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Seed structure in *Canavalia brasiliensis* Mart. ex Benth. (Leguminosae) and subcellular localization of ConBr lectin: Implications for ConBr biological functions

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ABSTRACT

Lectins are proteins capable of specific and reversible recognition of carbohydrates without modifying them. Many studies have isolated these molecules from Leguminosae seeds but little attention was given to subcellular localization and biological function of these molecules. Therefore, this work aimed to describe *Canavalia brasiliensis* seed structure and the subcellular localization of ConBr. In addition, we tested the affinity of anti-ConBr antibody for other lectins. To accomplish this, seed fragments were processed for light, scanning and transmission electron microscopy, as well as immunocytochemistry. The anti-ConBr affinity was also tested. Under SEM, the testa showed a regular contour of anticlinal walls without trichomes and inner integument of 10–20 cell layers. The cotyledons presented many parenchymatic cell layers with starch grains and cytoplasmic protein bodies. The immunological identity of anti-ConBr immunoglobulin to ConBr and other lectins was confirmed by immunodiffusion and Western blotting. Ultrastructurally, the cotyledons showed protein bodies characterized by heterogeneous appearance of irregularly formed electron-dense and electron-lucent areas. Immunolocalization showed lectin at both protein bodies and cell wall, however, more studies are required for the elucidation of ConBr functions. Moreover, the affinity of anti-ConBr for different lectins can make this molecule a useful biotechnological tool for lectin studies.

1. Introduction

Naturally occurring proteins with hemagglutinating activity, which, in later years, were shown to be sugar-specific and eventually termed lectins, have been known since the turn of the 19th century (Kilpatrick, 2002; Sharon and Lis, 2004). Lectins are a group of highly diverse proteins of nonimmune origin. These proteins contain at least one noncatalytic domain, which enables them to selectively recognize and reversibly bind to specific free sugars or glycans present on glycoproteins and glycolipids. One characteristic of this binding is the conservation of native carbohydrate structures (Peumans and Van Damme, 1995). Widely distributed in nature, these proteins can be found in almost all living organisms from plants to animals, including invertebrates, and microorganisms (Moreira et al., 1991).

Although many lectins have been described, one of the most studied is *Canavalia brasiliensis* lectin (ConBr). This protein was described in 1984 (Moreira and Cavada, 1984), and since then, it has been the subject of studies with different approaches, such as behavior during seed germination (Cavada et al., 1990), crystal structure (Sanz-Aparicio et al., 1997), antidepressant-like effect (Barauna et al., 2006), and stimulation of microorganismal growth (De Vasconcelos et al., 2012). Similar to many other lectins, ConBr is extracted from Leguminosae seeds.

The shape of seeds is usually regular and symmetric for Mimosoideae and Caesalpinioideae (Fawzi, 2011). The character-

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istics of the seed coat have been successfully employed to identify and classify different taxa (Akyol et al., 2012; Erol et al., 2006; Fawzi, 2011; Fawzi et al., 2010; Jafari et al., 2009).

In a typical angiosperm seed, the embryo is surrounded by the endosperm consisting of nutritive tissue and living cells and the testa consisting of seed coat, maternal tissue, and dead cells. The mature seeds of many angiosperm groups are endospermic. They have a more or less abundant endosperm layer, though the evolutionary trend is toward cotyledon storage and seeds without endosperms (Müller et al., 2006). In most cases, the cotyledon is the primary region for lectin accumulation in seeds. Although many studies have isolated lectins from Leguminosae seeds, the subcellular localization and biological functions of these proteins are still poorly explored. However, *Dioclea lehmanni* (Leguminosae) lectin was localized to the vacuoles of cotyledon cells or protein bodies, depending on seed maturation stage (Melgarejo and Pérez, 1997). In addition, *Cratylia mollis* (Leguminosae) shows intense and homogeneous reaction for lectin immunolocalization in protein bodies in the axis of the embryonic region (Santos et al., 2004).

Although many studies have traditionally focused on purification and characterization of lectins, recent works reporting on newer aspects of lectins are bringing the study of these molecules to another level. These include vasorelaxant properties (Bezerra et al., 2013), antinociceptive and anti-inflammatory effects (Leite et al., 2012), opioid-like antinociceptive effects (Pires et al., 2013), anti-inflammatory (Pinto et al., 2013) and pro-inflammatory effects (Rangel et al., 2011), and effects on the growth of microorganisms (Vasconcelos et al., 2013). These studies not only elucidate biotechnological applications of lectins but new plant physiological roles, as demonstrated by Delatorre et al. (2013). The immunolocalization of these proteins can provide data to improve purification methods and increase understanding of plant physiology. Thus, this work aimed at describing *C. brasiliensis* seed structure and the subcellular localization of ConBr, in addition to testing the affinity of anti-ConBr antibody for other lectins.

2. Methods

2.1. Plant material

Seeds of *C. brasiliensis* Mart. ex Benth. were supplied by B.S. Cavada of the Universidade Federal do Ceará. Measurements of seeds were performed on 100 samples with digital calipers (0–150 mm). Weight was performed on the same samples using automatic scales.

2.2. Scanning electron microscopy

Samples were frozen with liquid nitrogen for 1 h and crushed to obtain small testa and cotyledon fragments. Small fragments were fixed for four hours in a solution of 2.5% glutaraldehyde and 4.0% formaldehyde freshly prepared in 0.05 M cacodylate buffer, pH 7.2. Subsequently, the samples were rinsed in the same buffer and post-fixed for one hour at room temperature with 1.0% osmium tetroxide in 0.05 M cacodylate buffer, pH 7.2. The post-fixed samples were dehydrated in an ascendant acetone series. Afterwards, the samples were submitted to critical point drying by using CO_2 and sputter-coated with 10 nm gold, followed by observation under scanning electron microscopy (Evo 40-ZEISS/Inspect 50-FEI).

2.3. Histochemical methods

For histochemical tests, seeds were frozen and fragmented as described above. Cotyledon fragments were free-hand sectioned. Thin sections were stained with Sudan III (1% alcoholic solution) for lipids (Jensen, 1962), Coomassie blue (5% aqueous solution) for proteins (Southworth, 1973) and Lugol (1% aqueous solution) for starch. Samples were observed and photographed using an optical microscope (Eclipse 80i-NIKON).

2.4. Immunodiffusion assay

Immunochemical studies were performed to establish specific antibody reaction and the relationships between anti-*C. brasiliensis* lectin (ConBr) antibody and other lectins from the same subtribe. IgG polyclonal antibody against ConBr (supplied by B.S. Cavada) was raised on rabbit according to Moreira et al. (1993). Immunodiffusion tests were carried out on 1% agarose gels prepared with 0.05 M Tris-barbital buffer, pH 8.0, containing 0.02% sodium azide (Clausen, 1969). Antibody was disposed on central dots (2 mg/mL) and the antigens on lateral dots. Subsequently, the agarose gels were stained with an aqueous solution containing 50% methanol, 0.05% Coomassie blue R, and 10% acetic acid. After staining, the gels were destained in water and photographed.

2.5. Western blot analysis

To test antibody affinity by other Diocleinae lectins, as well as confirm the affinity to ConBr, purified lectins of *C. brasiliensis*, *Dioclea wilsonii*, *Canavalia boliviana* and *Dioclea virgata* were subjected to 15% SDS-PAGE (Laemmli, 1970). Following electrophoresis, the proteins were transferred to polyvinylidene fluoride (PVDF) membranes (GE Healthcare, USA) at a constant voltage of 25 V at room temperature for 2 h using a miniVE Blot Module apparatus (GE Healthcare, USA) and transfer buffer consisting of 25 mM Tris–HCl, pH 8.3, 192 mM glycine and 20% methanol (v/v). Nonspecific binding sites were blocked by incubating the membranes with 5% nonfat dry milk (w/v) in TTBS (100 mM Tris–HCl, pH 7.5, 150 mM NaCl and 0.1% Tween 20) overnight at 4 °C. Subsequently, the membranes were incubated with a 1:1500 (v/v) dilution of the polyclonal rabbit anti-ConBr serum for 150 min at room temperature. After washing three times with TTBS, the membranes were incubated for 60 min with a 1:10000 (v/v) dilution of anti-mouse IgG-alkaline phosphatase (Sigma–Aldrich, USA), followed by washing three times with TTBS and once with TBS (100 mM Tris–HCl, pH 7.5, 150 mM NaCl). The proteins were viewed by the addition of Sigma Fast NBT/BCIP buffered substrate (Sigma–Aldrich, USA), and the reaction was stopped with water.

2.6. Transmission electron microscopy

For transmission electron microscopy fragments obtained as described for scanning electron microscopy were fixed with 4% formaldehyde and 0.05% glutaraldehyde in 0.05 M sodium cacodylate buffer, dehydrated in a graded acetone series (50–100%), and embedded in LRGold resin. Polymerization was performed under UV light at 4 °C. Ultrathin sections were obtained using a diamond knife on an ultramicrotome (Reichert Ultracut E), collected on 300 mesh Formvar-covered Cu grids, and contrasted according to Reynolds (1963).

2.7. Immunocytochemistry assay

Immunolabeling was carried out at room temperature. Ultrathin sections collected on Formvar-covered Ni grids were incubated in ammonium chlorite, pH 8.0, and washed in PBS/BSA. Then, the sections were incubated for 1 h with rabbit immunoglobulin G (IgG) anti-ConBr 1:100 in PBS. Anti-ConBr antibody was produced according to Moreira and Cavada (1984). After rinsing in PBS, the sections were indirectly labeled for 1 h on drops of goat anti-rabbit gold conjugate (10 nm diameter gold nanoparticles, Sigma) and diluted 1:100 in PBS. The sections were washed in PBS, rinsed in

water, and stained with uranyl acetate (20 min), followed by lead citrate (2 min), according to Reynolds (1963). Control grids to detect nonspecific immunolabeling were made using IgG from nonimmunized rabbits or by omitting the primary antibodies. Observation and documentation were performed with an electron microscope (EM 900-ZEISS).

3. Results

3.1. Seed morphometry

C. brasiliensis seeds were ellipsoidal, with a smooth and brown to dark-brown shiny surface (Fig. 1A and B). The hilum was elliptical with no elevated outgrowths (Fig. 1C).

Table 1
Canavalia brasiliensis seed biometry. Minimum, mean, maximum and standard error for main axis, second axis, and short axis of seeds and weight for 100 seeds measured.

	Biometry			
	Main axis	Second axis	Short axis	Weight (g)
Minimum	13.17	9.70	4.52	0.52
Mean	16.09	11.48	6.73	0.91
Maximum	18.88	14.17	9.11	1.46
Standard error	1.21	0.96	0.80	0.2

Seed biometry revealed lengths of 16.09 ± 1.21 mm (mean ± standard error) in the main axis, 11.48 ± 0.96 mm

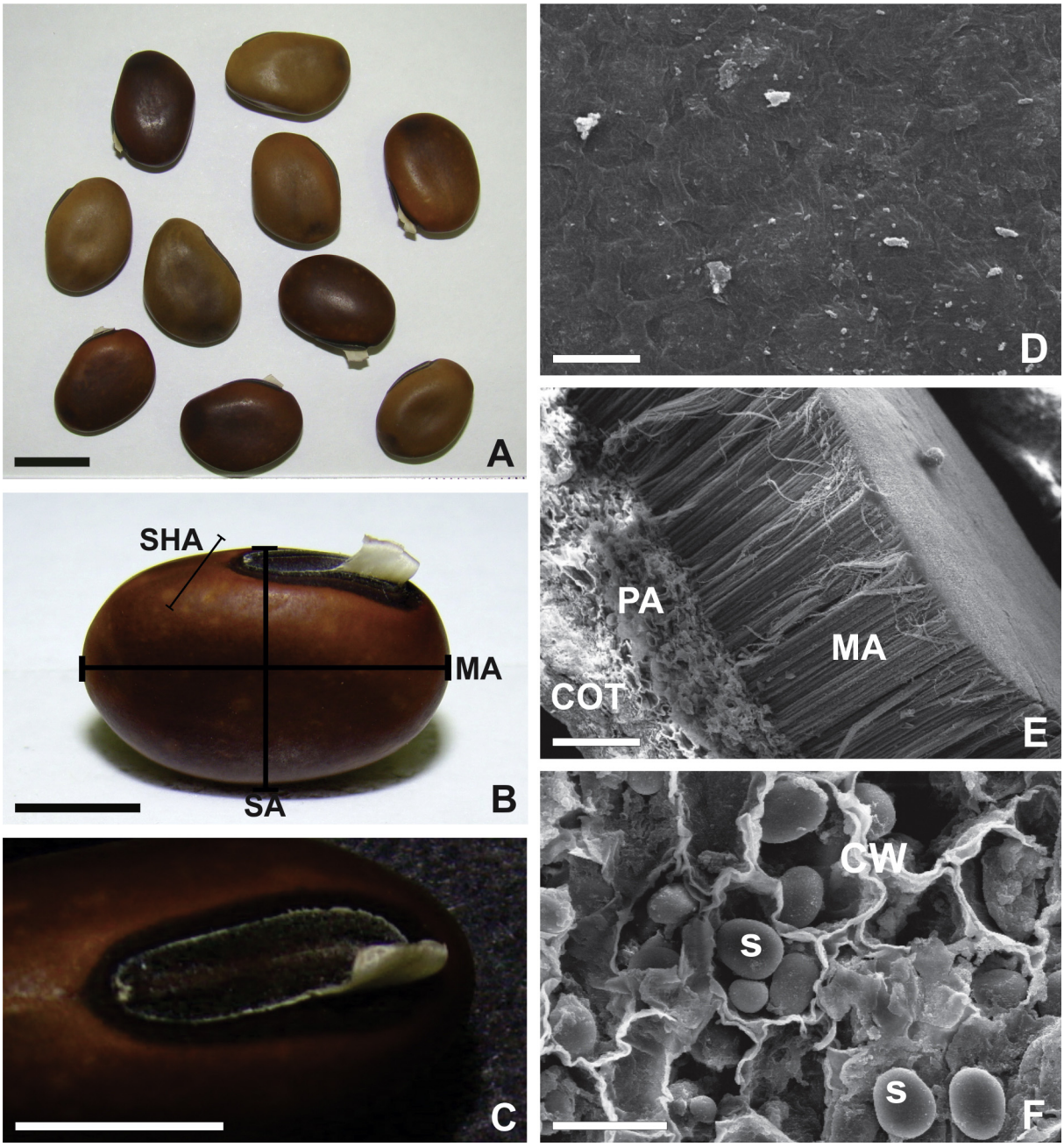

Fig. 1. *Canavalia brasiliensis* seeds overview (A–C) and scanning electron microscopy (D–F). (A) *C. brasiliensis* seeds overview. (B) Seed detail showing main axis (MA), second axis (SA) and short axis (SHA). (C) Hilum detail. (D) Testa micromorphology. (E) Seed transverse fragment showing macrosclereids (MA), parenchyma cells (PA) and part of cotyledon tissue (COT). (F) Cotyledon fragment exhibiting abundant starch grains (s) inside ruptured cells. Cw—cell wall. Bars: (A) 1 cm; (B) 0.5 cm; (C) 0.25 cm; (D) 10 μm; (E) 200 μm; (F) 50 μm.

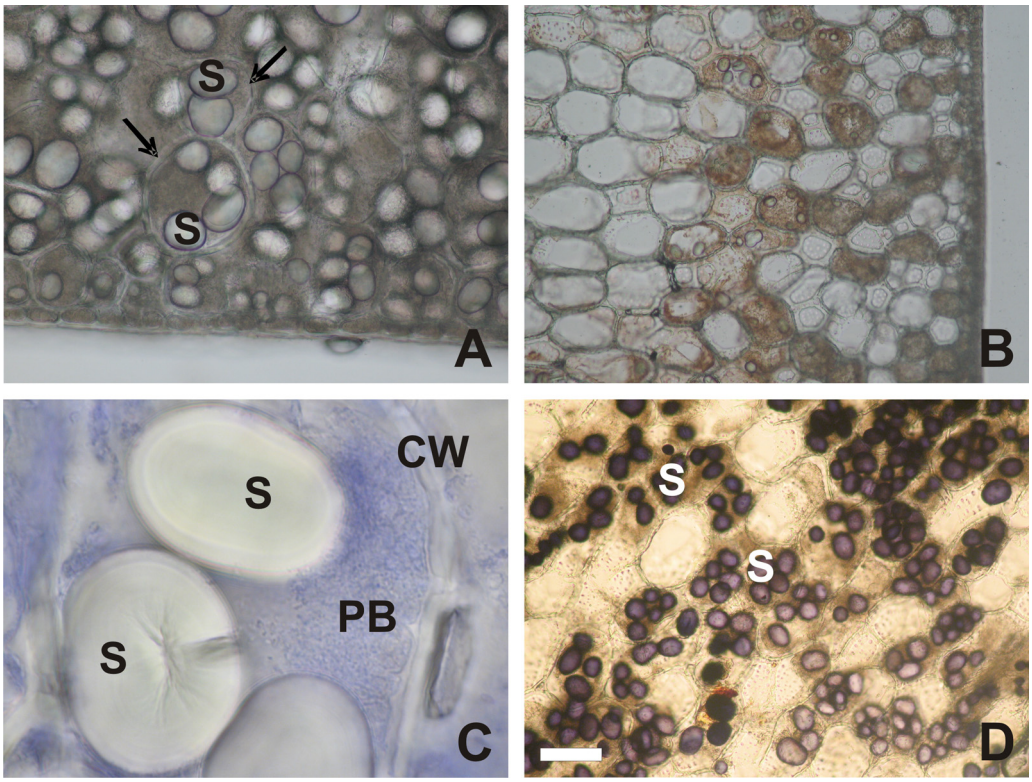

Fig. 2. Light microscopy of *Canavalia brasiliensis* cotyledons. (A) Section showing regular cell walls (arrows) and numerous starch grains (S). (B) Sudan-stained tissue section. Cytoplasm with strong staining. (C) Coomassie blue-stained section. Stained cytoplasm showing evident protein bodies (PB), but not at starch grains (S) or cell walls (CW). (D) Lugol-stained section. Dark-stained starch grains (S). Bars: (A and B) 20 μm; (C) 10 μm; (D) 50 μm.

at the second axis, and 6.73 ± 0.80 mm at the short axis (n = 100, Table 1).

3.2. Micromorphology

Under SEM, the testa showed a regular contour of anticlinal walls without trichomes (Fig. 1D). All seed regions presented the same surface pattern. In transverse seed sections, it was possible to observe macrosclereids which were elongated in the radial direction with thin cuticle. No intercellular spaces were observed on this tissue. The inner integument consisted of 10–20 layers of small isodiametric parenchymatic cells without intracellular spaces (Fig. 1E). The cotyledons presented many parenchymatic cell layers and abundance of starch grains at the cytoplasm (Fig. 1F).

3.3. Histochemistry assays

Free-hand unstained cotyledon sections observed under LM revealed multiple cell layers (Fig. 2A). Histochemical tests revealed the presence of lipids, at lipid droplets (Fig. 2B), proteins on protein bodies, dispersed throughout the cytoplasm (Fig. 2C), and numerous starch grains occupying most of the cytoplasm (Fig. 2D).

3.4. Immunodiffusion assay

Immunodiffusion assay confirmed the specificity and immunological identity of anti-ConBr immunoglobulin to ConBr (Fig. 3A), as well as other Diocleinae lectins, including CBol (*C. boliviana* lectin) (Fig. 3B), CMar (*Canavalia maritima* lectin) (Fig. 3C), DMeg (*Dioclea megacarpa* lectin) (Fig. 3D), DGra (*Dioclea grandiflora* lectin) (Fig. 3E), and DRos (*Dioclea rostrata* lectin) (Fig. 3F), but not HML (*Hypnea musciformis* lectin—Rhodophyta) (Fig. 3G) or BSA (bovine serum albumin).

3.5. Western blotting

SDS-PAGE showed typical Diocleinae pattern on different proteins (Fig. 4A, lines 3–8). Western blotting confirmed the immunodiffusion assay. The results demonstrated that polyclonal anti-ConBr antibody interacts with ConBr (Fig. 4B, line 3) and other proteins, such as CBol (Fig. 4B, line 4), CMar (Fig. 4B, line 5), DMeg (Fig. 4B, line 6), DGran (Fig. 4B, line 7), and DRos (Fig. 4B, line 8). On the other hand, the antibody was not able to recognize molecular mass markers (Fig. 4B, line 1) or BSA (Fig. 4B, line 2).

3.6. Cotyledon cell ultrastructure and lectin immunolocalization

Based on ultrastructural analysis, cotyledons showed polygonal cells with thick cell walls. The cytoplasm was dense and adhered closely to the cell wall (Fig. 5A and B). The nucleus was clearly delimited by a double membrane nuclear envelope and a spherical endoplasmic reticulum (ER). Mitochondria were present and presented typical morphology. Ribosomes were attached to the ER. The cell wall was thin.

Oval vesicles surrounded by a single membrane (protein bodies) were noted (Fig. 5B). These organelles varied in size and were surrounded by a single membrane. A closest and detailed observation revealed that the protein bodies were characterized by heterogeneous appearance of irregularly formed electron-dense and electron-lucent areas. Immunolocalization showed lectin at cytoplasmic protein bodies (Fig. 5C and D) and cell wall (Fig. 5E and F).

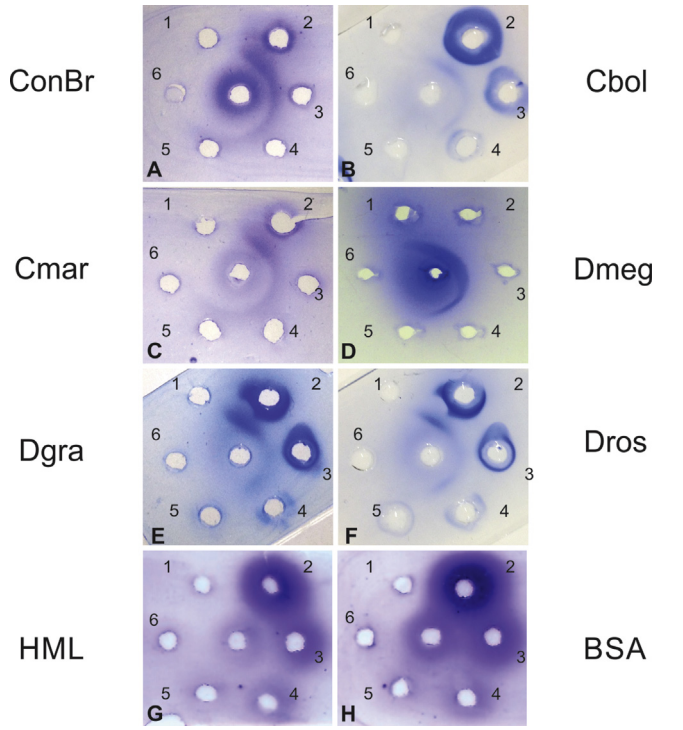

Fig. 3. Immunodiffusion of proteins against IgG anti-ConBr antibody 2 mg/mL (center dot) against ConBr (*Canavalia brasiliensis* lectin) (A); Cbol (*Canavalia boliviana* lectin) (B); Cmar (*Canavalia maritima* lectin) (C); Dmeg (*Dioclea megacarpa* lectin) (D); Dgra (*Dioclea grandiflora* lectin) (E); Dros (*Dioclea rostrata* lectin) (F); HML (*Hypnea musciformis* lectin) (G) and BSA (bovine serum albumin) (H). Dots: (1) 0.05 M Tris(hydroxymethyl) aminomethane-Barbital buffer, pH 8.0; (2) 2 mg/mL; (3) 1 mg/mL; (4) 0.5 mg/mL; (5) 0.25 mg/mL and (6) 0.012 mg/mL. Note: complete immunological identity (precipitation) in Figures A–F (Diocleinae lectins), but not in Figures G and H (algae lectin and unrelated protein).

4. Discussion

4.1. Seed morphometry

C. brasiliensis seeds were described morphologically and structurally. Seed biometry revealed lengths of 16 mm in the main axis, 11 mm at the second axis and 6.7 mm at the short axis. Because Leguminosae seeds are usually regular in shape, the irregular shape in *C. brasiliensis* probably results from the pressure of adjacent seeds on the fruit (Fawzi, 2011). The minor variation in seed size is common in polyspermic fruits from Leguminosae seeds (Ilkiu-Borges and Mendonça, 2009). On the other hand, the variable size is proportional to species number and taxonomic diversity. For example, *Bobgunnia madagascariensis* presents seeds of 4.9–7.3 mm × 2.3–3.3 mm × 4.3–4.8 mm (Lackey, 2009), while *Bauhinia monandra* presents seeds of 9.46–11.14 mm × 6.01–8.67 mm × 2.39–3.69 mm (Ilkiu-Borges and Mendonça, 2009).

Seed structure knowledge can be useful on different fields of study, including taxonomy (Akyol et al., 2012; Erol et al., 2006; Fawzi, 2011; Fawzi et al., 2010; Jafari et al., 2009; Oliveira and Paiva, 2005), as well as seed physiology, germination and lectin behavior during this process.

4.2. Micromorphology

Leguminosae seeds are micromorphologically characterized by regular testa, regular contour of epidermal anticlinal walls, absence of trichomes, elongated epidermis, presence of inner integument and endosperm with many isodiametric cells (Bobrov et al., 1999). However, testa organization on *C. brasiliensis* seeds was somewhat different to that described for other Leguminosae species, especially for some tissue cell size and intercellular spaces (Sefa-Dedeh and Stanley, 1979; Silva et al., 2008). However, seeds of plants from such families as Caryophyllaceae, the pink, or carnation, family (e.g., *Silene* L.) can present sculpted patterns on periclinal and irregular anticlinal walls (Fawzi et al., 2010).

4.3. Histochemistry assays

Multiple endosperm cell layers with starch grains in cytoplasm and the presence of proteins dispersed on cytoplasmic protein bodies were observed in the studied species. Cotyledon structure in Leguminosae is characterized by the presence of lipids, starch and proteins, mainly storage proteins, as previously described (Bobrov et al., 1999).

In Leguminosae seeds, reserve proteins are mainly confined to protein bodies (Pusztai et al., 1979), as ascribed to ConBr. Concanavalin A was also shown on cytoplasmic protein bodies in jack beans (Clarke et al., 1975). During germination, protein bodies break down into amino acids for the growing seedling, showing different patterns of protein mobilization. ConBr may have a role as storage protein, while seed lectins in legume plants may play other biological roles (Cavada et al., 1990; Moreira et al., 1993; Silva et al., 2000). This fact may justify the presence of ConBr at the endosperm.

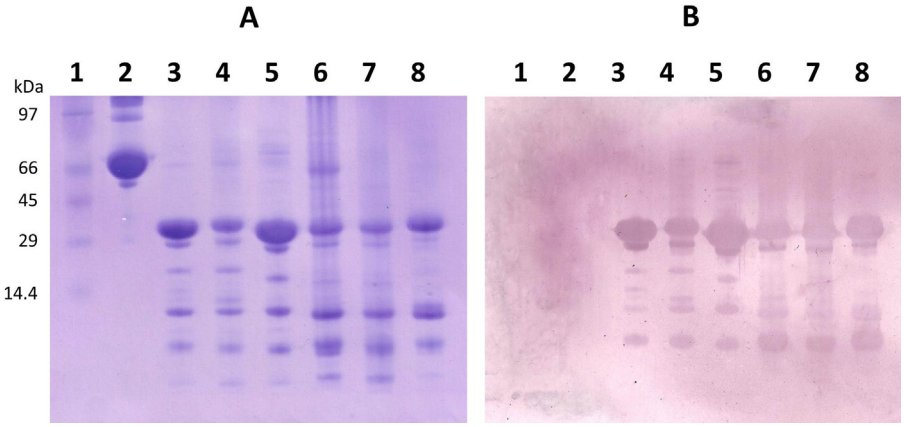

Fig. 4. SDS-PAGE profile and Western blotting of Diocleinae subtribe lectins. (A) SDS-PAGE. (B) Western blot analysis for the interaction of polyclonal rabbit anti-ConBr serum. Line 1: Molecular mass markers: phosphorylase b, 97 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa; and α-lactalbumin, 14.4 kDa; Line 2: Bovine serum albumin; Line 3: *Canavalia brasiliensis* lectin; Line 4: *C. boliviana* lectin; Line 5: *C. maritima* lectin, Line 6: *Dioclea megacarpa* lectin; Line 7: *D. grandiflora* lectin; Line 8: *D. rostrate* lectin.

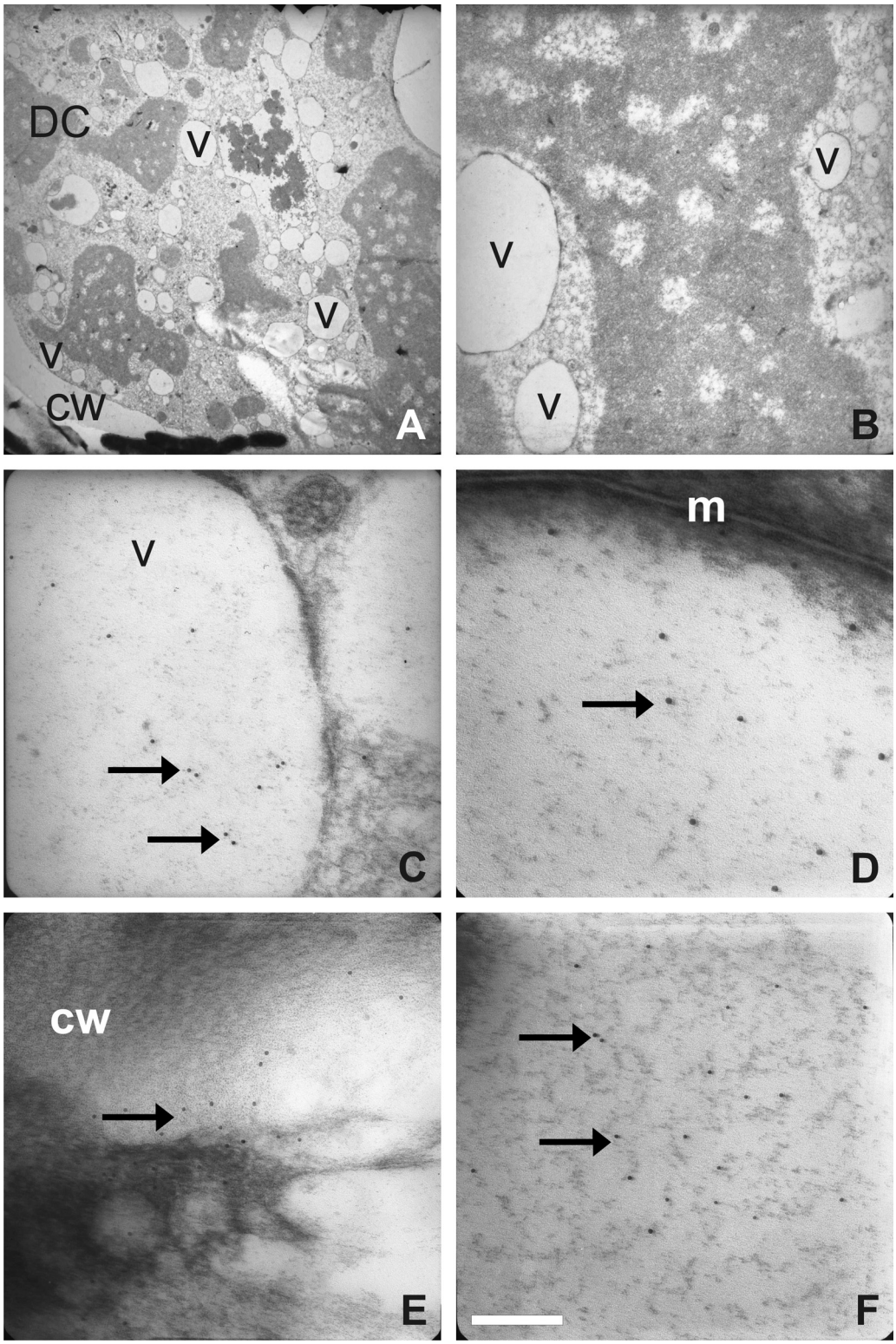

Fig. 5. Transmission electron microscopy of *Canavalia brasiliensis* endosperm. (A and B) General view of cell cytoplasm showing dense cytoplasm (DC), cell wall (CW) and different size and shape of vesicles (V), protein bodies; (C–F) Immunogold localization of *Canavalia brasiliensis* lectin at different cell compartments, such as vacuoles (Figures C and D), cell wall (Figure E) and subcellular space (Figure F). m—membrane. Bars: (A) 1 μm; (B) 1 μm; (C, E and F) 0.5 μm; (D) 0.8 μm.

However, the real dynamics of ConBr during germination is still unclear.

5. Immunodiffusion and Western blotting

Lectins of the Diocleinae subtribe (Leguminosae; Papilionoideae; Phaseoleae) are among the most extensively investigated, however, ConBr localization in plant cells has not previously been reported. As observed by several investigators, Diocleinae lectins are highly homologous in structure (Cavada et al., 2001), but they differ considerably with regard to biological activities (Assreuy et al., 2009; Figueiredo et al., 2009; Gadelha et al., 2005). Such homology of structure has resulted in the recognition of different proteins by anti-ConBr, as well as *C. boliviana* lectin (CBol), *C. maritima* lectin (CMar), *D. megacarpa* lectin (DMeg), *D. grandiflora* lectin (DGra), and *D. rostrata* lectin (DRos). Importantly, proteins lacking homology were not recognized, reinforcing antibody specificity for Diocleinae lectins.

5.1. Cotyledon cell ultrastructure and lectin immunolocalization

Ultrastructural characteristics observed in *C. brasiliensis* cotyledons, such as polygonal cells with thick cell walls and dense cytoplasm closely adhered to the cell wall, have already been described in legume seeds (Chen et al., 1998; Lackey, 2009; Oliveira and Paiva, 2005). The organelle termed protein bodies was also structurally similar to the one described for seeds (Pernollet, 1978).

Previous studies on seed structure have demonstrated on Leguminosae that storage proteins accumulate during seed development on storage vacuoles (Clarke et al., 1975; Elmer et al., 2003; Hei et al., 2001; Wang et al., 2009). Wang et al. (2009) described a membrane-associated protein that was specifically localized to the protein storage vacuole tonoplasts in developing mung bean seeds.

The subcellular localization of lectin in other Diocleinae species has already been described; however, ConBr subcellular localization has not been reported. The first record on Leguminosae lectin localization on protein bodies was reported in 1975 (Clarke et al., 1975); however, this study did not use immunocytochemistry as a supporting assay. Since then, some studies have reported on lectin localization in *D. lehmanni* (Melgarejo and Pérez, 1997), *Glycine max* (Horisberger and Vonlanthen, 1980), *Vigna radiata* cell walls (Haab et al., 1981), *Phaseolus vulgaris* (Manen and Pusztai, 1982), *Canavalia ensiformis* (Herman and Shannon, 1984) and *C. mollis* (Santos et al., 2004). These studies revealed the presence of lectin in storage parenchymatic cells, specialized vacuoles termed protein bodies, cytoplasm or cell wall. On the other hand, none of these authors suggested that lectin localization must be directly related to its function.

The presence of lectin on the cotyledon periphery, immediately below the tegument, may suggest a defense function (Santos et al., 2004). However, this region is the first to consume starch during germination, in contrast to lectin which is the last to be consumed during germination (Cavada et al., 1990). According to Chrispeels and Raikhelb (1991), lectin localization on the cell wall may indicate that this molecule is involved in the elongation of this structure. On the other hand, this molecule may be transported to degradation on another cell.

Locating the lectin at the seed dormancy stage is the first step toward revealing the real function of this protein on different physiological processes as germination.

Thus, while the present study has shown that ConBr is located on cotyledonary protein bodies, more studies are required to elucidate the fine function and dynamics of ConBr in plant physiology.

C. brasiliensis lectin (ConBr) was localized for the first time on cotyledons cells. The antibody against ConBr was not only able to recognize this protein but also other Diocleinae lectins. The presence of ConBr lectin in protein bodies and cell wall of cotyledon cells reflects a possible role in defense and storage. The recognition of different Diocleinae lectins by the anti-ConBr antibody encourages the use of this molecule to localize other lectins, avoiding the preparation of new antibodies. Thus, anti-ConBr can be a powerful biotechnological tool in the study of lectins.

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