in relation to the embryo, together with hydrolysable tannins, at 57.1 g kg^{-1} fresh mass, corresponding to 86.4% of the embryo value (figure 4B) and condensed tannins (figure 4C), at 25.2%, according to the Stiasny reaction.

From the biochemical analyses made of the seed reserves of *C. robustum* it was found that the lipids correspond to more than 68% of the reserves, both in the integument and the cotyledons. With regard to total soluble proteins in *C. robustum* seeds the highest level was found in the cotyledons with 21%, while the tegument had 11%. Other reserves such as polysaccharides, total sugars and starch, the levels found were relatively small, compared with lipids and proteins (figure 5A, B).

Discussion

The two outer layers of the outer integument, *i.e.* the macrosclereid with the typical light line, and the subjacent osteosclereid layer form seed coat constituted the seed coat in the mature seeds of *C. robustum*. Cell walls of the remaining tissues of the outer integument and all the cellular layers derived from the inner integument persist in the mature seed.

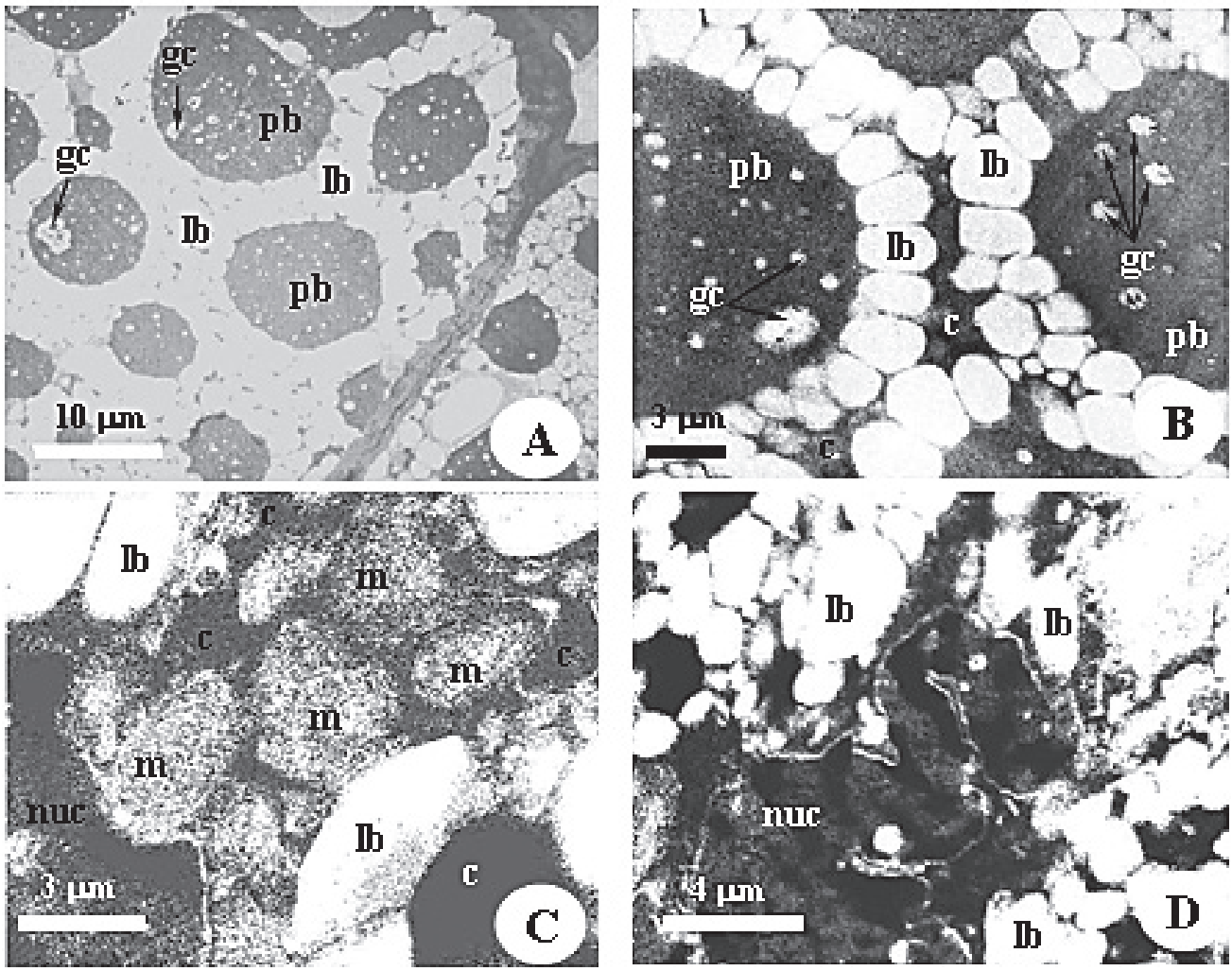

Figure 3. Transmission electron micrographs of sections of *Centrolobium robustum* embryo cells. Part of a cell showing lipid and protein bodies. Empty areas included in protein body matrix contained globoid crystals before they were chipped out during fixation or sectioning (A). In detail, protein bodies, lipid bodies and areas that had been contained globoid crystals (B). Detail of a group of de-differentiated mitochondria, lipid bodies, nucleus cointained in cytoplasm (C). Detail of part of a nucleus and lipid bodies (D). c = cytoplasm; gc = globoid crystals; pb = protein bodies; m = de-differentiated mitochondria; lb = lipid bodies; nuc = nucleus.

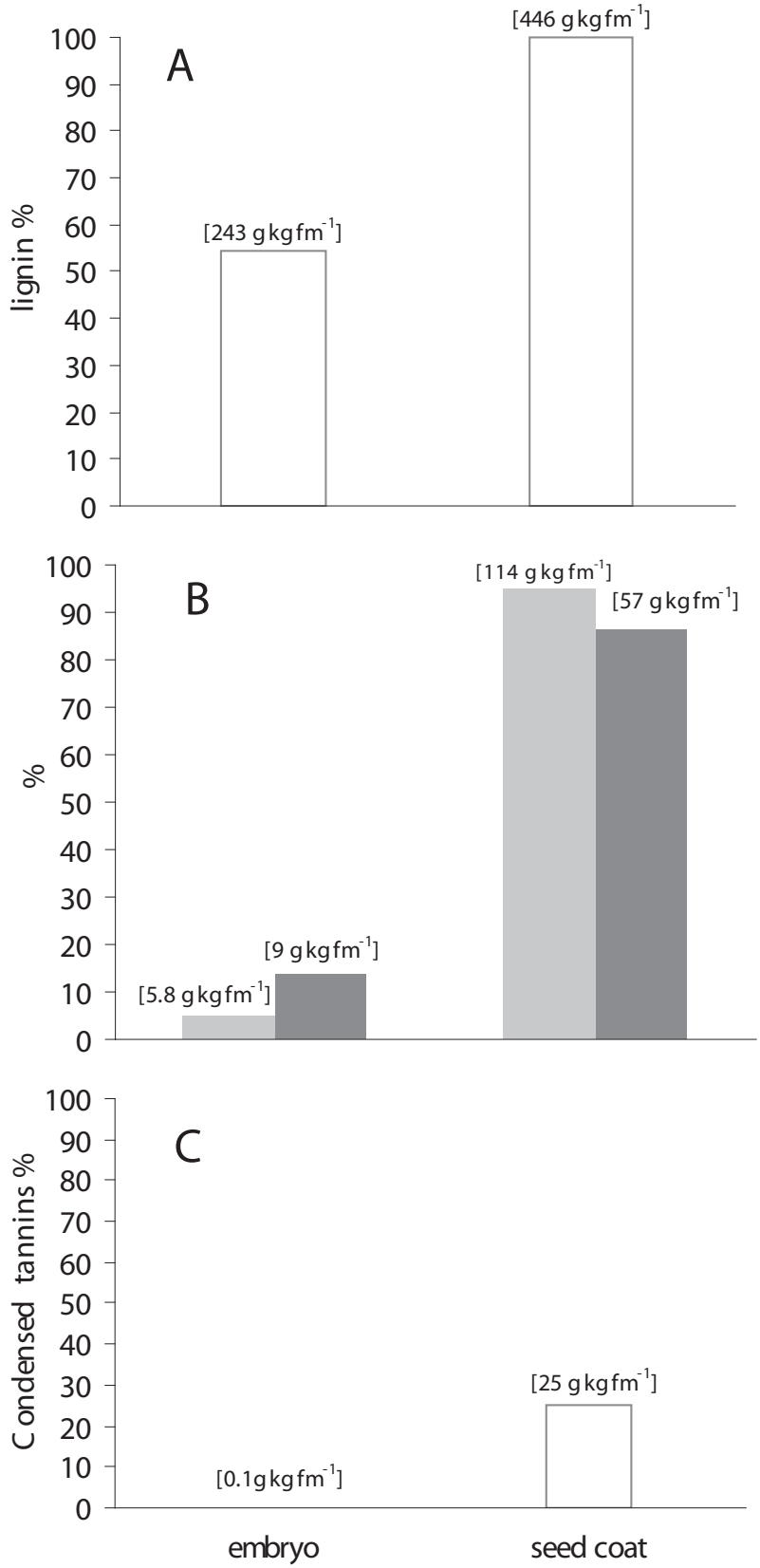

Figure 4. Relative percentage and concentrations of lignin, simple phenols, hydrolysable and condensed tannins, in the seed coat and embryo of *Centrolobium robustum* seed. Lignin (A). Simple phenols (■) and hydrolysable tannins (■) (B). Condensed tannins (C).

All these characteristics coincide with those known for seeds of legumes (Gunn 1981).

According to Beltrati & Paoli (2003), a seed can be denominated testal, when its main layer of mechanical tissue is present in the external epiderm and exotestal when the external epiderm forms a rigid palisadal layer made up of macrosclereid-type cells with thick lignified walls elongated longitudinally. Considering these characteristics together with the observations here made, the seed of *C. robustum* could be classified as belonging to the exotestal type of a testal seed.

The surface of the *C. robustum* seed coat was rough, undulated, with shallow depressions. According to the terms adopted by Zeng *et al.* (2004), this type of surface can be described as rugose-foveate.

Lersten (1979) reports that seeds of the Vicieae consistently show a papillose pattern on their testae. The SEM images of *C. robustum* testa revealed also a papillose patterns.

Stomata were observed on the external epidermis of the outer integument of the *C. robustum* seed. According to Beltrati & Paoli (2003), stomata are observed on the external epidermis of the seed coat of about 30 families of the angiosperms. Normally the stomata are localized on the leaves of plants, where they regulate gas exchange with the atmosphere and function as pores, but according Rugenstein & Lersten (1981) they are rarely found in seeds. Rugenstein & Lersten (1981) find stomata in the irregularly reticulate of the mature seed epidermis of eight *Bauhinia* species. Rosa *et al.* (2002) cite the presence of stomata on the epidermis of the seed coat close to the raphe of *Pisonia aculeata* L. (Nyctaginaceae), and Teixeira *et al.* (2004) mention that the presence of stomata and the absence of osteosclereids in the seed coat of the *Caesalpinia echinata* seed are related to a faster rate of germination. Nevertheless, little is known of the function of these stomata in seeds, but probably they allow gas exchange for photosynthesis during seed development, but before seed tissues degreening.

Endosperm - Although Vidal (1978) has classified *C. robustum* seed as exalbuminous, we observed an endosperm closely linked to the seed coat. The greater part of the endosperm was consumed during seed development. A reduced endosperm is also found in other angiosperm species that have also been classified as exendospermous, such as, species of all tribes of Asteraceae (Compositae), whose an endosperm of 1 to 3 layers envelops the embryo. Nevertheless, according to Grau & Hopf (1985), the occurrence of an endospermal tissue in this family in the taxonomical literature is widely neglected. In *Bulnesia* (Zygophyllaceae), whose endosperm is observed in different proportions and with different ultrastructural profiles (Maldonado *et al.* 1998); *Chenopodium quinoa* (Chenopiaceae), where the endosperm is present only in the micropylar region and consists of one or two layers of thick-walled cells surrounding the hypocotyl-radicle axis of the embryo (Prego *et al.* 1998); and other legume species. In Fabaceae *s.l.*, seeds may or may not possess a discernible endosperm that varies from a trace adnate to the inner integument and adjacent to the radicle, to an endosperm that encases the embryo and is as thick as or thicker than the cotyledons. A massive endosperm (often massive amounts) is phylogenetically associated with more primitive species (Gunn 1981). Therefore, a reduced endosperm is associated with more advanced species

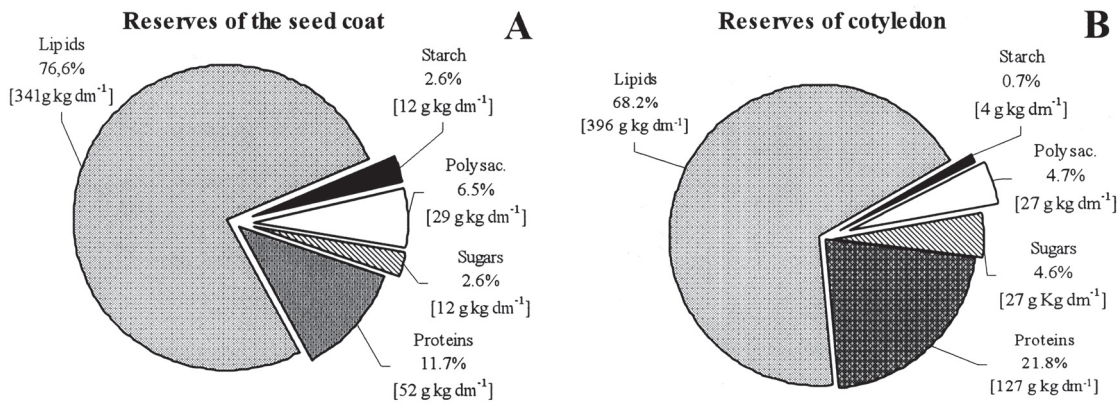

Figure 5. Relative content (%) and concentration of reserves present in the *Centrolobium robustum* seed. Seed coat (A). Cotyledons (B).

(Grau & Hopf 1985). On the other hand, the legume endosperm may be homogeneous or heterogeneous (Reid & Bewley 1979). In the homogeneous type, the endosperm is totally composed of living storage cells, while in the heterogeneous type only the outer layer is made up of living cells and the rest are dead cells with a lumen totally occupied by galactomanans (Reid & Bewley 1979, Reid 1985, Reid & Edwards 1995, Reid *et al*. 1995, Edwards *et al*. 2002). The endosperm of the *C. robustum* seed was the heterogeneous type.

Storage reserves in embryo and endosperm tissues - In *C. robustum*, the endosperm and embryo cells were observes filled with lipids and protein bodies and with nuclei irregular in form, pressured by protein and lipid bodies. In general, the mitochondrias and plastids were seen dedifferentiated. According to Vertucci & Farrant (1995) during the latter stages of storage reserves accumulation, in orthodox seeds there is a general dedifferentiation of subcellular organelles such as mitochondria, and chloroplasts. Degreening (loss of thylakoid membranes and chlorophyll) of orthodox embryos is coincident with the onset of extreme desiccation tolerance. The reduction of membrane surface area in organelles does not imply that these organelles become dysfunctional. The reduction in internal membrane surface may be, according to Vertucci & Farrant (1995), is a mechanism to slow metabolism and avoid the physical consequences of drying highly membranous system. In *C. robustum* embryo tissues, acquisition of desiccation tolerance (mature seeds with the very low water content, *i.e*. 5.2% (± 0.44)) together with subcellular feature (*i.e*. de-differentiation of organelles) are indicating desiccation tolerance, and anticipating an orthodox behaviour for these seeds. A detailed study of mitochondria of *Phaseolus vulgaris* cotyledons, made by Opik (1965), shows that mitochondria in the cotyledons are de-differentiated. The shape of the nucleus in embryo cells appears to depend on the tissue in which it occurs. In the storage cells the nuclei are usually large and lobed as in other species of Fabaceae, such as *Phaseolus vulgaris* (Opik 1965) and *Vicia faba* (Briarty *et al*. 1970).

As above-mentioned, endosperm and embryo cells of the *C. robustum* embryo accumulated lipids and proteins in the form of lipid and protein bodies and protein bodies enclosed globoid crystals. Embryos of *C. robustum* also stored starch, but in relatively lower quantities.

The lipid bodies of the cotyledonary cells of *C. robustum* seeds varied in size from 1.5 to 25 µm and are therefore within the size range reported by Murphy & Vance (1999), who observed values between 0.1 to 50 µm. The latter authors mention that the lipid bodies are dispersed in the cytoplasm or aggregated in large clumps, easily recognized as protoplasmatic inclusions. On the other hand, Huang (1992) suggests that the size of these lipid bodies depends on the species as well as environmental and nutritional factors. Vertucci & Farrant (1995) report that lipid bodies are deposited along the periphery of the plasmalemma in a number of species and suggest that they would serve as reservoirs for lipid that will be required when the plasmalemma expands during imbibition and germination.

According to Lott (1981), the seeds of angiosperms store most of their reserve proteins in storage vacuoles known as protein bodies, which enclose globoid crystals. As found for *C. robustum*, the presence of lipid bodies and protein bodies in the embryo is reported in the seeds of other legume species, such as *Caesalpinia pyramidalis*, *Senna spectabilis*, *Eritrina velutina*, *Canavalia brasiliensis*, *Poecilanthe ulei*, *Acacia bahiensis*, *A. farnesiana*, *Mimosa arenosa* (Mayworm *et al*. 1998); *Lupinus albus*, *L. angustifolius* and *L. luteus* (Pozuelo *et al*. 2001), and seeds of species of other families, such as *Amaranthus* (Amaranthaceae) (Coimbra & Salema 1994), *Bulnesia* (Zygophyllaceae) (Maldonado *et al*. 1998), *Chenopodium* (Chenopodiaceae) (Prego *et al*. 1998), *Myrsine laetevirens* (Myrsinaceae) (Otegui *et al*. 1998) and *Euterpe edulis* (Palmae) (Panza *et al*. 2004), as well as other species.

As above-mentioned, in seeds of *C. robustum*, the lipid and protein bodies were found both in the cotyledons and endosperm. Moloney (1999) observes that lipid bodies in endospermous seeds of dicotyledonous plants accumulate mainly in the endosperm while Huang (1992) reports that in exendospermous seeds of dicotyledons these lipid bodies are found mainly in the cotyledons and the embryo axis. In part, these observations would be valid for *C. robustum* seeds, except in that lipid bodies are present in both endosperm and embryo.

Globoid crystals are constituted by phytin that is the K, Mg salt of *myo*-inositol hexaphosphoric acid (Bewley & Black 1994). Coimbra & Salema (1994) report that protein body inclusions can vary in number and form, whether from one tissue to another or in the same cell. The presence of globoid crystals is also reported for embryonic cells of other legume seed, such as *Arachis hypogaea*, *Medicago sativa*,

Phaseolus lunatus, *Pisum sativum*, *Vigna unguiculata* (Lott 1981), *Lupinus albus*, *L. angustifolius* and *L. luteus* (Pozuelo *et al*. 2001), and seeds of species of other families, such as *Cucurbita maxima* (Cucurbitaceae), *Bidens cernua*, *Helianthus annuus*, *Lactuca sativa* (Asteraceae), *Capsella bursa-pastoris*, *Brassica campestris* (Brassicaceae), *Cucumis sativus* (Cucurbitaceae) (Lott 1981), among other species.

The occurrence of starch grains in cotyledons depends on the species. They occur rarely in the oil-storing seeds but are common in some cotyledons, notably of legumes. In legume seeds, cotyledons store protein, lipids and starch, although the proportions vary for different species. For example, in broad bean (*Vicia faba*) the ratio of protein:lipid:starch is 23:1:56, but in *Arachis hypogaea* (peanut) it is 31:48:12 (Bewley & Black 1994).

Biochemical analysis - Lignin was present in the seed coat, and according to Taiz & Zeiger (2006) is associated with hemicellulose, providing mechanical support for this structure. On the other hand, simple phenols are found to be present in the seed coat, as well as hydrolysable tannins and condensed tannins. Condensed tannins are a group of polymeric phenols that, together with lignins, confer defence properties towards attack by bacteria, fungi, viruses and insects (Temmink *et al*. 1989). According to Monteiro *et al*. (2005), the tannins have the function of inhibiting herbivorous organisms, since at high concentrations they become unpalatable to phytophages. The authors also suggest that the tannins, in combination with some proteins, render plant tissues strongly resistant to putrefaction. Furthermore, the tannin content can vary according to the environmental, climatic, and geographic conditions, as well as to the restriction of soil nutrients or effects of atmospheric pollution (Monteiro *et al*. 2005). According to Laurena *et al*. (1984), the condensed tannins are related to the colour of the testa or seed coat. This can vary from white to yellow, red, brown and black. They also report that in the testa of *Vigna unguiculata*, the content of condensed tannins in the mature seeds varies from 14.7% for a reddish seed coat to 5.5% for a white seed coat. On the other hand, Guzman-Maldonado *et al*. (1996) report that condensed tannins in the seed coat of *Phaseolus vulgaris* varied from 28.5% for black to 37.4% for yellow seed coats. The high level of condensed tannins in the integument of *C. robustum* appears to be responsible for its typical reddish colour.

The seed reserves guarantee the survival of the seedlings during the initial stages of growth, until a capacity for photosynthesis is attained. Physically, the lipid bodies present a large surface area that permits rapid mobilization of these reserves during seedling growth (Huang 1992). Murphy *et al*. (2001) observe that the mobilization of neutral lipids, stored within the lipid bodies, takes place after germination. Huang (1992) mention that the majority of angiosperm seeds, especially those of legumes, store high concentrations of lipids as reserve material. According to Barclay & Earle (1974) this type of seed is also rich in proteins. The high level of lipids in the embryo of *C. robustum* seeds was in the range of other seeds classified as oilseeds, as for example, *Bertholletia excelsa* (Lecythidaceae) (70% fat), *Prunus dulcis* (Rosaceae) (54% fat), *Arachis hypogaea* (Fabaceae) (50% fat), *Anacardium occidentale* (Anacardiaceae) (43.4% fat), *Cocos nucifera* (Arecaceae) (41.6% fat), *Myristica fragans* (Myristicaceae) (33% fat), *Glycine max* (Fabaceae) (17.7% fat) (http://pt.wikipedia.org; http://www.hort.purdue.edu).

Otegui *et al*. (1998) observe a protein content in the embryo of *Myrsine* of 3.4%, whereas Pozuelo *et al*. (2001) report that the embryo of *Lupinus* seeds contain between 30-40% of proteins. According to Taiz & Zeiger (2006), the main function of these reserve proteins, both in the seed coat and embryo, is to supply amino acids for the formation of enzymes during germination, which are utilized by the cells to digest their own protein and lipid reserves.

According to Barclay & Earle (1974), when a seed has a high lipid and protein content, the levels of starch are low, which corresponds to that found in seeds of *C. robustum*, as clearly shown by the biochemical and epifluorescent analyses. Beltrati & Paoli (2003) report that starch grains can be found in the endosperm associated with proteins in amorphous granules. Although chemical analysis of the tegument of *C. robustum* showed the presence of starch, this presumably was in the endosperm tissue that is closely associated with the tegument.

Acknowledgements

We thank Dr. Mary Anne Heidi Dolder, head of the "Laboratório de Microscopia Eletrônica", Dr. Paulo Pinto Joazeiro, of the "Departamento de Histologia e Embriologia, Instituto de Biologia, Universidade Estadual de Campinas", Brazil and Dr. Elliot Kitajima, of the "Escola Superior de Agricultura Luiz de Queiroz, Universidade de São Paulo", Brazil, for their collaboration with the Scanning Electron

Microscope. We thank Dr. Ladaslav Sodek for reviewing the manuscript, and CNPq and FAPESP for financial support.

Literature cited

Barclay, A.S. & Earle, F.R. 1974. Chemical analyses of seeds III. Oil and protein content of 1253 species. Economic Botany 28: 178-236.

Beltrati, C.M. & Paoli, A.A. 2003. Semente. *In*: B. Appezzato-da-Gloria & S.M. Carmello-Guerreiro (eds.). Anatomia vegetal. Universidade Federal de Viçosa, Viçosa, pp. 399-424.

Bewley, J.D. & Black, M. 1994. Seeds. Physiology of Development and Germination. 2 ed. Plenum Press, New York.

Bieleski, L.R. & Turner, N.A. 1966. Separation and estimation of amino acids in crude plant extracts by thin-layer electrophoresis and chromatographs. Analytical Biochemistry 17: 278-293.

Bradford, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principles of protein-dye binding. Analytical Biochemestry 72: 248-256.

Briarty, L.G., Coult, D.A. & Boulter, D. 1970. Protein bodies of germinating seeds of *Vicia faba*. Journal of Experimental Botany 21: 513-524.

Coimbra, S. & Salema, R. 1994. *Amaranthus hypocondriacus*: seed structure and localization of seed reserve. Annals of Botany 74: 373-379.

DeMason, D. 1986. Endosperm structure and storage reserve histochemistry in the palm, *Washingtonia filifera*. American Journal of Botany 73:1332-1340.

Edwards M.E., Marshall E.,Gidley M.G. & Grant Reid, J.S. 2002. Transfer specificity of detergent-solubilized fenugreek galactomannan galactosyltransferase. Plant Physiology 129: 1391-1397.

Feder, R.G. & O'Brien, T.P. 1968. Plant micro-techniques: some principles and new methods. American Journal of Botany 55: 123-142.

Gemmrich, A.R. 1977. Mobilization of reserve lipids in germinating spores of the fern *Anemia phyllitidis* L. Plant Science Letters 9: 301-307.

Graham, D. & Smydzuk, J. 1965. Use of anthrone in the quantitative determination of hexose phosphates. Analytical Biochemistry 11: 246-255.

Grau, J. & Hopf, H. 1985. Das Endosperm der Compositae. Botanisch Jahrbucher 107: 251-268.

Gunn, C.R. 1981. Seeds of Leguminosae. *In*: R.M. Polhill & P.H. Raven (eds.). Advances in Legume Systematics. Royal Botanical Garden, Kew, pp. 913-926.

Guzman-Maldonado, H., Castellanos, J. & Gonzalez-De-Mejía, E. 1996. Relationships between theoretical and experimentally detected tannin content of common beans (*Phaseolus vulgaris* L.). Food Chemistry 55: 333-335.

Harris, N., Spence, J. & Oparka, KJ. 1994. Plant cell biology: a practical approach. Oxford University Press, New York.

Hatfield, R.D., Jung, H.J., Ralph, J., Buxton, D.R. & Weimer, P.J. 1994. A comparison of the insoluble residues produced by the Klason lignin and acid detergent lignin procedures. Journal Science of Food Agriculture 65: 51-58.

Huang, A.H. 1992. Oil bodies and oleosins in seeds. Annual Review of Plant Physiology and Plant Molecular Biology 43: 177-200.

ISTA. 2005. International rules for seed testing. The International Seed Testing Association (ISTA), Bassersdorf.

Justiniano, M.J. & Fredericksen, T.S. 1998. Ecologia y silvicultura de espécies menos conocidas. Tarara Amarilla. *Centrolobium microchaete*. Papilionoideae. Editora El Pais, Santa Cruz.

Kuo, J. 2007. Processing Plant tissues for ultrastructural study. Methods in Molecular Biology 369: 35-45.

Laurena, A.C., Truong, V.D. & Mendoza, E.M. 1984. Effects of condensed tannins on the in vitro protein digestibility of Cowpea (*Vigna unguiculata* (L.) Walp.) Journal of Agricultural and

Food Chemistry 32: 1045-1048.

Lersten, N.R. 1979. A distinctive seed coat pattern in the Vivieae (Papilionoideae; Leguminosae). Proceedings of the Iowa Academy of Science 86: 102-104.

Lorenzi, H. 1992. As árvores brasileiras: manual de identificação e cultivo de plantas nativas do Brasil. 2 ed. Plantarum, Nova Odessa.

Lott, J.N. 1981. Protein body in seeds. Nordic Journal of Botany 3: 421-432.

Maldonado, S., Lima, C., Etchart, M., Lainez, V. & Lederkremer, R.M. 1998. Ultrastructural and chemical studies on seeds of *Bulnesia schickendantzii* and *Bulnesia bonariensis*. Acta Botanica Neerlandica 47: 285-297.

Mayworm, M.A., Nascimento, A.S. & Salatino, A. 1998. Seeds of species from the 'caatinga´: proteins, oils and fatty acid contents. Revista Brasileira de Botânica 21: 299-303.

Moloney, M.M. 1999. Seed oleosins. *In*: P.R. Shewry & R. Casey (eds.). Seed Proteins. Kluwer, Dordrecht, pp. 781-806.

Monteiro, J.M., Albuquerque, U.P., Araújo, E.L. & Amorim, E.L. 2005. Taninos: uma abordagem da química à ecologia. Química Nova 28: 892-896.

Murphy, D.J. & Vance, J. 1999. Mechanisms of lipid-body formation. Trends in Biochemical Sciences 24: 109-115.

Murphy, D.J., Pinzón, I.H. & Patel, K. 2001. Role of lipid bodies and lipid-body proteins in seeds and other tissues. Journal of Plant Physiology 158: 471-478.

Oliveira, D.M.T. 1999. Morfo-anatomia do embrião de leguminosas arbóreas nativas. Revista Brasileira de Botânica 22:413-427.

Opik, H. 1965. Respiration rate, mitochondrial activity, and mitochondrial structure in the cotyledons of *Phaseolus vulgaris* L during germination. Journal of Experimental Botany 16: 667-682.

Otegui, M., Lima, C., Maldonado, S. & Lederkremer, R.M. 1998. Histological and chemical characterization of *Myrsine laetevirens* seed. International Journal of Plant Sciences 159: 762-772.

Panza, V., Láinez, V. & Maldonado, S. 2004. Seed structure and histochemistry in the palm *Euterpe edulis*. Botanical Journal of the Linnean Society 145: 445-453.

Pozuelo, J.M., Lucas, M.M., Lorenzo, C., Fernández-Pascual, M., Maldonado, S. & Felipe, M.R. 2001. Immunolocalization of alkaloids and X-ray microanalysis of elements in lupin seeds. Protoplasma 218: 104-111.

Prego, I., Maldonado, S. & Otegui, M. 1998. Seed structure and localization of reserves in *Chenopodium quinoa*. Annals of Botany 82: 481-488.

Reid, J.S.G. & Bewley, J.D. 1979. A dual role for the endosperm and its galactomannan reserves in the germinative physiology of fenugreek (*Trigonella foenum-graecum* L.), an endospermic leguminous seed. Planta 147:145-150.

Reid, J.S.G. & Edwards, M. 1995. Galactomannans and other cell wall storage polysaccharides in seeds. *In*: A.M. Stephen (ed.). Food polysaccharides and their applications. Marcel Dekker, New York, pp. 55-186.

Reid, J,S.G., Edwards, M., Gidley, M.J. & Clark, A.H. 1995. Enzyme specificity in galactomannan biosynthesis. Planta 195: 489-495.

Reid, J.S.G. 1985. Cell wall storage carbohydrates in seeds: biochemistry of the seed "gums" and "hemicelluloses". Advances in Botanical Research 11: 125-155.

Rojas, C.G. 2005. Checklist das plantas do Nordeste. Versão I. 2005. http://www.umbuzeiro.cnip.org.br/db/pnechk/bib.html#465 (access in 18.07.2005).

Rosa, S.M., Souza, L.A. & Moscheta, I.S. 2002. Morphology and anatomy of the development of the anthocarp and fruit of *Pisonia aculeata* l. (Nyctaginaceae). Acta Científica Venezolana 53: 245-250.

Rugenstein, S.R. & Lersten, N.R. 1981. Stomata on seeds and fruits of *Bauhinia* (Leguminosae: Caesalpinioideae). American Journal of Botany 68: 873-876.

Singleton, V.L., Orthofer, R. & Lamuela-Ravenos, R.M. 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Methods Enzymology 299: 152-178.

Taiz, L. & Zeiger, E. 2006. Plant Physiology. 4 ed. Sinauer Associates, Sunderland.

Teixeira, S.P., Carmello-Guerreiro, S.M. & Machado, S.R. 2004. Fruit and seed ontogeny related to the seed behavior of two tropical legumes. Botanical Journal of the Linnean Society 146: 50-70.

Temmink, J.H., Field, J.A., Vanhaastrecht, J.C. & Merkelbach, R.C. 1989. Acute and sub-acute toxicity of bark tannins in carps (*Cyprinus carpio* L.). Water Research 23: 341-344.

Vertucci C.W. & Farrant J.M. 1995. Acquisition and loss of desiccation tolerance. *In*: M. Negbi & J. Kigel (eds.). Seed development and germination. Marcel Dekker, New York, pp. 237-271.

Vidal, W.N. 1978. Considerações sobre as sâmaras que têm ala para-nuclear. Rodriguésia 47: 109-168.

Wissing, A. 1955. The utilization of bark II. Investigation of the Stiasny-reaction for the precipitation of polyphenols in Pine bark extractives. Svensk Papperstidning 58: 45-750.

Yiu, S.H., Altosaar, I. & Fulcher, R.G. 1983. The effect of commercial processing on the structure and microchemical organization of rapeseed. Food Microstructure 2: 165-173.

Zeng, C.L., Wang, J.B., Liu, A.H. & Wu, X.M. 2004. Seed coat microsculpturing changes during seed development diploid and amphidiploid *Brassica* species. Annals of Botany 93: 555-566.