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Ultrastructural and chemical studies on seeds of *Bulnesia schickendantzii* and *Bulnesia bonariensis*

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SUMMARY

Bulnesia, a genus of the Zygophyllaceae, comprises two subgenera, subgenus *Bulnesia* and subgenus *Gonopterodendron*. One species of each subgenus was studied: *B. schickendantzii* and *B. bonariensis*, respectively. *B. schickendantzii* endosperm consists of four to six cell layers. Cell walls are composed of a middle lamella, a very thick outer wall and a thin inner wall. *B. bonariensis* endosperm is reduced to one layer of cells. The cell walls are formed by a middle lamella and a thinner wall. Protein bodies are observed in the embryo of both species and in the endosperm of *B. schickendantzii*. Lipid vesicles are present in the embryo and endosperm of both species. Phytin crystals, present in the protein bodies of *B. schickendantzii*, are rare in the protein bodies of *B. bonariensis*. *B. schickendantzii* endosperm contains the majority of sugars and proteins. In *B. bonariensis* the higher percentage of carbohydrate and protein is found in the embryo. Determination of neutral sugars was performed after acid hydrolysis. Sugars were analysed as alditol acetates by gas-liquid chromatography. Arabinose, xylose, galactose and glucose are the main monosaccharides but the ratio differs in tissues of both species. Soluble proteins were analysed by polyacrylamide gel electrophoresis, showing a different profile in tissues of both species. Very few bands are glycosylated. The ultrastructural and chemical differences observed between both species suggest the segregation of *Gonopterodendron* from the genus *Bulnesia* as a separate genus and encourage further studies of the other six species in order to confirm this assertion.

Key-words: *Bulnesia*, carbohydrates, seed proteins, seed ultrastructure, Zygophyllaceae.

INTRODUCTION

Bulnesia Gay, a South American genus of Zygophyllaceae, includes eight species, five of them native of Argentina. The genus has been divided into two subgenera: the subgenus *Bulnesia* consists of four species, *B. retama* (Gill. ex Hook & Arn.) Griseb., *B. chilensis* Gay, *B. foliosa* Griseb., and *B. schickendantzii* Hieron., mainly characterized

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by endospermous seeds, and the subgenus *Gonopterodendron* Griseb. composed by *B. arborea* (Jacq.) Engl., *B. carrapo* Killip & Dugand, *B. sarmientoi* Lorentz, and *B. bonariensis* Griseb., characterized by non-endospermous seeds (Palacios & Hunziker 1984). A preliminary study on *Bulnesia* seeds presented anatomical evidence indicating strong similarity between *B. foliosa, B. retama* and *B. schickendantzii*, on one hand, and *B. bonariensis* and *B. sarmientoi* on the other hand (unpublished results). Comas *et al.* (1979, 1984) studied the seed proteins in the eight species of the genus, but their results did not include separate analyses of the embryo and the endosperm.

Other studies on species of the genus *Bulnesia* have been carried out by Poggio (1977), Poggio & Hunziker (1986) and Poggio *et al.*, (1986), who reported chromosomal and DNA studies. Cozzo (1948) studied the wood anatomy of five species; Crisci *et al.* (1979) used morphological characters in a numerical–taxonomic study of the genus, and Poggio (1980) analysed the pattern of phenolic compounds by chromatography. Most of those studies recognized three pairs of closely related species: *B. retama–B. chilensis, B. schickendantzii–B. foliosa* and *B. arborea–B. carrapo*. However, the results are confusing and do not allow rigorous taxonomic conclusions.

We now report an histological and chemical analysis of embryo and endosperm from seeds of one species from each subgenus, i.e. *B. schickendantzii* (subgenus *Bulnesia*) and *B. bonariensis* (subgenus *Gonopterodendron*). Ultrastructure and chemical analyses for total carbohydrate, cell wall neutral sugars, and soluble proteins in embryo and endosperm do not support the inclusion of both species in a same genus. We think that *Gonopterodendron* should be segregated as a new genus of Zygophyllaceae.

MATERIALS AND METHODS

Materials

Bulnesia bonariensis. Argentina, Prov. La Rioja, Dep. Independencia. JHH 12491. Seeds from 30 individuals.

Bulnesia schickendantzii. Argentina. Prov. La Rioja, Dep. San Blas de los Sauces. JHH 11989. Seeds from 12 individuals. Prov. Catamarca, Dep. Tinogasta. JHH 11991. Seeds from 21 individuals. Voucher specimens of both species are deposited in SI.

Preparation for light and electron microscopy

Five seeds from different individuals were used for each species. Mature and dry seeds were used. Small blocks of tissue cut from different regions of seeds were fixed for 4–8 h in 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.5 at 4°C. For light microscopy the fixed tissue was dehydrated through an ethanol series and embedded in 2-butoxyethanol methacrylate (JB4 Polyscience, Inc. Warrington, PA, USA). Both fresh unfixed tissue and tissue that had been embedded for TEM were also used for light microscopy. The tissue sections were stained with acid fuchsin and toluidine blue O (Feder & O'Brien 1968); Coomassie brilliant blue (Pearse 1985); fast green FCF (Fulcher *et al.* 1972); iodine, potassium iodide; and periodic acid-Schiff (PAS) (O'Brien & McCully 1981), or Sudan black B (Bronner 1975).

For TEM, tissue fixed in glutaraldehyde was post-fixed in 1% OsO_4 in the same buffer for 4 h and then dehydrated and embedded in Spurr's resin. Sections were mounted on grids coated with Formvar and then carbon, stained in uranyl acetate

followed by lead citrate, and examined in a JEOL 1200EX II transmission electron microscope.

For SEM, whole seeds or pieces of seeds cut longitudinally or transversely were mounted onto aluminium stubs, gold-coated and viewed with a JEOL 35 scanning electron microscope. For each species, five seeds from different individuals were used.

Preparation of material for chemical analysis

The endosperms and embryos from 46 seeds of *B. schickendantzii* and 36 seeds of *B. bonariensis* previously soaked in 95% ethanol were separated mechanically. The tissues were air-dried until they reached a constant weight. The dried material was ground into a fine powder in a mortar then defatted by extraction with chloroform:methanol (2:1), sonication for 10 min, and centrifugation for 15 min at 8000 *g*. This extraction step was repeated three times. The pellet was air-dried, suspended in 0.1 M citrate buffer (pH 6) 2M NaCl, sonicated for 10 min, stirred for 1 h at room temperature and centrifuged at 8000 *g* for 20 min. The treatment was repeated twice. The supernatants were dialyzed against water for 36 h and concentrated under reduced pressure. The extraction procedure is summarized in Fig. 1.

Protein analysis

Protein content was determined using bovine serum albumin as standard (Bradford 1976). Proteins were analysed by SDS–PAGE in 12% polyacrylamide slab gels containing 0.1% SDS (Studier 1973). A sample containing 40 μg of protein was loaded into each well. Gels were stained with Coomassie blue R 250 (Korn & Wright 1973). BIO-RAD prestained SDS–PAGE standards (low range) were used. Glycoproteins were detected by staining gels with periodic acid-Schiff reagent (Korn & Wright 1973).

Sugar analysis

Carbohydrates were determined by the phenol–sulphuric acid method with glucose as standard (Dubois *et al.* 1956).

Neutral monosaccharides were determined after acid hydrolysis of the samples containing myo-inositol as an internal standard with 2 M trifluoroacetic acid (TFA, 5 mg/ml) at 105°C for 3 h. The residue was separated by centrifugation at 8000 *g* for 10 min. The solution was evaporated under reduced pressure, with repeated additions of water to remove TFA.

The residue from the first treatment was stirred with 1.3 ml TFA (99%) at 37°C for 18 h. An aliquot (0.5 ml) was transferred to a glass vial, diluted with water (1 ml) and myo-inositol (2 mg/ml, 0.5 ml) was added. This sample, for hemicellulose hydrolysis, was heated at 100°C for 30 min and then evaporated to dryness. A second aliquot (0.5 ml) was diluted with water (0.1 ml), myo-inositol was added as above and heated for 30 min. The addition of water (0.1 ml) was repeated at 30 min intervals until a total volume of 0.5 ml was added, and heating continued for a further 2 h before evaporation to dryness (Morrison 1988).

Sugars were analysed as alditol acetates (Sloneker 1972) by capillary gas-liquid chromatography (glc) with a Hewlett Packard 5890 gas chromatograph with nitrogen

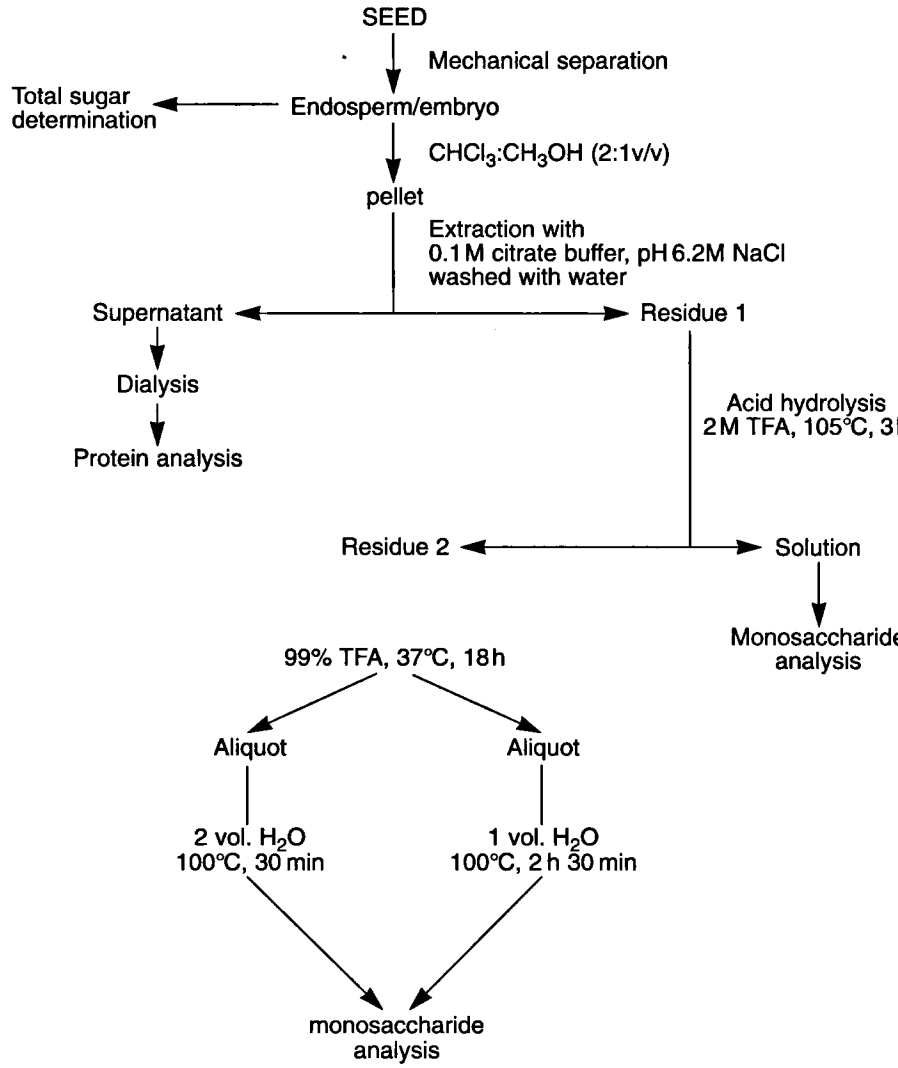

Fig. 1. Extraction procedure of endosperm and embryo of *B. schickendantzii* and *B. bonariensis*.

as the carrier gas. The following conditions were used: SP 2330 fused silica column (0.25 mm × 15 m), flow rate 4 ml/min, ti 250°C, tc 220°C, td 250°C. An HP3395 Integrator was used.

RESULTS

Ultrastructural studies

Endosperm. The proportions of endosperms in the two *Bulnesia* species were very different. In *B. schickendantzii* the endosperm consisted of four to six layers of living cells (Figs 2, 4a,b) whereas in *B. bonariensis* there was only one layer of living cells in this tissue (Figs 3, 4c).

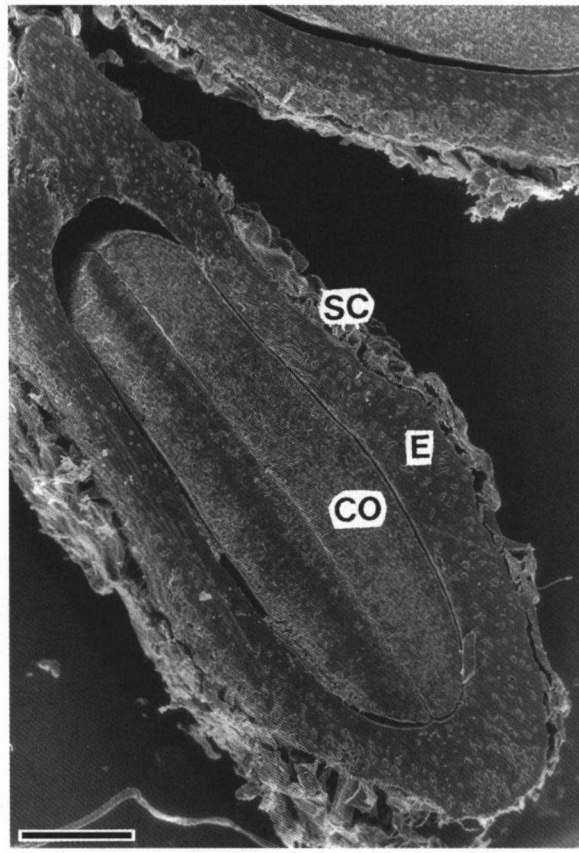

Fig. 2. Scanning electron micrograph of a transverse section of a *B. schickendantzii* seed. CO, cotyledon; E endosperm; SC, seed coat. Scale bar = 200 μm.

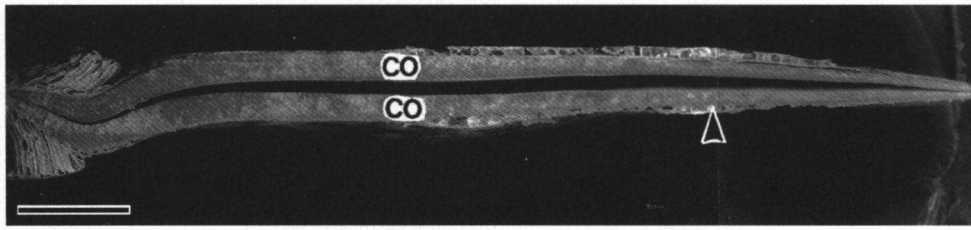

Fig. 3. Scanning electron micrograph of a transverse section of a *B. bonariensis* seed. CO, cotyledon; black arrow indicates seed coat + endosperm. Scale bar = 1 mm.

In *B. schickendantzii*, the endosperm cell walls are very thick except in the areas of primary pit fields. At the electron microscope level, there are three distinct wall layers: middle lamella, thickened outer wall and thin granular inner wall (Figs 4a,b, 5b,c). Cell walls are the site of polysaccharide storage. The cells themselves store abundant lipids and proteins in the form of lipid vesicles and protein bodies (Figs 4a,b, 5b,c). Protein bodies have a proteinaceous matrix containing one or more globoid crystals (Fig. 5b, c). The number and size of globoid crystals vary between different protein bodies in

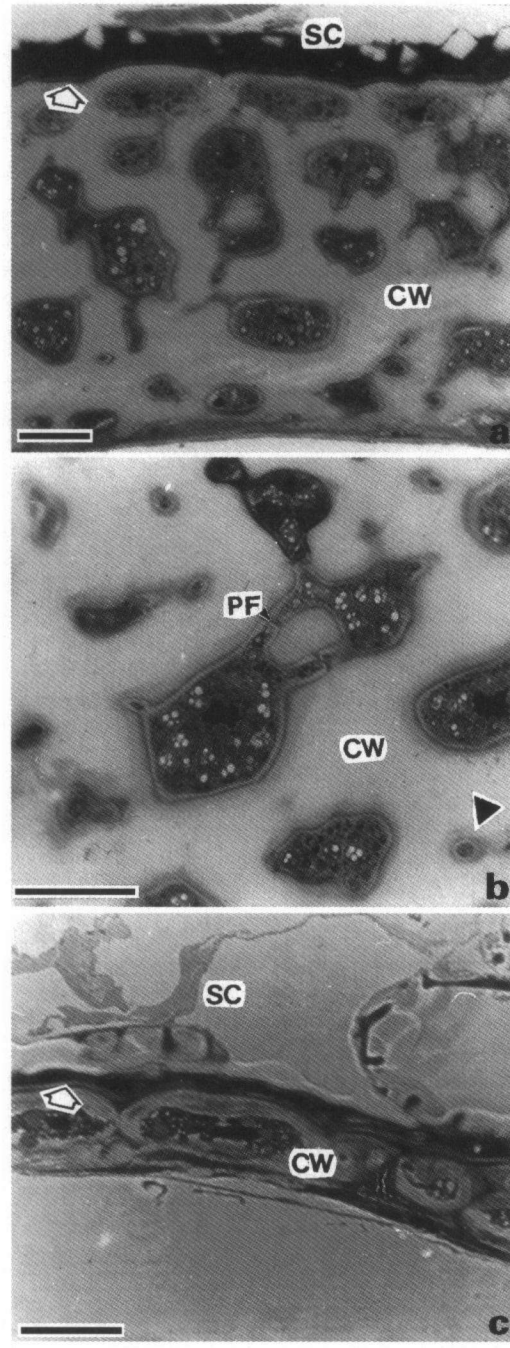

Fig. 4. (a) and (b) Light microscopy of transverse sections of the endosperm of *B. schickendantzii*. CW, cell wall; PF, primary pit field; SC, seed coat. Endosperm cell shows protein bodies and nucleus. On the outer cell layer both the cuticle layer (white arrow) and the cell walls are present. The hard globoid crystals may have chipped out during sectioning, thereby forming a hole inside protein bodies. (a) Scale bar = 25 μm. (b) Scale bar = 25 μm. (c) Light microscopy of a section of the endosperm of *B. bonariensis*. CW, cell wall; SC, seed coat; white arrow indicates cuticle layer. Scale bar = 10 μm.

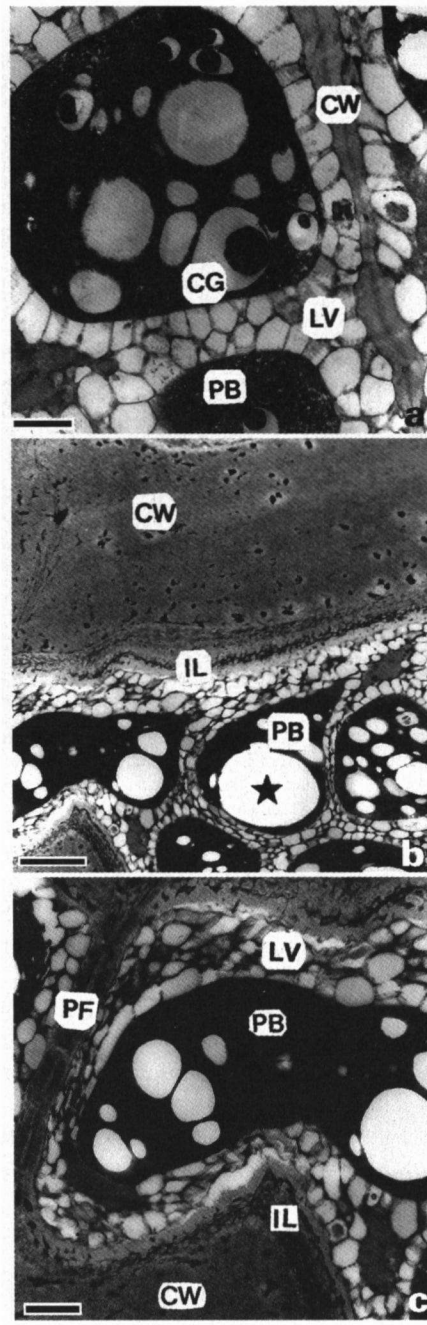

Fig. 5. Transmission electron micrographs. (a) Cell of the palisade tissue of a cotyledon of *B. schickendantzii*. CG, crystal globoid; CW, cell wall; LV, lipid vesicle; PB, protein body: Scale bar = 1 μm. (b) and (c) Cell of the endosperm of *B. schickendantzii*. CW, cell wall; IL, inner layer of the cell wall; LV, lipid vesicle; PB, protein body; PF, primary pit field. Globoid crystals have chipped out during sectioning, forming a hole in the section (asterisk). (b) Scale bar = 2 μm. (c) Scale bar = 1 μm.

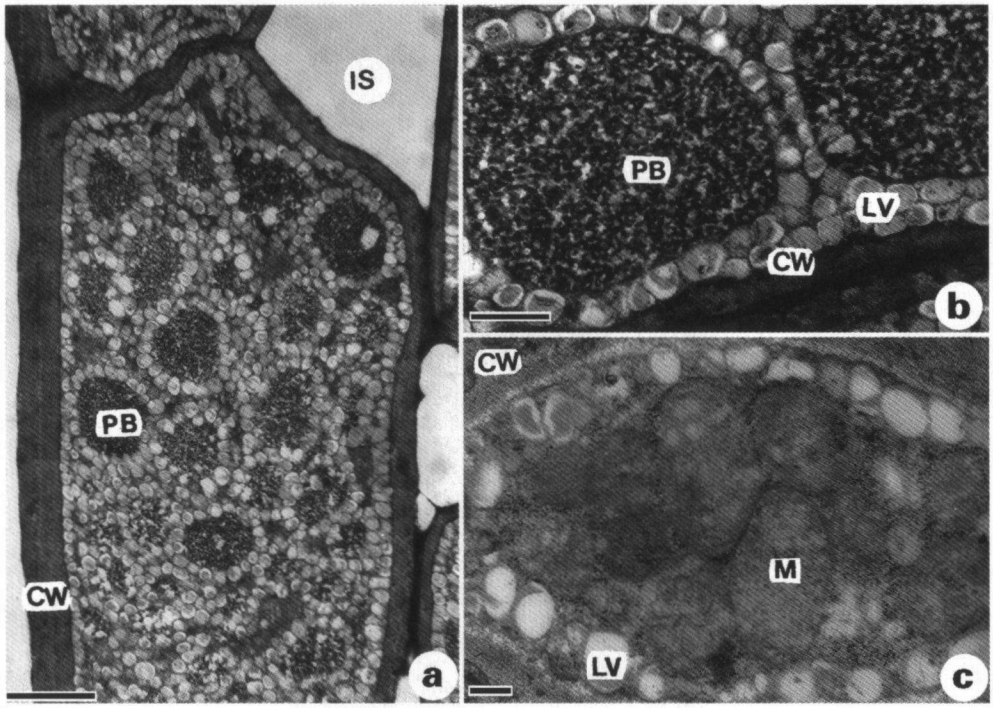

Fig. 6. Transmission electron micrographs. (a) and (b) Cell of the palisade tissue of a cotyledon of *B. bonariensis*. CW, cell wall; IS, intercellular space; LV, lipid vesicle; PB; protein body. (a) Scale bar = 10 μm. (b) Scale bar = 2 μm. (c) Cell of the endosperm of *B. bonariensis*. CW, cell wall; LV, lipid vesicle; M, mitochondrion. Ribosomes are seen in the cytoplasm. Scale bar = 1 μm.

the same endosperm cell. Globoid crystals, which are hard and do not infiltrate well with resin, were often removed during sectioning, leaving a hole in the section (Figs 4a,b, 5b,c). Mitochondria but no ribosomes or endomembranes are visible. Vacuoles are present occasionally.

In *B. bonariensis*, cell walls are two-layered: middle lamella and a relatively thin cell wall. The cells store lipids in the form of lipid vesicles (Fig. 6c). Cytoplasm stains positively for protein but protein bodies are absent. Mitochondria and ribosomes are visible (Fig. 6c).

In both species, there is a cuticle on the outer cell walls, of the endosperm layer next to the seed coat (Fig. 4a,c).

Cell walls stained positively with the PAS, fast green and toluidine blue reagents. The proteinaceous regions stained positively with the Coomassie blue, fast green FCF and acid fuchsin. Globoid crystals stained metachromatically with toluidine blue. The cuticle and the storage lipid vesicles stained with Sudan black.

Embryo. The embryos of both *Bulnesia* species consist of a hypocotyl–radicle axis and two cotyledons. The position and cytological characteristics of the tissues of the cotyledons and axis indicate their fate in the developing seedling. The mesophyll (palisade and spongy tissues), protoderm and procambium are distinguished in the cotyledons and the protoderm, procambium and ground meristem are distinguished in the axis.

Table 1. Fractions obtained from *B. schickendantzii* and *B. bonariensis* (mg/seed). Percentages are given in parentheses

	Bulnesia schickendantzii		*Bulnesia bonariensis*	
Seed tissue	Endosperm	Embryo	Endosperm	Embyro
Dry weight	2.5 (100)	0.7 (100)	1.9 (100)	25.0 (100)
Total neutral sugars[a]	0.73 (29)	0.096 (14)	1.7 (90)	5.47 (22)
Protein[a]	0.049 (2)	0.035 (5)	0.12 (6)	1.90 (8)

[a]Values are the average of triplicate determinations.

All embryo cells have thin primary cell walls (Figs 5a, 6a,b) that stain with the PAS reaction, fast green and toluidine blue. No starch was detected in the embryo, as determined by a lack of staining with iodine-potassium iodide. Protein bodies and lipid vesicles occupy most of the cytoplasm of all embryo tissues (Figs 5a, 6a,b). In *B. schickendantzii* protein bodies contain two or more phytin crystals in a dense proteinaceous matrix (Fig. 5a). In *B. bonariensis* the proteinaceous matrix is loose and frequently lacking phytin crystals (Fig. 6a,b). Protein crystalloids are absent from the protein bodies of both species.

Chemical analysis

Sugar analysis. The yield (dry weight) of the different seed fractions, neutral sugars and protein for *B. schickendantzii* and *B. bonariensis* are shown in Table 1.

Hydrolysis with 2 M TFA at 105°C for 3 h released most of the neutral sugars (Fig. 7A and B). Although in very different proportions, both species showed the same main monosaccharides: arabinose, xylose, galactose and glucose. Minor amounts of mannose and the 6-deoxy hexoses rhamnose and fucose were also detected. Only a small amount of sugar was released by further hydrolysis (Fig. 7, C and D). This is in agreement with the previous report (Morrison 1988), showing that 2 M TFA hydrolyzes most of the hemicelluloses. The third TFA treatment which is reported to hydrolyze cellulose only resulted in increased glucose from the *B. bonariensis* samples (cf. Fig. 7E for *B. schickendantzii* and Fig. 7F for *B. bonariensis*).

Protein analysis. The SDS–PAGE study of the soluble proteins showed a different profile for both species (Fig. 8). In *B. schickendantzii* the embryo and the endosperm had in common the bands a, b, c, d, e, h and i (Fig. 8 lanes A and B). The endosperm profile included strong bands at around 30 kDa and 40 kDa which did not appear in the embryo. The embryo also showed a low molecular weight band at 22 kDa. Only two bands were glycosylated, as detected with the PAS reagent, one at 53 kDa in the embryo and one at 40 kDa in the endosperm (not shown).

The *B. bonariensis* profiles showed a strong double band at 40 kDa in the embryo (lane C) and in the endosperm (lane D). The protein at 28 kDa was the only glycosylated one, appearing in both tissues but strongly in the endosperm. Low molecular weight polypeptides appeared as non-resolved strong bands in the front of the gel in all samples. Proteins of high molecular weight seem more abundant in *B. schickendantzii.*

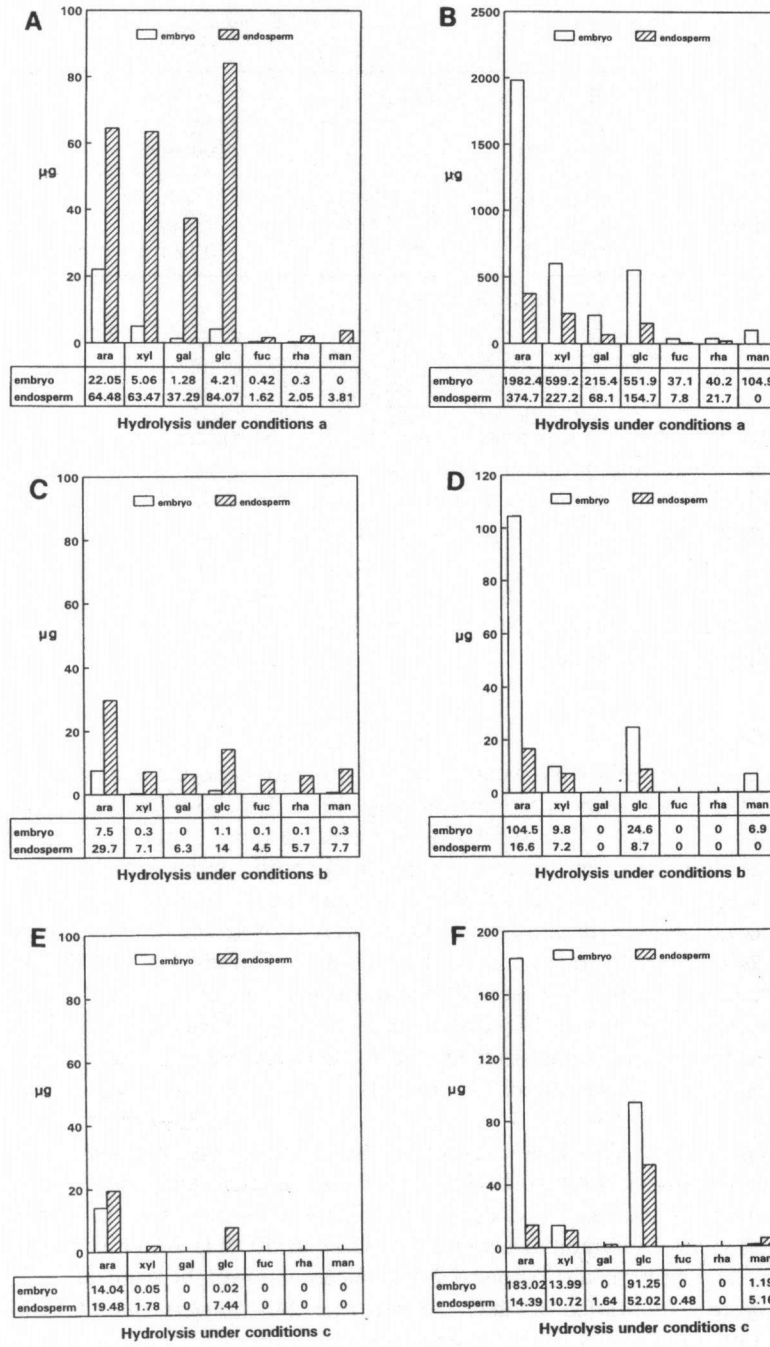

Fig. 7. Determination of cell wall neutral sugars (µg/seed) after acid hydrolysis under three conditions: a, 2 M trifluoroacetic acid (TFA) at 105°C, 3 h; b, TFA 99%, 37°C, 18 h (1); an aliquot of 1 was diluted with 2 volumes of water and heated at 100°C, 30 min; c, an aliquot of 1 was diluted and hydrolyzed at 100°C as described for hydrolysis of cellulose (see Methods). Sugars were analysed as alditol acetates by gas-liquid chromatography. Inositol was used as inner standard. Figs A, C, E, *B. schickendantzii*. Figs B, D, F. *B. bonariensis*. All values are the average of triplicate determination.

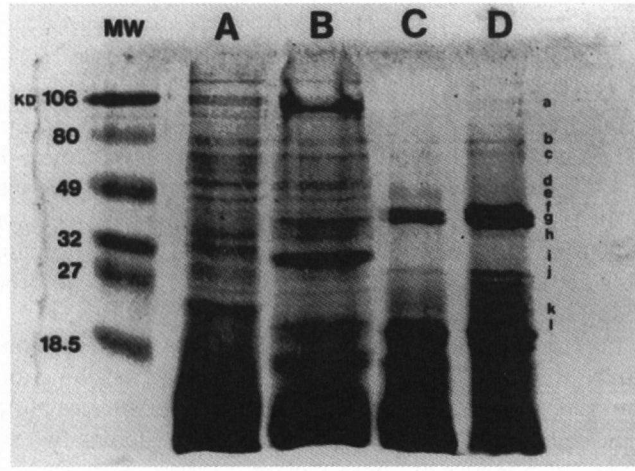

Fig. 8. Polyacrylamide gel electrophoresis performed on 12% polyacrylamide with 0.1% SDS. The gel was stained with Coomassie blue. Lane MW: molecular weight standards; lane A: *B. schickendantzii* embryo proteins; lane B: *B. schickendantzii* endosperm proteins; lane C: *B. bonariensis* embryo proteins and lane D: *B. bonariensis* endosperm proteins.

DISCUSSION

This is the first ultrastructural and chemical report of seeds differing in structure and the type of reserve accumulation in species classified as belonging to the same genus in Angiosperms. We have studied two species, *B. schickendantzii* and *B. bonariensis* and found that both have endosperm in the mature seeds, although in a very different proportion and with very different ultrastructural features. In *B. schickendantzii* the cell walls of the endosperm are very thick and consist of three distinct layers: middle lamella, thickened outer wall and thin granular inner wall. In *B. bonariensis* the endosperm cell walls differentiate only two layers: middle lamella and a comparatively thinner cell wall.

In the embryo and endosperm of *B. schickendantzii* and in the embryo of *B. bonariensis* protein bodies and lipid vesicles occupy most of the cytoplasm; the major extractable proteins are derived from the protein bodies in those tissues, cytoplasm being very reduced. Protein bodies are absent in the cells of the endosperm of *B. bonariensis* but cytoplasm, with ribosomes and mitochondria, stains positively with the protein stains. We infer that proteins would be extracted from organelles and cytoplasm as suggested by Chandra Sekhar & De Mason (1988) for *Phoenix dactylifera.*

Whereas protein bodies of embryo and endosperm of *B. schickendantzii* contained two or more phytin crystals in a dense proteinaceous matrix, those from *B. bonariensis* embryo lacked phytin crystals and the proteinaceous matrix was loose.

According to De Mason (1985), the presence of endosperm-specific proteins is consistent with the hypothesis that the endosperm is a possible source of hydrolytic enzymes involved in germination. Some protein bands were specific to each species: *B. schickendantzii* showed proteins a, d, e, h, i, which did not appear in *B. bonariensis* and protein f and j were present only in *B. bonariensis* (Fig 8). These observations suggest that these proteins could be taxonomic-specific markers. No common bands were found between the embryos of the two species.

Sugar analysis also showed striking differences between both species, suggesting a different polysaccharide composition of the cell walls. The large amount of glucose, xylose and arabinose in the endosperm of *B. schickendantzii* could arise from an arabinoxyloglucan. The low amount of mannose in both species would indicate the absence of galactomannan. On the other hand, arabinose could be part of an arabinogalactan. In contrast, glucose is a minor component of the endosperm of *B. bonariensis* and is neither found in a large amount in the embryo. Interestingly, arabinose is a main component in both endosperm and embryo. Glucose, probably arising from cellulose, was also detected in this species. As far as we know, no other studies have been performed on the sugars from seeds of the Zygophyllaceae.

According to Takhtajan (1991) the main storage function of seeds has evolved from the endosperm to the embryo. The reduction of the endosperm is functionally correlated with increased embryo size and represents an evolutionary advance in flowering plants. Palacios & Hunziker (1984) revised the genus *Bulnesia* to include two subgenera based on the seed size and the presence or absence of endosperm. The subgenus *Bulnesia* was characterized by small and albuminate seeds and the subgenus *Gonopterodendron* by large and exalbuminate seeds. Porter (1974) proposed that the southern South American species of *Bulnesia* are derived from tropical ancestors, today represented by *B. arborea* and *B. carrapo*, both of them belonging to *Gonopterodendron*. Since *B. arborea* and *B. carrapo* have seeds with advanced characters, we infer that they cannot represent the living ancestors of the genus.

We have studied one species from each of the two subgenera proposed by Palacios & Hunziker (1984) and have found that the histological, ultrastructural and chemical properties of the seeds in both species would support the segregation of *Gonopterodendron*. Further seed studies of the remaining six species in the genus and in closely related genera of Zygophyllaceae are required to strengthen this assertion and determine phylogenetic relationships.

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