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The mannan from *Schizolobium parahybae* endosperm is not a reserve polysaccharide

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Abstract

In higher plants, mannans are found as dominant reserve material in the endosperm of Arecaceae seeds and also in some species from Apiaceae, Rubiaceae and Asteraceae. A linear β(1 → 4)-D-mannan was now isolated from the endosperm of *Schizolobium parahybae*, family Caesalpiniaceae, a native of Southern Brazil. Its seeds were germinated and the consumption of polysaccharides from the endosperm, namely galactomannans and β(1 → 4)-D-mannan, was analysed at differents stages of germination. At the 6th day after germination no residual 3:1 Man:Gal galactomannan was found, indicating that complete degradation of galactomannan had been reached. However, after 12 days of germination, the mannan was recovered from the remaining endosperm. Its presence in the endosperm after germination demonstrated that it is not a reserve material as described for seeds of other species.

Keywords: Mannan; Galactomannan; Germination; *Schizolobium parahybae*; Structural polysaccharide

1. Introduction

In plants, pure mannans are defined as essentially linear β(1 → 4)-D-mannopyranosyl chains containing less than 5% of galactose. Mannans are the main reserve material in the seed endosperm of the monocotyledonous Arecaceae family (Avigad & Dey, 1997). They have been characterized in *Phytelephas macrocarpa* (ivory nut), *Phoenix dactylifera* (date), *Phoenix canariensis, Iriartea ventricosa* and *Cocos nucifera* (Meier, 1958; Percival, 1966; Timell, 1957).

Two mannans, A and B, having different molecular weight have been isolated from ivory nuts. Mannan A is extractable with alkali and mannan B cannot be extracted directly. Mannan B is separated from cellulose by precipitation from cuprammonium solution (Meier, 1958; Timell, 1957).

Seeds from *Carum carvi*, Apiaceae, also store mannan in the endosperm (Hopf & Kandler, 1977). However, the mannan of *C. carvi* differs from those of Arecaceae in its low content of the alkali-soluble mannan A, which is dominant in Arecaceae (45% of the endosperm of ivory nut seeds).

Several studies have been performed on ultrastructural aspects of mannans. These homopolysaccharides were analysed by electron microscopy, electron-diffraction (Chanzy, Grosrenaud, Vuong, & Mackie, 1984; Chanzy, Dubé, Marchessault, & Revol, 1979), and X-ray diffraction (Nieduszynski & Marchessault, 1972; Yui, Miyawaki, Yada, & Ogawa, 1997). Mannans exhibit polymorphism, whose forms are named I and II. Mannan I corresponds to the native state and mannan II to the alkali-treated form. Chanzy et al. (1984) demonstrated that mannan A corresponds to mannan I and mannan B to mannan II.

Investigations on conformational properties of mannan chains and their oligosaccharides by molecular modelling have also been performed due the interest in their basic structure, which is widespread in thickening heteropolysaccharides used for food and nonfood applications, such as galactomannans and glucomannans (Mackie, Sheldrick, Akrigg, & Perez, 1986; Petkowicz, Mazeau, & Reicher, 1998; Tvaroska, Perez, Noble, & Taravel, 1987; Yui et al., 1997).

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Wolfrom, Laver, and Patin (1961) isolated a pure mannan from coffee beans. More recent studies have shown that mannans are present in green and roasted coffee beans as well as in hot water coffee extracts (Fischer, Reimann, Trovato, & Redgwell, 2001; Oosterveld, Voragen, & Schols, 2003; Redgwell, Trovato, Curti, & Fischer, 2002). On roasting, mannans undergo moderate degradation, increasing their extractability from the bean (Redgwell et al., 2002). It has been pointed out that mannans are responsible for the high viscosity of coffee extracts, which could negatively affect the technological processing of instant coffee (Sachslehner, Foidl, Foidl, Gübitz, & Haltrich, 2000). According to Navarini et al. (1999), coffee extracts contain an arabinogalactomannan instead of a simple linear mannan. The isolation of an endo-β-mannanase from germinating coffee (*Coffea arabica*) grains suggest that in seeds, the mannan is also a reserve material (Marraccini et al., 2001).

The mannans are known as important biological response modifiers, which can exhibit a remarkable range of immunopharmacological and therapeutic activities. Aloe species contain O-acetyl β(1 → 4)-D-mannans, which have been reported to show antiviral and antiproliferative properties *in vivo* (Krizková et al., 2006). Sampedro et al. (2004) showed by flow cytometry that the mannan from *Aloe saponaria* inhibits the proliferative response in normal and tumoral cells. Recently, Liu et al. (2006) observed that a mannan from *Aloe vera* exhibits *in vitro* tumoricidal properties.

Schizolobium parahybae (guapuruvu) and *Schizolobium amazonicum* (pinho cuiabano) are Caesalpiniaceae spp. which grow in southern Brazil and the Amazon region, respectively (Ernani, 1994). However, Rizzini (1986) has considered the two species identical. Isolated endosperms from both species yielded ~50% of galactomannan with 3.0:1 Man:Gal ratios, having the same galactose distribution along the main chain (Ganter, Heyraud, Petkowicz, Rinaudo, & Reicher, 1995; Petkowicz, Sierakowski, Ganter, & Reicher, 1998). Seed coats from both *Schizolobium* spp. furnished a similar, unusual, neutral, linear (1 → 5) linked α-L-arabinofuranan (Petkowicz et al., 1998; Zawadzki-Baggio, Sierakowski, Corrêa, & Reicher, 1992). In a previous paper (Petkowicz, Reicher, Chanzy, Taravel, & Vuong, 2001), a pure mannan was obtained from the endosperm of *S. amazonicum.* This mannan II, described for the first time in Leguminosae spp., was characterized by chemical analyses, ^{13}C NMR, X-ray and electron diffraction.

We now report the isolation of a pure mannan from the seeds of *S. parahybae*, to investigate whether it plays some nutritional role in the seeds, by following its consumption after germination.

2. Materials and methods

2.1. Polysaccharide source

Seeds of *S. parahybae* were purchased from EMBRAPA (Empresa Brasileira de Agropecuária).

2.2. Polysaccharide isolation

Seeds of *S. parahybae* (200 g) were treated with boiling water (500 mL) for 30 min and then at 4 °C until swelling took place. The seed coats were then removed and each side of the endosperm was separated from the embryo. Isolated endosperms were kept in water at 4 °C for 24 h. Each isolated endosperm was shown to be constituted by three different parts (interior section, exterior section and a mucilaginous fraction).

The exterior section of the endosperm was isolated, rinsed with water, and submitted to exhaustive hot aqueous (65 °C) extraction. The insoluble residues from aqueous extractions were successively extracted twice with 2 M NaOH (100 °C) in the presence of $NaBH_4$.

At each step of disencrustation, samples were collected, thoroughly washed or dialyzed against distilled water until neutrality, and the monosaccharide composition determined.

2.3. Monosaccharide composition

Samples were dissolved in 72% (w/w) aqueous H_2SO_4 (1 h, 0–4 °C). Water was then added to a final concentration of 0.25 M (5 h, 100 °C) (Saeman, Moore, Mitchell, & Millet, 1954). The hydrolyzates were neutralized with $BaCO_3$ and the insoluble material removed. The hydrolyzates were then reduced with $NaBH_4$, and the products acetylated with pyridine-acetic anhydride (1:1 v/v, 16 h, 25 °C). The resulting alditol acetates were examined by gas–liquid chromatography – mass spectrometry (GC-MS) using a Varian model 3300 gas chromatograph coupled to a Finnigan Ion-Trap, model 810 R-12 mass spectrometer, using a DB-225 capillary column (30 m × 0.25 mm i.d.), programmed from 50 to 220 °C at 40 °C/min, then hold. Helium was the carrier gas.

2.4. Nuclear magnetic resonance spectroscopy

^{13}C NMR spectra were recorded at 50 °C with a DRX-500 Bruker Avance spectrometer, using 50% urea in D_2O as solvent. Chemical shifts are expressed as δ (ppm) relative to the resonance of internal DSS (sodium 4,4-dimethyl-4-silopentane-1-sulfonate).

2.5. High performance size exclusion chromatography of galactomannans

The high performance size exclusion chromatography (HPSEC) apparatus used was a Waters unit coupled to a refractive index (RI) and a multi-angle laser light scattering Wyatt Technology Dawn-F MALLS. Four Waters Ultrahydrogel columns (2000; 500; 250; 120) were connected in series and coupled with the multi-detection instrument. A solution of 0.1 M $NaNO_2$, 0.02 % NaN_3 was used as eluent at a flux of 0.6 mL/min. Samples of galactomannan (1 mg/mL) were filtered through a 0.22 μm nitrocellulose membrane. HPSEC data were col-

lected and analyzed by a Wyatt Technology ASTRA program. All the analyses were carried out at 25 °C. The refractive index increment of the solvent–solute solution, with respect to a change in solute concentration (dn/dc) was determined using a Waters 2410 differential refractometer.

2.6. Seed germination

Seeds of *S. parahybae* were washed and sterilised by immersing them for 20 min in aqueous sodium hypochlorite (0.75 % active chlorine) and then washed exhaustively with sterile distilled water. They were then placed to hot water, which was kept at room temperature for 24 h . They were then placed in a layer of sterile vermiculite (7.3 cm of height) for germination at 23 °C with a photoperiod of 12 h. The vermiculite was kept moist with sterile water addition at intervals of 24 h.

2.7. Isolation of polysaccharides from germinated seeds

After radicle protrusion, seeds were harvested daily and the remaining endosperm removed. The different parts (interior section, exterior section and the mucilaginous fraction) of each isolated endosperm were separated. These components were submitted to enzymatic inactivation by boiling in water for 2 min. All materials were dried, weighted and submitted to exhaustive hot aqueous (65 °C) extraction. For the interior and exterior sections of the endosperm, the insoluble residues from aqueous extractions were successively extracted with aqueous alkali as already described. At each step, samples were collected, thoroughly washed and the monosaccharide composition analyzed. Aqueous extracts from the mucilaginous fraction were treated with ethanol (2 v) and the precipitaded polysaccharides analyzed.

3. Results and discussion

Many storage polysaccharides of seeds such as mannans, galactomannans, glucomannans, xyloglucan and galactans are present in the endosperm or cotyledon for mobilization after germination. Galactomannans are similar in structure to the mannans, but the main chains are substituted at O-6 by single α-D-galactopyranosyl units. The Man:Gal ratios are characteristic of each species and can vary from 1:1 to 4:1 (Avigad & Dey, 1997).

Seeds of *S. parahybae* were previously analyzed to determine the fine structure of the galactomannan (Ganter et al., 1995; Ganter, Zawadzki-Baggio, Leitner, Sierakowski, & Reicher, 1993; Petkowicz et al., 1998); the presence of an unusual arabinan in the seed coats was also found (Zawadzki-Baggio et al., 1992).

When isolated endosperms from the seeds of *S. parahybae* were kept in water at 4 °C for 24 h they had three different parts (interior section, exterior section and a mucilaginous fraction) as previously described for *S. amazonicum* (Petkowicz et al., 2001). The interior section was originally in contact with the embryo and the exterior section, which has a relative high mechanical resistance, corresponding to the seed coat side. Between these two parts is the mucilaginous fraction which contains a galactomannan with a 3:1 Man:Gal ratio and is the main polysaccharide in the seeds of *S. parahybae*. However, a less substituted galactomannan was found in interior sections (3.9:1 Man:Gal ratio). The exterior sections were submitted to aqueous extraction (65 °C) and then two alkali extractions (100 °C) and the composition of the resulting residues determined. A pure mannan (97% mannose) was obtained from the insoluble residue of exterior section from the endosperm of *S. parahybae*. The ^{13}C NMR spectrum of the mannan (Fig. 1) consisted of 6 signals, at δ 101.8 (C-1), 78.1 (C-4), 76.5 (C-5), 73.0 (C-3), 71.6 (C-2) and 62.0 (C-6), identical to those of a

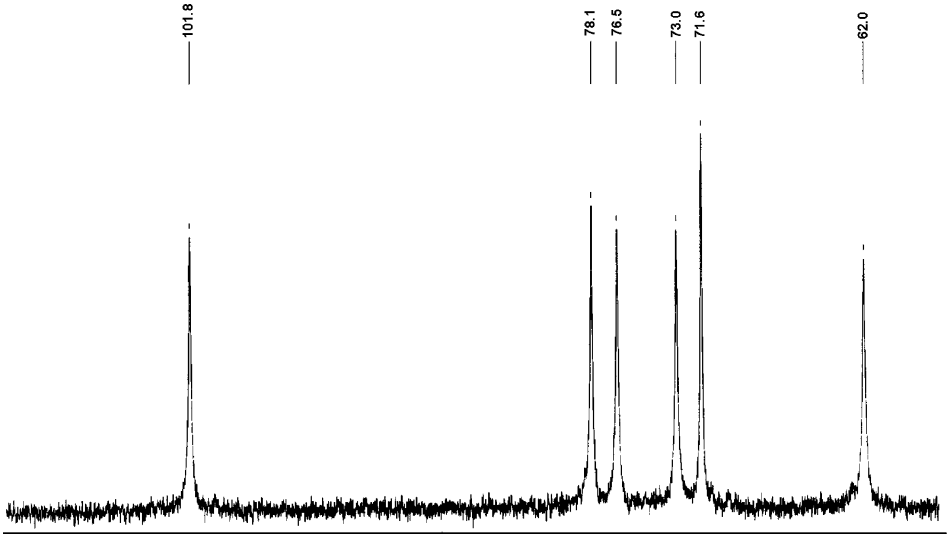

Fig. 1. ^{13}C NMR spectrum of the β-D-mannan isolated from endosperm of *Schizolobium parahybae*; solvent 50% urea in D_2O at 50 °C (chemical shifts are expressed in δ ppm).

linear (1 → 4)-linked β-D-mannan (Gorin, 1973; Jarvis, 1990; Petkowicz et al., 2001). Thus, both *S. parahybae* and *S. amazonicum* (Petkowicz et al., 2001) seeds contain pure mannans, which is unusual for seeds of Fabales. This finding along with similarities already described, reinforce the hypothesis that *S. parahybae* and *S. amazonicum* are the same species.

The seeds of *S. parahybae* were germinated and the consumption of polysaccharides from the three parts of the endosperm, determined at differents stages of germination. Samples were collected after germination over 12 days and named T_1 (first day after germination), T_2 (second day after germination), …, T_{12} (12 days after germination). After 12 days from germination the interior section was present with the same Man:Gal ratio as determined for ungerminated seeds (3.8:1) and no mucilaginous fraction was observed. The breakdown of galactomannan was followed by HPSEC of polysaccharides isolated from mucilaginous fraction. Profiles from HPSEC analyses (Fig. 2) showed that the mobilisation process started three days after germination. After T_1 and T_2, the elution profile was the same as that of T_0 showing that no degradation of galactomannan took place. After 3 days a slight change was observed, which become more intense at T_4. The T_5 profile indicated that the rate of degradation was greater. The cumulative distribution of molar masses for each fraction can be

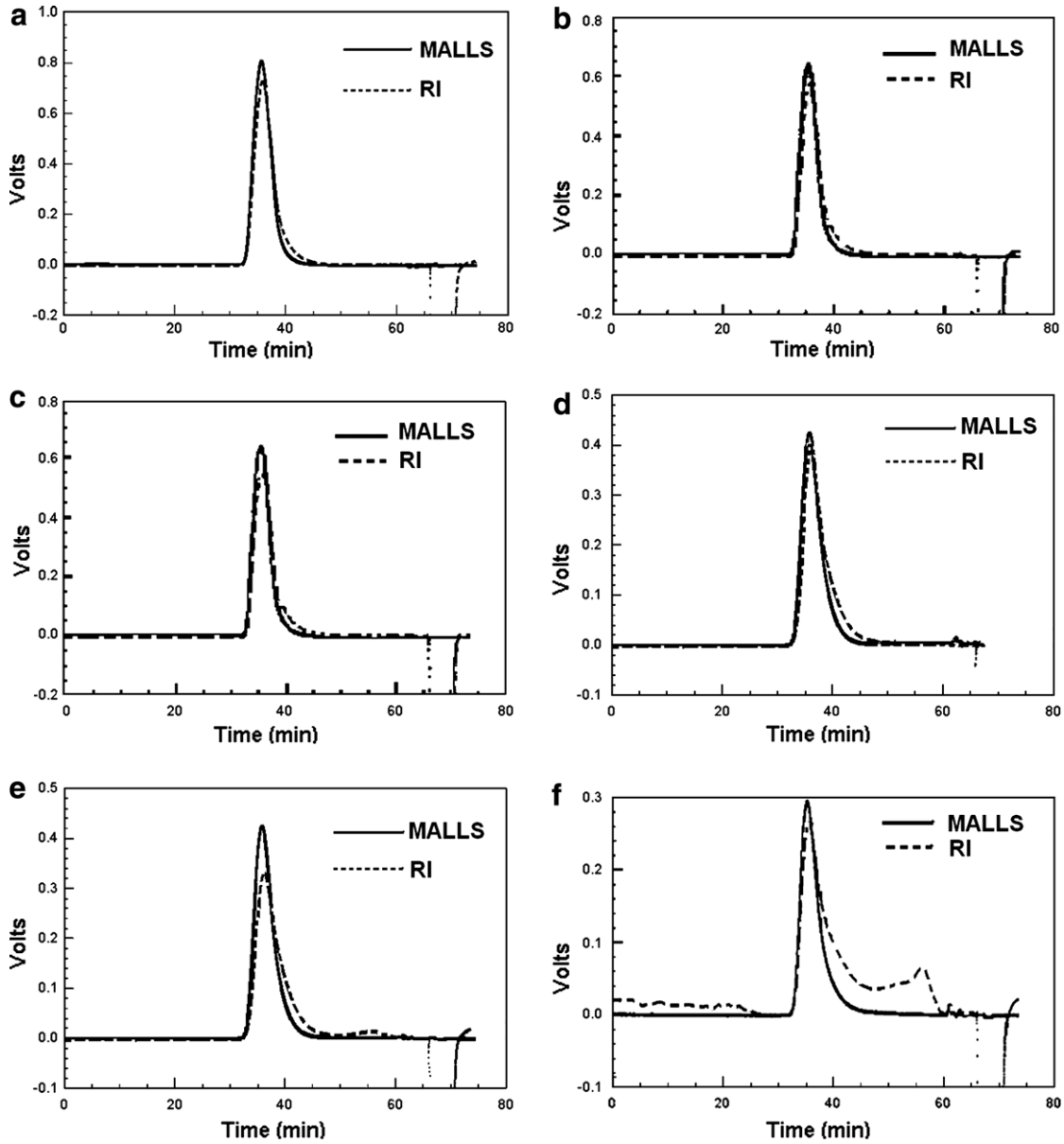

Fig. 2. High performance size exclusion chromatography (HPSEC) of 3:1 Man:Gal galactomannan from mucilaginous fraction from the endosperm of *Schizolobium parahybae*, with multi angle laser light scattering detection (MALLS; 90° is shown) and refractive index (RI) detection. (a) Before germination (T_0); (b) One day after germination (T_1); (c) Two days after germination (T_2); (d) Three days after germination (T_3); (e) Four days after germination (T_4); (f) Five days after germination (T_5).

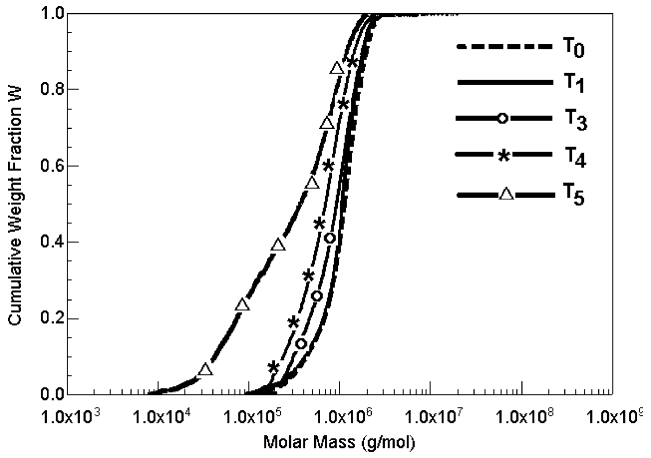

Fig. 3. Cumulative distribution of molar mass for 3:1 Man:Gal galactomannan from mucilaginous fraction from the endosperm of *Schizolobium parahybae* before germination and after one day (T_1), three days (T_3), four days (T_4) and five days (T_5) from germination.

compared in Fig. 3. A large displacement of molar mass (M_w) range can be observed from T_0 to T_5. The average M_w of the isolated galactomannans ranged from 1,057,000 g/mol for T_0–515,000 g/mol for T_{12} (dn/dc of 0.127 and 0.169, respectively). Reid (1971) has shown for *Trigonella foenum-graecum* that in the earliest stages of germination the raffinose family oligosaccharides are metabolised and there was no change in the appearance, amount or composition of galactomannans. For *Sesbania marginata*, the maximum velocity of galactomannan degradation was reached between the 3rd and 4th day of germination (Buckeridge & Dietrich, 1996). At T_6, no residual galactomannan in the mucilaginous fraction could be detected, indicating that complete degradation of the 3:1 Man:Gal galactomannan had been reached. The results are consistent with a rapid and complete hydrolysis of main reserve polysaccharide, as pointed out by McCleary and Matheson (1975) for different legume seeds. The Man:Gal ratio of the galactomannan present in the mucilaginous fraction remained constant (3:1) until it was completely consumed. This result is in agreement with those obtained for other Caesalpiniaceae spp. indicating a concerted action of enzymes (McCleary & Matheson, 1975; Reid, 1971). However, for *Sesbania marginata*, *Medicago sativa* and *Cyamopsis tetragonolobus*, which belong to the Fabaceae family, an increase in Man:Gal ratios was detected at the end of the degradation process (Buckeridge & Dietrich, 1996; McCleary & Matheson, 1975). These data suggest that the mechanism of galactomannan mobilization should be different among the three taxonomic groups of Fabales.

The monosaccharide composition of polysaccharide samples from the exterior section at differents stages of germination and before germination (T_0) are shown in Table 1. Mannose and galactose contents along the 12 days after germination are typical of mannans. Glucose is only a minor component suggesting the absence of cellulose in this material; this is in agreement with the previous X-ray and

Table 1
Monosaccharide composition of exterior section of the endosperm of *Schizolobium parahybae* at different stages of germination

Samples[b]	Monosaccharide composition[a] (%)				
	Ara	Xyl	Man	Gal	Glc
T_0	4.5	Tr	85.1	6.1	1.7
T_1	7.7	Tr	83.7	8.5	Tr
T_2	6.3	0.7	83.3	8.1	1.5
T_3	8.5	1.7	80.7	7.2	1.9
T_4	5.8	0.9	85.2	6.1	1.9
T_5	5.5	1.3	84.9	7.1	1.3
T_{10}	10.5	4.9	74.3	6.8	3.2
T_{12}	6.8	3.7	84.3	3.2	2.0

[a] By GC-MS of alditol acetate derivatives.
[b] Subscripts refer to days after germination.

Table 2
Comparison of the ^{13}C chemical shifts (ppm) for mannans isolated before and after germination of *Schizolobium parahybae* seeds (solvent 50% urea in D_2O at 50 °C)

Mannan	δ (ppm)					
	C-1	C-2	C-3	C-4	C-5	C-6
Before germination	101.8	71.6	73.0	78.1	76.5	62.0
After germination	101.7	71.6	73.0	78.1	76.6	62.1

electron diffraction studies (Petkowicz et al., 2001). The mannan isolated at T_{12} was examined by ^{13}C NMR spectroscopy. The ^{13}C NMR data for the polysaccharide confirmed a linear mannan. The chemical shift assignments (Table 2) are in close agreement with those reported for ivory nut mannan (NaOD solution) and *Phoenix dactylifera* (solid state) and are in accord with those obtained for ungerminated seeds. The presence of the mannan at T_{12} demonstrated that is not a reserve material, as described for seeds of Arecaceae, coffee (Rubiaceae) and lettuce (Asteraceae) (Avigad & Dey, 1997; Halmer, 1989; Marraccini et al., 2001). The lettuce endosperm contained 60% of a mannan, which is completely mobilized in germinating seeds before the depletion of the main embryonic reserves, lipid, and protein (Halmer, 1989).

Mannose differs from glucose by the stereochemistry of the OH-2 group and pure mannans resemble cellulose in the conformation of its individual chains. The mannan from *S. parahybae* seeds has probably a structural role by replacing cellulose.

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