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Diverse patterns of cell wall mannan/galactomannan occurrence in seeds of the Leguminosae

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ABSTRACT

Endosperms from seeds of different subfamilies of Leguminosae were submitted to sequential aqueous and alkaline aqueous extractions. The extractions from species belonging to the Mimosoideae and Faboideae subfamilies yielded galactomannans with constant Man:Gal ratios, whereas the extractions from Caesalpinioideae seeds gave rise to galactomannans with increasing values of the Man:Gal ratio. The presence of a family of galactomannans within the same species may be a trait found only in Caesalpinioideae subfamily. The final insoluble residues that were obtained after the removal of galactomannans from the Caesalpinioideae and Faboideae subfamilies are composed of pure mannans and do not contain cellulose, while those from the Mimosoideae subfamily are composed of cellulose. A mannan was isolated from the unripe endosperm of *Caesalpinia pulcherrima*, suggesting no developmental relationship between galactomannan and mannan. These results are consistent with the presence of a distinctive cell wall pattern in the endosperms of Leguminosae species.

1. Introduction

Plant cell walls are highly complex and dynamic cell compartments that support many aspects of cell development, provide the protoplast with structural rigidity and directly determine the size and shape of plant cells. Thus, they play an important role in the regulation of cell growth and morphogenesis (Cosgrove, 2000; Hoson, 2002). They are mainly composed of polysaccharides, including cellulose, which is the most important and normally the most abundant wall component. Cellulose is found at the core of plant cell walls, where it serves as a scaffold onto which other wall components bind. Pectins and hemicelluloses are components of the cell wall matrix, within which the cellulosic microfibrils are embedded. Both primary and secondary cell walls contain cellulose and hemicelluloses. Primary walls also contain pectin, as well as many enzymes and structural proteins, whereas secondary walls contain little protein or pectin, but contain lignin (Carpita & McCann, 2000).

Secondary cell walls are deposited when cells stop growing. These cell walls often exhibit elaborate specializations for which the incorporation of lignin is usually the most distinguishing feature. However, the secondary walls of cotyledon and endosperm cells in the seeds of many species are unlignified and contain very little cellulose. They are thickened with massive deposits of a non-cellulosic polysaccharide that is typically found in the primary cell wall. The polysaccharides of these secondary walls are digested during germination and are known as "cell wall storage polysaccharides" (Reid & Edwards, 1995).

The major classes of cell wall storage polysaccharides are pure mannans, galactomannans, glucomannans, xyloglucans and galactans (Bacic, Harris, & Stone, 1988; Carpita & McCann, 2000). Among these polymers, native and modified galactomannans have a long history of use for many different applications, such as thickening, emulsifying, gelling, flocculating and film forming capacity (Dea & Morrison, 1975; Mikkonen & Tenkanen, 2012; Singh et al., 2000; Singh, Tiwari, Tripanthi, & Sanghi, 2005).

Galactomannans are the largest group of cell wall storage polysaccharides and consist of a $\beta(1\rightarrow4)$ D-mannan backbone with a single $\alpha(1\rightarrow6)$ substitution of D-galactose (Dea & Morrison, 1975). They are typically found in the endosperm of the Leguminosae species. On the other hand, 'pure mannans' or 'true mannans' are polysaccharides consisting of at least 85% or 95% mannose, depending on the study (Aspinall, 1959; Stephen, 1983).

The endosperm of a *Schizolobium* sp. seed contains galactomannans with a range of different Man:Gal ratios. These galactomannans function as cell wall storage polysaccharides. After the extraction of galactomannans from *Schizolobium* sp. endosperm, the alkali insoluble residue is a pure mannan that is not mobilized during germination (Petkowicz, Reicher, Chanzy,

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Taravel, & Vuong, 2001; Petkowicz, Schaefer, & Reicher, 2007; Petkowicz, Sierakowski, Ganter, & Reicher, 1998). The authors did not find cellulose in *Schizolobium* sp. endosperm, hence they proposed that in these seeds, mannan performs the structural role that cellulose performs in most of other species.

In this work, the seeds of other Leguminosae species that store galactomannans were investigated to check whether a linear pure mannan occurs exclusively in the genera *Schizolobium* or whether it occurs in other species from the different Leguminosae subfamilies. For this purpose, two species belonging to each Leguminosae subfamily were chosen, endosperms from each species were submitted to sequential extractions, and the final insoluble residues were characterized. The selected species were: *Cassia fastuosa* and *Delonix regia* (Leguminosae-Caesalpinioideae), *Adenanthera pavonina* and *Leucaena leucocephala* (Leguminosae-Mimosoideae) and *Crotalaria juncea* and *Sesbania virgata* (Leguminosae-Faboideae). The presence of pure mannans in the endosperm of immature seeds from a Leguminosae-Caesalpinioideae species was also investigated. The results bring new findings which showed that the Leguminosae seeds belonging to the Mimosoideae and Faboideae subfamilies have only a single type of galactomannan in the endosperm while species from Caesalpinioideae subfamily have galactomannans with different Man:Gal proportions. Besides, for the first time it is shown that seeds from the Caesalpinioideae and Faboideae subfamilies have mannans instead of cellulose as microfibrillar component of the endosperm cell wall and the mannan also was found in the immature seeds.

2. Materials and methods

2.1. Materials

Dimethyl sulfate, trifluoroacetic acid (TFA), sodium borodeuteride, monosaccharide standards and dialysis tubing were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Deuterium oxide (D_2O) was supplied by Cambridge Isotope Laboratories (Andover, MA, USA). All other chemicals used were of analytical grade.

2.2. Polysaccharide source

Seeds of *D. regia* (lot number PI384), *L. leucocephala* (lot number PI375), *Hymenaea courbaril* (lot number PI347) and *Copaifera langsdorffii* (lot number AB94) were purchased from IPEF (Institute of Forestry Studies and Research, Piracicaba – São Paulo), record of producer seeds CATI 1498P – IBAMA (Brazilian Institute of Environment and Renewable Natural Resources) 1/35/90/2273-0). Seeds of *S. virgata* were kindly supplied by Dr. Sérgio Miana de Faria from EMBRAPA (Brazilian Agricultural Research Corporation, Rio de Janeiro) and were identified by the same Institution. Seeds of and *C. juncea* (RNC – National Register of cultivars in the Ministry of Agriculture 27669) were kindly supplied by the agronomist José Aparecido Donizeti Carlos from Piraí Seeds (Piracicaba – São Paulo). Seeds of *C. fastuosa* were collected from specimens used for landscaping in the Curitiba metropolitan area and a voucher specimen is deposited in the Herbarium UPCB (Federal University of Parana), under the number 5904. Seeds from *A. pavonina* and *Caesalpinia pulcherrima* were collected at the Federal University of Ceara (UFC), Fortaleza and identified in the Biology Department of UFC. *A. pavonina* voucher was deposited in the Herbarium Prisco Bezerra – EAC (UFC), under the number 34.872.

2.3. Polysaccharide isolation

The seeds were treated with water at 100 °C for 30 min and then kept at 4 °C until swelling occurred (~24 h). Afterwards, the seeds coats were removed, and the whole dried endosperms (50 g) were submitted to sequential aqueous extractions at 4 °C and 25 °C for 16 h, giving rise to fractions I and II, respectively. The resulting residues were then extracted with water at 60 °C (2×) for 16 h, giving rise to fractions III and IV, respectively. Subsequently, the endosperms were submitted to alkaline extractions at 25 °C with 2 M NaOH and 4 M NaOH (2×), in the presence of $NaBH_4$, for 8 h, affording fractions V, VI and VII, respectively. The seven extracts were each precipitated with 2 volumes of EtOH, and the polysaccharides were collected by centrifugation, dried under vacuum and weighed. The alkaline fractions were resuspended, dialyzed against water and lyophilized. The final insoluble residues obtained after the sequential extractions were thoroughly washed with water until neutrality was reached. For *C. lagsdorffii* and *H. courbaril*, the seeds were dehulled, and the cotyledons were milled and defatted with toluene:EtOH (2:1, v/v) in a Soxhlet apparatus. Milled and defatted cotyledons (50 g) were submitted to sequential extractions, as described above.

2.4. Investigation of the presence of mannans and galactomannans in developing Caesalpinioideae seeds

C. pulcherrima seeds were washed and then placed in plastic bags containing humus as a substrate for germination. After 30 days, the plants were transplanted to the soil. The plants start flowering at about six months after planting. To investigate the presence of mannans, the *C. pulcherrima* seeds were harvested at 25, 29, 35, 39, 45 and 49 days after anthesis. The endosperm was removed from the seeds and treated with methanol:water (4:1, v/v) under reflux for 20 min, cooled to room temperature and centrifuged. The residues were dried and used for sequential extractions with water (25 °C and 60 °C), 2 M NaOH (25 °C) and 4 M NaOH (25 °C and 60 °C). Alkaline extractions were performed in the presence of $NaBH_4$. After the sequential extractions with water and diluted alkali, the monosaccharide composition of the final insoluble residues was determined. The same procedure was used for mature seeds at 51 days after anthesis.

The extracts obtained with water at 25 °C were treated with ethanol (3:1, v/v) to obtain galactomannans that were then washed three times with ethanol and dried under a vacuum. The Man:Gal ratio of these galactomannans, which had been isolated from immature seeds of *C. pulcherrima*, was determined. For comparison, galactomannans from mature *C. pulcherrima* seed endosperms were also isolated under the same conditions, and the Man:Gal ratio was determined.

2.5. Monosaccharide composition

Polysaccharides were hydrolyzed with 1 M TFA for 5 h at 100 °C (Adams, 1965) or with 72% H_2SO_4 for 1 h at 0 °C and then diluted to 8% for 6 h at 100 °C (Seaman, Moore, Mitchell, & Millet, 1954). Monosaccharides were reduced with $NaBH_4$ and acetylated with Ac_2O–pyridine (1:1, v/v, 16 h, at 25 °C) (Wolfrom & Thompson, 1963a, 1963b). The resulting alditol acetates were analyzed by gas-liquid chromatography (GLC) using a 5890 A II HP gas chromatograph at 220 °C (flame ionization detector and injector temperature, 250 °C) with a DB-210 capillary column (0.25 mm i.d × 30 m), a film thickness of 0.25 μm, and N_2 (2.0 mL/min) as the carrier gas.

2.6. Polarimetric analysis

Specific rotations were determined at 25 °C, in 0.5% (w/v) solutions, using water or 50% urea as the solvent.

2.7. Methylation analysis

The final residues were methylated twice by the Haworth method (Lindberg, 1972). Briefly, the final residues were solubilized in 40% NaOH (w/v) in the presence of $NaBH_4$. Dimethyl sulfate (1 mL) was added 10 times at intervals of 30 min and after the last addition, the solution was stirred overnight at 25 °C. After that, the dimethyl sulfate addition process was repeated. Then, water was added and the system was boiled under reflux for 30 min, neutralized with acetic acid and evaporated to dryness. The per-O-methylated products were hydrolyzed, reduced and acetylated as described above. The products were analyzed by gas-liquid chromatography–mass spectrometry (GLC–MS) as partly derived O-methylated alditol acetates. The GLC–MS analysis was performed using a Varian chromatograph model 3300 equipped with an OV-225 capillary column (0.25 mm i.d. × 30 m) linked to a Finningan-MAT mass spectrometer. The products were identified by their typical retention times and electron impact profiles.

2.8. High performance size exclusion chromatography

The high performance size exclusion chromatography (HPSEC) apparatus used was a Waters unit coupled to a refractive index (RI) and a Wyatt Technology Dawn F multiangle laser light scattering (MALLS) detector. Four Waters Ultrahydrogel columns (2000, 500, 250, and 120) were connected in series and coupled to the multi-detection instrument. A solution of 0.1 M $NaNO_2$ with 0.02% NaN_3 was used as an eluent at a flux of 0.6 mL/min. Samples of galactomannan (1 mg/mL) were filtered through a 0.22 μm nitrocellulose membrane. All the analyses were carried out at 25 °C.

2.9. Nuclear magnetic resonance spectroscopy

The ^{13}C NMR spectrum was recorded at 70 °C with a Bruker AC-300 spectrometer at 75 MHz after dissolving the fractions with 50% (w/w) urea–D_2O or S1 solvent (8% NaOH, 6.5% thiourea, 8% urea and D_2O) using acetone as an internal standard (Jin, Zha, & Gu, 2007; Petkowicz et al., 2001).

3. Results

3.1. Galactomannan fractions

Previously, we have shown that the seed endosperm from the Leguminosae *S. amazonicum* consist of three different parts: (i) the embryo side or interior section; (ii) the seed coat side or exterior section; and (iii) an intermediate zone rich in soluble galactomannan with a 3:1 Man/Gal ratio located between the two sides of the endosperm. A β(1→4) microfibrillar mannan was identified as the final insoluble residue from the exterior section after sequential extractions (Petkowicz et al., 2001). In the present work the whole endosperm of Leguminosae seeds were submitted to sequential extractions and the final insoluble residues were also investigated.

According to Áquila, Braga, and Dietrich (2012), the major difference between galactomannans from different seed species is the ratio of D-Gal to D-Man. In the present work, sequential extractions from the endosperms from Caesalpinioideae subfamily members *C. fastuosa* (fractions C) and *D. regia* (fractions D) gave rise to galactomannans with increasing Man:Gal ratios (Table 1). The galactomannan obtained by aqueous extraction at 4 °C had a Man:Gal ratio of 3.0:1 for both species.

The residue from the first extraction was submitted to aqueous extractions at higher temperatures (24 °C and 60 °C), thus yielding less-substituted galactomannans. The Man:Gal ratio of galactomannans obtained by aqueous extractions ranged from 3.0:1 to 4.8:1 for *C. fastuosa* and ranged from 3.0:1 to 4.2:1 for *D. regia*.

When residues from the final aqueous extractions were submitted to alkaline extractions, galactomannans with lower galactose contents were obtained. Galactomannans obtained in the last alkaline extraction had Man:Gal ratios of 10:1 and 6.8:1 for *C. fastuosa* and *D. regia*, respectively.

In contrast to the galactomannans extracted from *C. fastuosa* and *D. regia*, galactomannans obtained by sequential extractions from the endosperms from Mimosoidae subfamily members *Adenanthera pavoniva* (fractions A) and *L. leucocephala* (fractions L) had constant Man:Gal ratios that were independent of the temperature or solvent used in the extraction (Table 1). The Man:Gal ratio was 1.6:1 for both *A. pavonina* and *L. leucocephala*.

After sequential extractions of the endosperm, Faboideae subfamily members *C. juncea* and *S. virgata* also yielded galactomannans with constant Man:Gal ratios in fractions R and S, respectively (Table 1). For all temperatures and all extraction solvents used in this study, the Man:Gal ratio was 2.0:1 for *C. juncea* and 1.5:1 for *S. virgata*.

The galactomannans were analyzed by high-performance size-exclusion chromatography (HPSEC) equipped with a refractive index (RI) and a multiangle laser light scattering (MALLS) detector. All the chromatograms showed a unimodal molar mass distribution. Fig. 1 displays the elution profiles of galactomannans C I–C VII using the RI signal.

Table 1
Mannose:galactose ratio for galactomannans obtained from the endosperm of Leguminosae species.

Leguminosae-Caesalpinioideae			
Cassia fastuosa		*Delonix regia*	
Fraction	Man:Gal	Fraction	Man:Gal
C I	3.0:1	D I	3.0:1
C II	3.2:1	D II	3.4:1
C III	4.0:1	D III	4.0:1
C IV	4.8:1	D IV	4.2:1
C V	6.7:1	D V	4.8:1
C VI	6.8:1	D VI	5.2:1
C VII	10:1	D VII	6.8:1
Leguminosae-Mimosoideae			
Adenanthera pavoniva		*Leucaena leucocephala*	
Fraction	Man:Gal	Fraction	Man:Gal
A I	1.6:1	L I	1.6:1
A II	1.6:1	L II	1.6:1
A III	1.6:1	L III	1.6:1
A IV	1.6:1	L IV	1.6:1
A V	1.5:1	L V	1.6:1
A VI	1.7:1	L VI	1.5:1
A VII	1.6:1	L VII	1.6:1
Leguminosae-Faboideae			
Crotalaria juncea		*Sesbania virgata*	
Fraction	Man:Gal	Fraction	Man:Gal
R I	2.0:1	S I	1.5:1
R II	2.0:1	S II	1.5:1
R III	2.0:1	S III	1.5:1
R IV	2.0:1	S IV	1.5:1
R V	2.2:1	S V	1.6:1
R VI	2.2:1	S VI	1.6:1
R VII	2.0:1	S VII	1.5:1

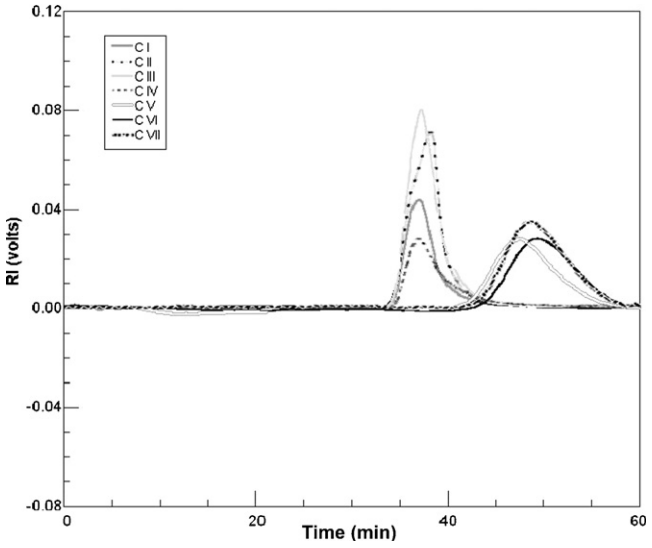

Fig. 1. Elution profile by high-performance size-exclusion chromatography of galactomannans (CI–CVII) obtained by sequential extraction from the endosperm of *C. fastuosa*. Detection by refractive index (RI).

3.2. Final insoluble residues

Hydrolysis of the final insoluble residues obtained after sequential extractions from *C. fastuosa* and *D. regia* showed that mannose is the main monosaccharide (95%) in these species (Table 2), suggesting the presence of a pure mannan. In addition, glucose was not found in these residues, suggesting the absence of cellulose. As shown in Table 2, the final insoluble residues obtained for *C. juncea* and *S. virgata* after the removal of galactomannans also revealed mannose to be the main monosaccharide (96% and 95%, respectively) in these species. After a methylation analysis of these fractions, 2,3,6-Me_3-Man was the main derivative (≥90%), indicating that the backbone chain of mannose is 1→4 linked. Minor proportions of derivatives 2,3-Me_2-Man and 2,3,4,6-Me_4-Gal from the substituted mannose units were also detected, as well as 2,3,4,6-Me_4-Man from the nonreducing ends. The chemical data indicated that the insoluble material from *C. fastuosa*, *D. regia*, *C. juncea* and *S. virgata* endosperm cell walls consist of 1→4 linked mannans. The negative optical rotation of these polysaccharides, −111.9° for *C. fastuosa*, −51.0° for *D. regia*, −49.1° for *C. juncea* and −50.8° for *S. virgata*, is consistent with the presence of β-D-mannans. According to some empirical methods of configurational analysis applied to asymmetric anomeric centers of monosaccharides, the specific rotation is positive for α-D and β-L compounds and negative for β-D and α-L derivatives (Collins & Ferrier, 1995).

Table 2
Monosaccharide composition of the final insoluble residues from the endosperm of *C. fastuosa* and *D. regia* (Leguminosae-Caesalpinioideae), *A. pavoniva* and *L. leucocephala* (Leguminosae-Mimosoideae), *C. juncea* and *S. virgata* (Leguminosae-Faboideae) and the final insoluble residues from the cotyledon of *Hymenaea courbaril* and *Copaifera langsdorffii* (Leguminosae-Caesalpinioideae).

	Man	Gal	Glc (%)	Ara	Xyl
Cassia fastuosa	95.1	4.9	–	–	–
Delonix regia	95.1	4.9	–	–	–
Adenanthera pavoniva	23.0	4.8	72.2	–	–
Leucaena leucocephala	17.7	–	82.3	–	–
Crotalaria juncea	96.1	3.9	–	–	–
Sesbania virgata	94.8	5.2	–	–	–
Hymenaea courbaril	2.4	1.3	90.7	4.0	1.6
Copaifera langsdorffii	3.9	2.8	80.0	9.5	3.8

Table 3
^{13}C NMR chemical shifts for mannans obtained from the endosperm of different Leguminosae species.

→4)-βMan-(1-	δ (ppm)					
	C1	C2	C3	C4	C5	C6
C. fastuosa	100.3	70.8	71.7	76.6	75.1	60.7
D. regia	100.9	70.6	72.1	77.2	75.0	61.0
C. juncea	100.3	70.3	71.7	77.1	75.0	60.7
S. virgata	100.3	70.3	71.7	76.6	75.2	60.8

The presence of pure mannans was also confirmed by ^{13}C NMR experiments. Six major signals corresponding to the carbons of a β(1→4) linked D-mannan were identified for each of the samples (Table 3). All assignments were based on literature values (Jarvis, 1990; Petkowicz et al., 2001). Fig. 2A depicts the ^{13}C NMR spectrum of the mannan from *C. fastuosa* in 50% urea–D_2O at 70 °C.

For the final insoluble residues from *A. pavonina* and *L. leucocephala* extractions, glucose was the predominant monosaccharide (72.2% and 88.3%, respectively). Smaller amounts of mannose and galactose were also detected (Table 2). These data suggest that these insoluble residues are composed mainly of cellulose, as is typical for the insoluble components of cell walls (Carpita & McCann, 2000). These materials were analyzed by ^{13}C NMR spectroscopy using solvent S1, as described in Jin et al. (2007). The spectrum obtained for the insoluble residue from *L. leucocephala* in S1 solvent at 25 °C is shown in Fig. 2B. Six signals at 103.4, 78.7, 75.3, 75.1, 76.3 and 60.5 ppm, corresponding to C-1, C-4, C-3, C-5, C-2 and C-6 of cellulose, respectively, were identified. These assignments were compared with those already described for solutions and solid state spectra of cellulose (Bardet, Emsley, & Vincendon, 1997; Jin et al., 2007; Princi, Vinci, Proietti, & Capitani, 2005).

X-ray diffraction confirmed the presence of pure mannans in the final insoluble residues from Caesalpinioideae and Faboideae seeds and the absence of mannan and the presence of cellulose for *A. pavonina* and *L. leucocephala* (data not shown).

The insoluble fractions obtained after sequential extractions from *H. courbaril* and *C. langsdorfii* cotyledons were compared (Table 2). *H. courbaril* and *C. langsdorfii* also belong to the Leguminosae family, but they store xyloglucan (Rosário et al., 2008). After the removal of the xyloglucans, an analysis of the insoluble final residue showed that glucose was its main component. *H. courbaril* was shown to contain 91% glucose, and *C. langsdorfii* was shown to contain 80% glucose, indicating the presence of cellulose in both of these seeds. Mannose was found to be a minor component, with values of 2% and 4% for *H. courbaril* and *C. langsdorfii*, respectively. These data could indicate that a pure mannan with a structural role only occurs in Leguminosae seeds that contain galactomannans as cell wall storage polysaccharides.

3.3. Mannan and galactomannans in developing Caesalpinioideae seeds

As expected, mature seeds from *C. pulcherrima* of the Leguminosae Caesalpinioideae subfamily also contain a pure mannan. After sequential extractions of the endosperm with water and diluted alkali, the final insoluble residue was shown to be 95% mannose. Immature *C. pulcherrima* seeds were used to investigate whether the mannan in Caesalpinoideae is a product of galactose group removal during maturation. Maturing *C. pulcherrima* seeds were harvested at 25, 29, 35, 39, 45 and 49 days after anthesis. Unripe, gelatinous endosperm was removed from the seeds, and after sequential extractions with water and diluted alkali, the monosaccharide composition of the polysaccharides from the first aqueous extraction and the final insoluble residues were determined.

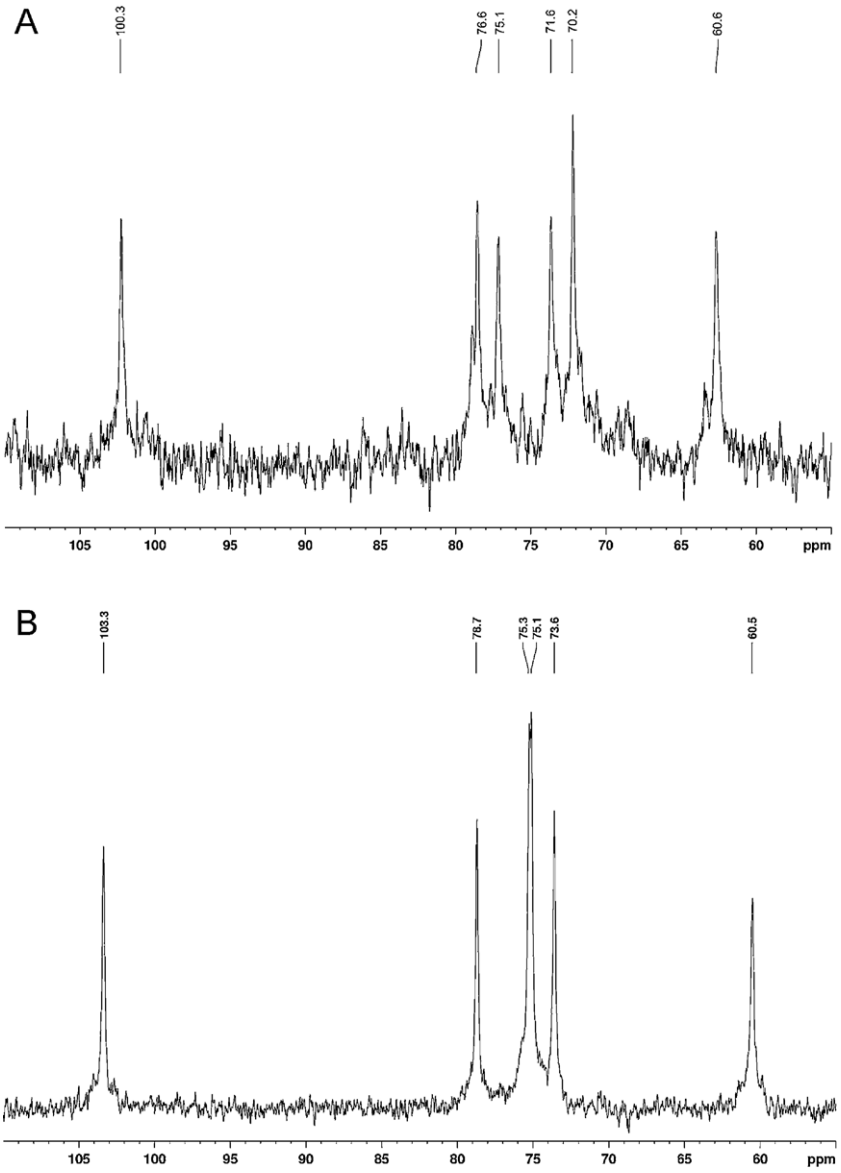

Fig. 2. ^{13}C NMR spectra of the final insoluble residue from *C. fastuosa* in 50% urea–D_2O at 70 °C (A) and the final insoluble residue from *L. leucocephala* in S1 solvent at 25 °C (B).

The extractions with water at 25 °C from the endosperms of seeds harvested at 25, 29, 35, 39, 45 and 49 days after anthesis gave rise to galactomannans whose Man:Gal ratios are depicted in Table 4. At the earliest stages of deposition, the Man:Gal ratio remained constant (2.4:1). From 39 to 49 days after anthesis, the Man:Gal ratio increased to 3.0:1, which is almost the same ratio found for the galactomannans that had been isolated from mature seeds (2.9:1) under the same conditions.

Mannose was the main component (>90%) of the final insoluble residues isolated from the endosperms of seeds harvested at 25, 29, 35, 39 and 45 days after anthesis (Table 5). Only traces of glucose were observed in these insoluble residues (<1%), suggesting the absence of cellulose. A methylation analysis (Table 6) showed that the final insoluble residues isolated from the endosperms of seeds harvested at 25 and 45 days after anthesis contained a (1→4)-linked Man backbone. The analysis also detected 2,3-Me_2-Man and 2,3,4,6-Me_4-Gal from substituted residues. The relatively high yield of 2,3,4,6-Me_4-Man (nonreducing ends) suggests a low molar mass of the polymers. The methylation analysis data indicated that the insoluble material isolated from the endosperms of seeds harvested

Table 4
Man:Gal ratios of galactomannans obtained from extraction with water at 25 °C from the endosperm of seeds of *C. pulcherrima* harvested at 25, 29, 35, 39, 45 and 49 days after anthesis.

Days after anthesis	Man:Gal
25	2.4:1
29	2.4:1
35	2.4:1
39	2.7:1
45	3.1:1
49	3.0:1

Table 5
Monosaccharide composition of final insoluble residues isolated from the endosperm from seeds of *C. pulcherrima* harvested at 25, 29, 35, 39 and 45 days after anthesis.

Days after anthesis	Man (%)	Gal (%)
25	94.4	5.6
29	93.8	6.2
35	94.5	5.5
39	91.1	8.9
45	94.4	5.6

Table 6
Methylation analysis of final insoluble residues isolated from the endosperm from seeds of *C. pulcherrima* harvested at 25 and 45 days after anthesis.

Partially *O*-methylated alditol acetate	25 days after anthesis (%)	45 days after anthesis (%)
2,3,4,6-Me_4-Man	5.6	3.1
2,3,4,6-Me_4-Gal	6.3	5.2
2,3,6-Me_3-Man	80.1	86.2
2,3-Me_2-Man	8.0	5.5

at 25 and 45 days after anthesis is comprised of 1→4 linked mannans.

4. Discussion

It has been reported that galactomannans from different leguminous taxonomic groupings differ in their degrees of substitution and that the Man:Gal ratio is genetically controlled within a given species (Reid & Edwards, 1995). However in the present work we have shown that seeds from a given species from Leguminosae Caesalpinioideae subfamily have galactomannans with different Man:Gal proportions.

The Man:Gal ratio of galactomannans obtained by aqueous extractions from endosperm of *C. fastuosa* ranged from 3.0:1 to 4.8:1. Man:Gal ratio of 2:1 was found for galactomannans from seeds of *Cassia javahikai* and *Cassia pleurocarpa* extracted with 1% (v/v) acetic acid for 24 h at room temperature (Singh, Sethi, & Tiwari, 2009; Singh, Srivastava, & Tiwari, 2009).

The decrease in the galactose content of galactomannans obtained by sequential extractions indicates the presence of a family of galactomannans in *C. fastuosa* and *D. regia* seeds. The HPSEC elution profiles suggest that *C. fastuosa* and *D. regia* endosperms contain a heterogeneous and polydisperse population of galactomannans with respect to both the degree of substitution and the molar mass. This is one of the reasons for the different Man:Gal ratios reported for galactomannans isolated from *D. regia*: 4.28:1 (Anderson, 1949) and 2:1 (Kapoor, 1972).

In general, within the same species, galactomannans have constant sugar ratios, and the Man:Gal ratio and galactomannan yield are believed to have taxonomic meaning for Leguminosae (Dea & Morrison, 1975). However, the presence of galactomannans with different Man:Gal ratios within the same species was also observed for *S. amazonicum*, *S. parahybae* (Petkowicz et al., 1998) and *Gleditisia triacanthos* (Manzi, Mazzini, & Cerezo, 1984), which all belong to the same subfamily as *C. fastuosa* and *D. regia*. According to Manzi et al. (1984), the endosperm of *G. triacanthos* contains, at least, four different populations of galactomannans, having Man:Gal ratios ranged from 2.0:1 to 4.2:1.

In contrast to the species studied within the Caesalpinioideae subfamily, the species belonging to the Mimosoideae and Faboideae subfamilies have only a single type of galactomannan in the endosperm. A Man:Gal ratio of 1.6:1 was found for galactomannans from *A. pavonina* and *L. leucocephala* (Mimosoidae subfamily). The same value was reported by Lombardi and Mercê (2003) for a galactomannan isolated from *L. leucocephala*. However, Cerqueira et al. (2009) found a Man:Gal ratio of 1.4:1 for a galactomannan isolated from *A. pavonina*.

C. juncea and *S. virgata*, which belong to the Faboideae subfamily, yielded galactomannans with constant Man:Gal ratios of 2.0:1 and 1.5:1, respectively. Anderson (1949) isolated a galactomannan from *C. juncea*; however, the Man:Gal ratio was not determined.

As expected the lowest degrees of substitution were found for galactomannans isolated from Leguminosae-Caesalpinioideae species. The Man:Gal ratio in the galactomannans varies widely throughout the Leguminosae family, from 3 to 5:1 in the primitive Caesalpinioideae subfamily to around 1.1:1 in the more advanced Leguminosae-Faboideae species. These variations are not due to inter-species differences in the biosynthetic pathway for the synthesis of the primary galactomannan, but have been attributed instead to the presence of differing ratios of mannosyl to galactosyl transferases between species and to post-deposition modifications (Davis, Hoffmann, Russell, & Debet, 1995; Dea & Morrison, 1975; Edwards, Scott, Gidley, & Reid, 1992). A combination of the activities of α-1,6-galactosyltransferase and α-galactosidase determine the Man:Gal ratio of a galactomannan (Edwards et al., 1992, 2004).

Pollard, Eder, Fischer, & Windhab (2010) isolated galactomannans from legume seed endosperms and reported that galactomannans obtained from Caesalpinioideae are less substituted and have much lower molar mass than those obtained from the Faboideae subfamily, which are more substituted. These authors proposed that mannosyl transferase may be involved in chain length regulation or chain termination during biosynthesis of less-substituted galactomannans in Caesalpinioideae, but not in more-substituted galactomannans from Faboideae legumes, resulting in longer chains in the latter.

The presence of a single type of galactomannan in the endosperms of species belonging to the Mimosoideae and Faboideae subfamilies, in addition to the results obtained for the Caesalpinioideae seeds of *S. amazonicum* (Petkowicz et al., 2001), *S. parahybae* (Petkowicz et al., 2007) and *G. triacanthos* (Manzi et al., 1984) and those from the present work, suggest that the presence of a family of galactomannans within one species may be a trait existing exclusively in the Caesalpinioideae subfamily, given that no species from the Faboideae and Mimosoideae subfamilies studied herein have this trait. Considering the evolutionary relationships between the subfamilies of Leguminosae, in which Mimosoideae and Faboideae are derived from Caesalpiniodeae, these data could suggest that there was a reduction in galactomannan polydispersity during leguminous evolution. In addition, the results obtained in this study indicate that the synthesis of a family of galactomannans, as observed in the Caesalpinioideae subfamily, was replaced in evolution with the synthesis of a single type of polysaccharide, as observed in the Mimosoideae and Faboideae subfamilies.

Petkowicz et al. (2001, 2007) isolated pure mannans from the insoluble fractions obtained after sequential extractions from *S. amazonicum* and *S. parahybae* endosperms. For seeds of *S. amazonicum*, it was demonstrated that the mannan is located in a specific region of the endosperm next to the seed coat (Petkowicz et al., 2001).

In the present work, a pure mannan was isolated from *C. fastuosa* and *D. regia*, which belong to the same subfamily as *S. amazonicum* and *S. parahybae*. However, glucose was the main monosaccharide detected in the insoluble residues from the cotyledons of *H. courbaril* and *C. langsdorfii*, which are also members of the Leguminosae-Caesalpiniodeae subfamily, but store xyloglucan. These results indicate that the mannan might be a characteristic of species from the Caesalpinioideae subfamily that contain galactomannan as a storage carbohydrate. A linear pure mannan was also isolated from the insoluble fractions that were obtained after sequential extractions from *C. juncea* and *S. virgata* (Faboideae) endosperms. In the Mimosoideae subfamily, cellulose was obtained as the insoluble fraction after the removal of galactomannans.

The structural diversity of mannan based polysaccharides allows for a wide range of physicochemical properties, which in turn contributes to their in-planta functionality. Pure mannans are insoluble and the water-solubility of the galactomannans increases with the galactose substitution (Schröder, Atkinson, & Redgwell, 2009). The biological importance of the Man:Gal ratio in legume seed galactomannans is not well-established. It has been proposed that this ratio may be related to the interaction of galactomannans with water. Galactomannans can absorb large amounts of water, thus protecting the germinating embryo against desiccation (Reid

& Bewley, 1979). It has been pointed that galactomannans with different levels of substitution can interact with cellulose by different ways. Low galactose galactomannans induces a coalescence of cellulose fibrils and reduction of crystallinity. Medium galactose galactomannans form cross-links of varying lengths between cellulose fibrils and higher galactosyl substitution presents a significant barrier to the interaction (Whitney, Brigham, Darke, Reid, & Gidley, 1998).

Mannans are the main reserve material in the seed endosperm of Palmae species (Avigad & Dey, 1997). Although the mannans found in the endosperms of seeds from the Caesalpinioideae and Faboideae subfamilies could be used as storage polysaccharides, these seeds also contain soluble galactomannans, the presence of which is probably related to the drought-avoidance mechanism described by Reid and Bewley (1979). Seeds with deposits of pure mannans in their endosperm are hard and resistant to mechanical damage and retain their resistance even after being exposed to water, due to its high insolubility in water (Reid & Edwards, 1995; Stephen, 1983).

The presence of galactomannans that have different degrees of substitution only in the endosperms of the Caesalpinioideae species can be explained by differences in the control of galactosylation during galactomannan formation in these seeds. The deposition of galactomannans in seeds was first described for *Trigonella phoenum-graecum* by Meier and Reid (1977). Since then there are only a few detailed accounts of the deposition process occurring during seed development (Áquila et al., 2012). Edwards et al. (1992) investigated galactomannan deposition in the developing endosperms of species from the Caesalpinioideae and Faboideae subfamilies. These authors observed that the Man:Gal ratio in the Faboideae species did not change during or after deposition. However, for *Senna occidentalis* (Caesalpinioideae), the genetic control of galactosylation was the result of a post-depositional modification. In these seeds, the authors observed galactose removal from the primary biosynthetic product by endogenous α-galactosidase activity in the endosperm during late deposition. For *Senna macranthera* and *G. triacanthos*, both Caesalpinioideae, it was also observed increase in the Man:Gal ratio during seed development (Áquila et al., 2012; Mallet, McCleary, & Matheson, 1987). Similar results were found in the present work for *C. pulcherrima.* The Man:Gal ratio of galactomannans present in the *C. pulcherrima* endosperm at different stages of seed development increased in relation to seed development reaching ~3.0:1 for mature seeds. The same Man:Gal ratio was found by Unrau and Choy (1970) for a galactomannan extracted with hot water from seeds of *C. pulcherrima.*

The removal of galactosyl residues from polymeric galactomannans by an α-galactosidase might give rise to polysaccharides with different Man:Gal ratios, as well as to a pure mannan. However, pure mannans replacing cellulose in the endosperms of Caesalpinioideae species is probably not related to a post-depositional modification of galactomannans, given that in this work, pure mannans were also found in Faboideae seeds. This hypothesis is corroborated by the results of Edwards et al. (1992), who reported constant and fairly low levels of α-galactosidase throughout galactomannan deposition in Faboideae seeds. Additionally, our results showed the presence of mannans in immature *C. pulcherrima* seeds. The isolation of mannans from unripe *C. pulcherrima* endosperm indicates that there is no developmental relationship between galactomannan and mannan in Caesalpinioideae seeds.

The presence of mannans in immature *C. pulcherrima* seeds suggests that this polysaccharide is not a product of an enzymatic modification of galactomannans during the course of maturation. This hypothesis has been suggested for some Palmae species whose immature seeds contain galactomannans, while mannans are found at maturity (Kooiman, 1971). To our knowledge, this is the first time that the presence of a pure mannan in immature Leguminosae seed endosperms has been reported. It seems that mannan, instead of cellulose, is deposited during the development of *C. pulcherrima* seeds as structural polysaccharide. Mannose differs from glucose by the stereochemistry of the OH-2 group and pure mannans resemble cellulose in the conformation of its individual molecular chains. Like cellulose mannans also displays crystalline polymorphism whose forms are named I and II. (Chanzy, Grosrenaud, Vuong, & Mackie, 1984; Nieduszynski & Marchessault, 1972). For seeds of *S. amazonicum* it was demonstrated by X-ray and electron diffraction that the mannan found in the endosperm is a microfibrillar crystalline mannan II (Petkowicz et al., 2001).

It is interesting that in addition to these differences in some of the biochemical features of their galactomannans, seeds from the Caesalpinoideae and Faboideae subfamilies also show anatomical differences. Seeds from some Faboideae species contain an aleurone layer, which is thought to be responsible for the production of hydrolytic enzymes for the mobilization of galactomannan. In contrast, in seeds from Caesalpinoideae, there is no clear distinction between the endosperm and aleurone layer, and the enzymes are probably produced and liberated by the endospermic cells (Buckeridge & Dietrich, 1996; Reid, 1971).

The isolation of pure mannans from species from the Caesalpinioideae and Faboideae subfamilies, the absence of cellulose in these species, and the data obtained by Petkowicz et al. (2007) showing that the mannan from *S. parahybae* is not consumed during seed germination, suggest that in these subfamilies, there might be a distinctive cell wall pattern, specifically one for which a mannan, rather than cellulose, might function as the skeletal component of the cell wall.

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