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Interaction between cellulose and storage xyloglucans: the influence of the degree of galactosylation

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Abstract

Xyloglucan (Xg) is a polymer found in the primary cell walls of growing tissues and also in the seeds of many dicotyledons as a storage polysaccharide. In this study the interaction with cellulose and Xgs with different fine structures was studied. pH and temperature did not have a marked effect on the interaction, except for a slightly higher interaction at pH 6.0. Ultrastructural analysis showed that the binding capacity depends on the surface area of the cellulose. Among different sources of Xg, the binding capacity varied significantly. HPAEC-PAD analysis of the Xg cellulase-limit digest oligosaccharides of bound Xg suggested that there might be a certain pattern of galactosyl substitution which is related to a higher Xg binding capacity.

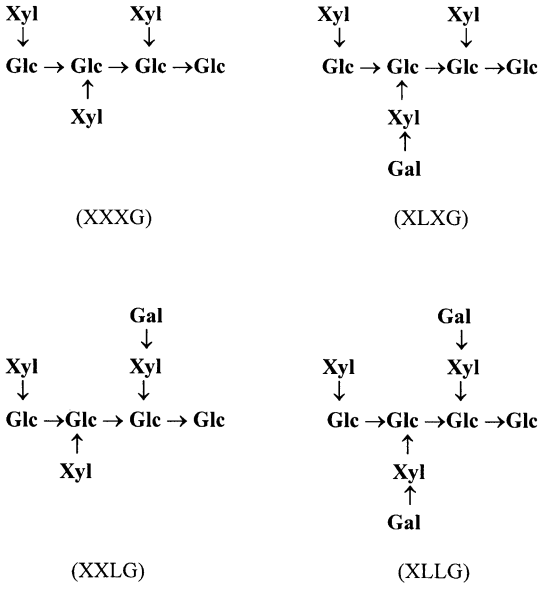
Keywords: Cellulose; Galactose; Xyloglucan; Storage cell wall

1. Introduction

Xyloglucans (Xgs) are cell wall polysaccharides which have a cellulose-like β-(1,4)-glucan backbone to which single-units of α-(1,6)-D-Xyl*p* substituents are attached (branching point designated **X**). Some xylosyl residues are further substituted at O-2 by β-D-Gal*p* residues (branching point designated **L**) and some of the galactosyl residues may be substituted at O-6 by α-D-Fuc*p* (branching point designated **F**) (Hayashi, 1989; Hayashi & Maclachlan, 1984).

Some seeds (especially from legumes) are known to contain large amounts of Xg, which are thought to function as storage compounds (Buckeridge, Santos & Tiné, 2000; Reid, 1985). In this type of Xg, fucose is thought to be absent.

In tamarind seed Xg the pattern of Xyl-substitution is remarkably regular, virtually the whole molecule being composed of repetitive units of Glc_4:Xyl_3 with variable galactosyl substitution (York, Halbeek, Darvill & Albersheim, 1990). A comparative study of the fine structure of seed storage Xgs (*Tamarindus indica*, *Tropaeolum majus* and *Copaifera langsdorffii*) has shown that they are similar in structure, being composed almost entirely of the Glc_4 subunits XXXG, XLXG, XXLG and XLLG (see schedule below):



These subunits are combined in different proportions to give a fine structure that varies according to the species and even within the same species, resulting in conformational differences (Buckeridge, Rocha, Reid & Dietrich, 1992).

More recently, Buckeridge, Crombie, Mendes, Reid, Gidley & Viera (1997) reported that the Xg found in

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seeds of the tropical legume *Hymenaea courbaril* displays unique structural features. Instead of being based on XXXG only, approximately 50% of the *H. courbaril* Xg is composed of a family of oligosaccharides based on XXXXG.

Xyloglucans are known to interact specifically with cellulose (Hayashi, Marsden & Delmer, 1987; Valent & Albersheim, 1974; Vincken, Keizer, Beldman & Voragen, 1995). The basis for this interaction is thought to be the similarity of the backbone of Xgs with cellulose (both are β-(1,4) linked polymers). It is reasonably well accepted that branching with xylosyl residues does not give enough conformational difference to prevent the interaction (Levy, Maclachlan & Staehelin, 1997; Levy, York, Struike-Prill, Meyer & Staehelin, 1991). These and other authors suggested that the degree of fucosylation of primary wall Xgs has an important role in rendering Xg more interactive with cellulose. It is thought that fucose residues flatten the main chain of Xg molecules, making the conformation more similar to cellulose and as a result increasing the interaction (Levy et al., 1991, 1997; Vincken et al., 1995).

It has been shown that fucosylated Xgs are comparatively more interactive with cellulose than unfucosylated ones, although the latter vary in the degree of galactosylation depending on the source. Even though these unfucosylated storage Xgs show a lower degree of interaction with cellulose, this may be partly explained by different degrees of galactosylation, or distributions along the main chain.

In the present work, we used storage cell wall Xgs from seeds that were extracted under identical conditions and had their fine structure studied, to compare their behaviour under different conditions of interaction with cellulose (paper fibres and microcrystalline).

2. Materials and methods

2.1. Polysaccharides

Microcrystalline cellulose powder was obtained from Avicel-SF; cellulose fibres were kindly provided by "Aracruz Papel e Celulose", Espírito Santo, Brazil. Analysis by acid hydrolysis followed by HPAEC-PAD showed that the composition of the fibres is 99% glucose and 1% xylose. Xyloglucan from primary cell walls of *Phaseolus vulgaris* was kindly supplied by Professor William York from CCRC/University of Georgia, USA. Storage xyloglucans were obtained from seeds of *H. courbaril* (from immature legumes), *C. langsdorffii*, *T. indica* and *T. majus*. Galactomannan was obtained from seeds of *Sesbania marginata*. The polysaccharides were extracted from cotyledon powders (or the endosperm in the case of galactomannan) with water (1% w/v) at 80°C for 8 h with constant stirring. After filtration, three volumes of ethanol were added to the aqueous extracts, kept overnight at 5°C and centrifuged (12,000*g* for 15 min at 5°C). The pellet was partially dried at room temperature and freeze-dried after resuspension in water.

2.2. Binding assays

Assays were performed in 25 mM sodium acetate buffer, 600 μg of Xg and 20 mg of microcrystalline cellulose. Before incubation, cellulose was washed five times with distilled water followed by centrifugation. The parameters that were varied during the experiment are pH (2–8) and temperature (5–60°C). After incubation the samples were centrifuged at 13,000*g* and the amount of unbound Xg present in the supernatant was quantified by the I_2/KI method (Kooiman, 1960). The proportion of adsorbed Xg was calculated from the difference between the amounts of Xg in the supernatant before and after interaction.

2.3. Scanning electron microscopy (SEM)

For the examination of the complexes produced between the polysaccharides, the samples (Galactomannan- and Xg-cellulose) were mounted on stubs, freeze-dried, coated with gold (Baltec SCD 050 coater), examined, and photographed in a Philips Scanning Electron Microscope XL20 at an acceleration voltage of 10 kV.

2.4. Analysis of adsorbed xyloglucan

The complexes *Hymenaea* and *Tamarindus* Xgs–cellulose formed during the binding assays were washed (three times) with distilled water and resuspended with 25 mM NaOAc, pH 6.0. Subsequently, they were subjected to digestion with endo-β-(1, 4) glucanase "cellulase" (from *Trichoderma viride* — Megazyme, Australia) for 24 h at 30°C. The reaction was stopped by boiling for 2 min followed by brief centrifugation at 13,000*g*. Samples of the supernatants were analysed for oligosaccharides by HPAEC-PAD and compared with standards obtained by cellulase hydrolyses of native xyloglucans. HPAEC was performed in a Dionex system DX-500 using a CarboPak PA100 column and detected by pulsed amperometric detection (PAD). The samples were eluted with a gradient of sodium acetate (from 35 to 75 mM) in sodium hydroxide (88 mM) and with a flow of 0.9 ml/min. In order to certify that the enzyme attacked all bound Xg, we performed a further extraction with alkali (NaOH, 4 M) and confirmed that there was no further recovery of Xg. This procedure (treatment with fungal cellulase) permitted an evaluation of the fine structure of the Xg bound to microcrystalline cellulose.

The distribution of galactose residues (galactose distribution index) on either side of the backbone of Xg (see Section 1) was estimated by the ratio among the peak areas of subunits (XLLG + XLXG)/(XLLG + XXLG). The closer to 1 the more uniform is assumed to be the distribution of galactose in the polysaccharide (Buckeridge et al., 1992).

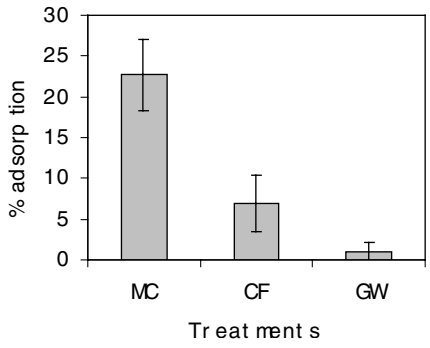

Fig. 1. Interaction between *T. indica* Xg with: microcrystalline cellulose (MC), cellulose fibres (CF) and glass wool (GW). The binding assays were performed (four times) with 600 μg of Xg and 20 mg of cellulose at pH 5.0. The bars represent the standard deviation.

3. Results and discussion

3.1. Xyloglucan binding specificity

Fig. 1 shows the interaction of the storage cell wall Xg from *T. indica* with microcrystalline cellulose (MC), cellulose fibres (CF) and glass wool (GW). A marked difference was observed in the amount of Xg adsorbed to each type of material. MC adsorbed approximately three times the amount of Xg adsorbed to CF. The absence of binding to GW showed the high specificity of the adsorption of the Xg to cellulose.

The higher binding capacity of Xg to MC compared with CF might be explained by the differences in surface area and/or chemical composition of the two types of cellulose. It has already been demonstrated that the binding of Xg to cellulose was dependent on fibre diameter (Hayashi et al., 1987).

3.2. Optimisation of binding assay conditions

Experiments were performed to determine the optimum pH and temperature of incubation using *T. indica* Xg and microcrystalline cellulose. For convenience, incubations were performed for 15 min, although binding was already maximal within 3 min (data not shown).

Within a range from pH 2.0 to 8.0, pH 6.0 showed a slightly better binding capacity (Fig. 2A). Below and above this pH the interaction decreased slightly. Although these data corroborate Vincken et al. (1995), Valent and Albersheim (1974) did not observe changes in the amount of Xg fragments bound to cellulose as the pH varied from 2 to 7.

Although some conformational changes to Xg with temperature can be inferred on the basis of the interaction of Xg with iodine (Minhoto, Tiné & Buckeridge, unpublished results), experiments performed using a range from 5 to 60°C did not alter significantly the Xg–cellulose binding capacity (Fig. 2B).

On the basis of the results described above, the optimum conditions for the Xg–cellulose microcrystalline interaction were pH 6, a temperature of 30°C and 15 min incubation. These conditions were used for further experiments.

3.3. Visualisation of polysaccharide complexes

Fig. 3 shows an ultrastructural analysis by scanning electron microscopy of Xg binding to cellulose. Galactomannan was used as a control. Fig. 3C and D shows the complexes of galactomannan from seeds of *S. marginata* (Fig. 3C) and Xg from seeds of *T. indica* with cellulose fibres (Fig. 3D). These fibres have an average width of 14 μm compared with 10 nm for in vivo microfibrils (Baba, Sone, Misaki & Hayashi, 1994). The complexes formed with MC were indistinguishable from the control samples.

Clusters of hemicellulosic polysaccharide not bound to cellulose (self-association plates) were visualised amidst cellulose fibres for galactomannan (arrows in Fig. 3C), whereas in a complex formed between Xg and cellulose (Fig. 3D) they are not visible. In this case only some "bridges" of fibre-like Xg (arrows) between cellulose fibres can be seen. These bridges are also present in galactomannan–cellulose complexes, but in much lower proportions.

The high degree of self-association for galactomannan molecules, illustrated in Fig. 3C, suggests that there is a relatively lower affinity between galactomannan and cellulose fibres compared to Xg. The same conditions for binding were used for both polysaccharides. However, we

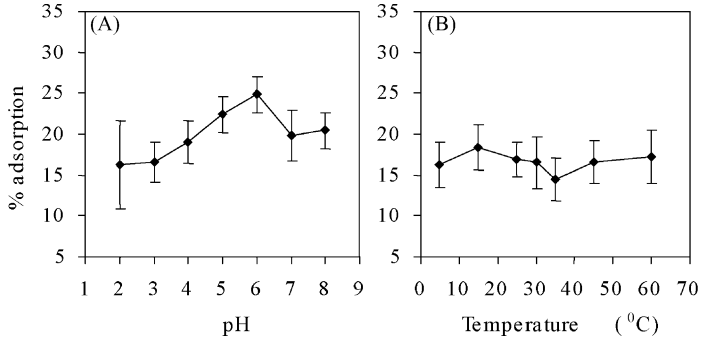

Fig. 2. Effect of pH (A) and temperature (B) on binding of *T. indica* xyloglucan to microcrystalline cellulose. The assays were performed (four times) in 25 mM NaOAc, 600 μg Xg and 20 mg of cellulose during 15 min. The bars represent the standard deviation.

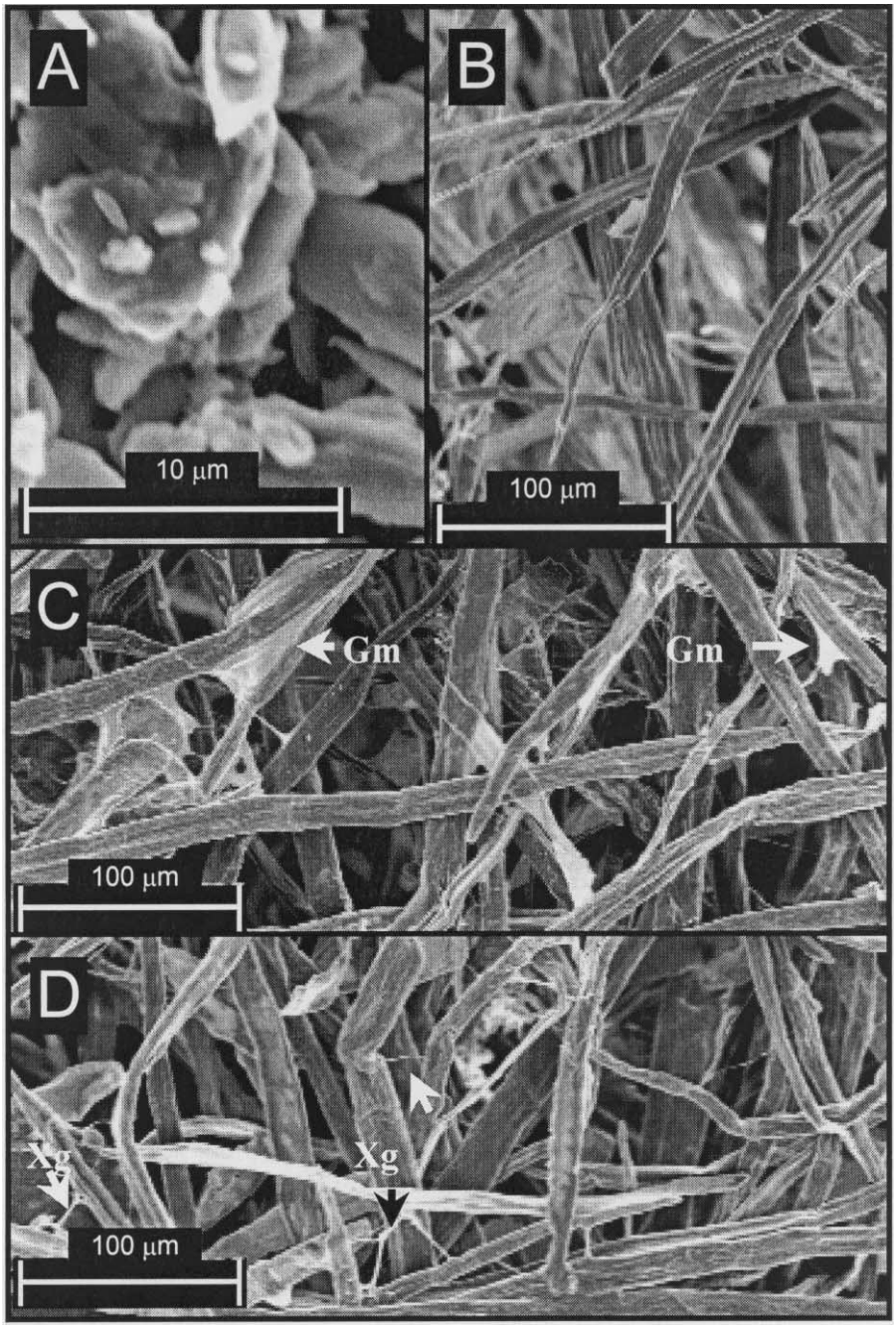

Fig. 3. Scanning electron micrography of microcrystalline cellulose (A); cellulose fibres (B); *S. marginata* galactomannan–cellulose complex (C); *T indica* xyloglucan–cellulose complex (D). Arrows in (C) show the self-association plates; in (D) they show "bridges" of Xg between the fibres.

were not able to detect the structures of self-association for Xg–cellulose complexes (Fig. 3D). This confirms that the interactivity of galactomannan with cellulose is lower than with Xg (Mishima, Hisamatsu, York, Teranishi & Yamada, 1998). In fact, the self-association becomes predominant when galactomannan is in excess. Whitney, Brigham, Darke, Reid and Gidley (1998) showed that when galactomannan concentration is increased from 0.2 to 0.5% in *Acetobacter* (a bacterium that produces cellulose) culture medium, the galactomannan–cellulose composite was less apparent in spite of an increase in self-association.

3.4. Comparative study of interaction between different seed storage xyloglucans and cellulose

Xyloglucans obtained from the seeds of *C. langsdorffii*, *H. courbaril*, *T. majus* and *T. indica* were tested for adsorption to microcrystalline cellulose. Table 1 shows

Table 1
Comparative binding capacity among different sources of xyloglucan with microcrystalline cellulose. Within rows, means followed by different letters are statistically different according to the Tukey test ($P < 0.05$)

Added Xg (μg/mg cellulose)	Species used			
	H. courbaril	*T. indica*	*C. langsdorffii*	*T. majus*
	Adsorbed Xg (%)			
10	39.3a	42.1a	33.0a	35.8a
20	32.1a	27.8ab	20.7b	29.8a
30	29.5a	27.6ab	23.5b	24.9ab
40	32.2a	27.9b	24.0c	20.1c
50	35.9a	29.0b	31.8b	23.9c

the response in binding capacity of increasing Xg concentrations from different species. It can be seen that the percentage of adsorbed Xg within the species vary slightly, independently of the amount of Xg added. An exception is shown at the initial level (10 μg Xg/mg cellulose) where the percentage of adsorption was higher. In general *H. courbaril* Xg showed a greater average binding capacity (33.8%) followed by *T. indica* (30.9%), *T. majus* (26.9%) and *C. langsdorffii* (26.6%).

The primary cell wall Xg from *P. vulgaris* had an interaction of about 70% (data not shown) against an average of 29% for the storage Xgs studied. The higher binding capacity of the former Xg is related to the presence of fucosyl residues (Levy et al., 1991, 1997).

Buckeridge et al. (1992) analysed the structure of seed storage Xgs from *T. indica*, *C. langsdorffii* and also *H. courbaril* (Buckeridge et al., 1997) and showed that the fine structures of these polymers are composed of four structural basic subunits, with the exception of *H. courbaril* that shows a new family of oligosaccharides (XXXXG plus different degrees of galactosylation in this oligosaccharide). It was also shown by these authors that the proportions among those subunits are combined differently among species, reflecting the differences in the distribution and degree of

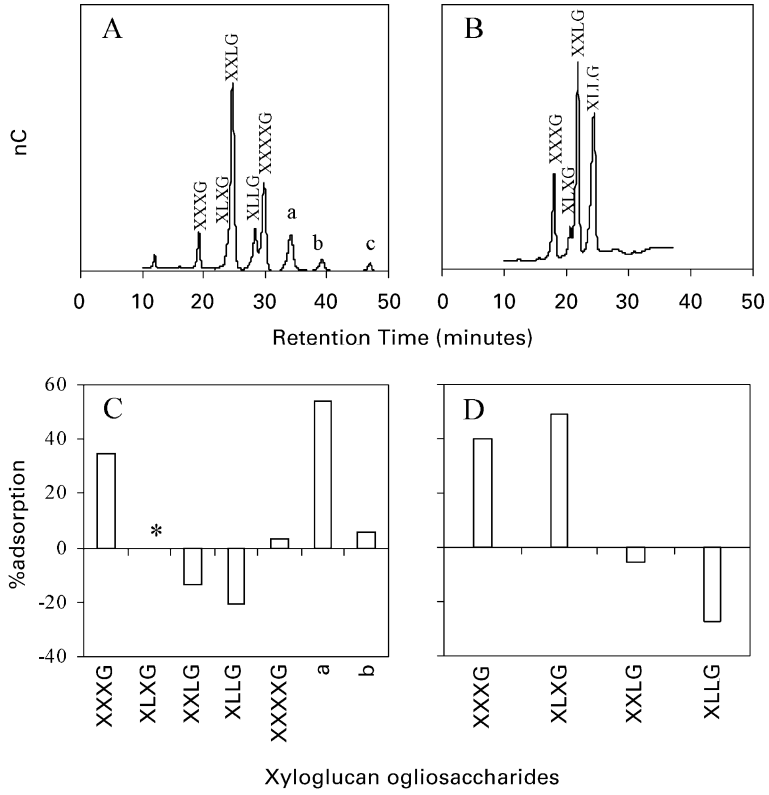

Fig. 4. Comparison of the fine structures of native and bound Xgs from *H. courbaril* and *T. indica*. Typical profiles of HPAEC-PAD analysis of *H. courbaril* (A) and *T. indica* Xg (B). (C) and (D) show the variation in the proportion of limit-digest oligosaccharides derived from bound and native Xgs of *H. courbaril* (C) and *T. indica* (D). The peaks *a*, *b* and *c* are subunits with a Glc:Xyl ratio of 5:4. Peak *c* was not detected after interaction with cellulose. (*) The percentage of adsorption of XLXG could not be calculated because it is not detectable in native samples of *H. courbaril* being present only after binding assays.

Table 2
Comparative binding capacity between Xgs from small and big seeds of *H. courbaril* with microcrystalline cellulose. Their galactosylation degree was estimated by the galactose/xylose ratio. The binding assays were performed (four times) with 20 mg of cellulose and 600 μg in 25 mM NaOAc pH 6.0 at 30°C. Within the column, means followed by different letters are statistically different according to the Tukey test ($P < 0.05$)

Xg sources	Adsorbed Xg (%)	Gal/Xyl ratio
Small seeds	28.8a	0.22
Big seeds	24.3b	0.46

galactosylation of the molecule. These differences in fine structure might be affecting, at some level, the binding capacity of storage Xgs with cellulose.

Vincken et al. (1995) studied the interaction of cellulose with Xg fragments with different degrees of polymerisation and reported that substitution with galactose probably decreases the ability of fragments to penetrate the smaller pores in cellulose. Whitney et al. (1998) found similar results. They observed that galactomannan and cellulose interaction presented a tendency of increase as a function of decrease in the galactosyl residues in galactomannan. Further analyses were performed here to evaluate the influence of galactose on Xg binding to cellulose.

3.5. The influence of galactose branching on the binding of xyloglucan to cellulose

HPAEC-PAD analyses showed the patterns of elution profiles of limit digest oligosaccharides from *H. courbaril* (Fig. 4A) and *T. indica* Xgs (Fig. 4B). Fig. 4C and D show the variations in the levels of each oligosaccharide after binding to cellulose. The percentages indicate the increase or decrease of the bound subunits in relation to native ones.

This fine structural analysis of the xyloglucan that remains bound to cellulose revealed that there might be domains in these storage polysaccharides that are relatively more interactive with cellulose. These regions appear to be richer in XXXG and XLXG (about 30–50% higher than in native Xg samples) and with a decreasing proportion of XXLG and XLLG (Fig. 4C and D). These results suggest that the degree of galactosylation might be associated with Xg–cellulose binding. The presence of oligosaccharides *a*, *b* and *c* from *H. courbaril* Xg (Fig. 4A) are also associated with a higher interactivity with cellulose. They are subunits from the XXXXG based polymer (Buckeridge et al., 1997) with a Glc:Xyl:Gal ratio of the 5:4:1 with galactose in different positions (Tiné, Cortelazzo & Buckeridge, 2000).

Table 3
Comparative binding capacity among Xgs from different sources and their respective galactose distribution index expressed by the ratio (XLLG + XLXG)/(XLLG + XXLG). Within the column, means followed by different letters are statistically different according to the Tukey test ($P < 0.05$). The percentages of interactions are averages of the data from Table 1

Species	Galactose distribution index (XLLG + XLXG)/(XLLG + XXLG)	Adsorbed Xg (%)
H. courbaril	0.20	33.8a
T. indica	0.65	30.9b
T. majus	0.75	26.9c
C. langsdorffii	0.92	26.6c

Recently, Santos and Buckeridge (unpublished) found that Xgs from small seeds of *H. courbaril* have lower amounts of galactose than Xg from big seeds. Here, we performed a comparison of the cellulose binding capacity of these two Xg types. The results shown in Table 2 indicate that a higher degree of galactosylation of Xg is associated with lower interactivity. Apart from the degree of galactosylation, the distribution of galactosyl residues along the main chain (i.e. the fine structure) seems to play a role in the binding to cellulose as well. The subunit XXLG was found to be relatively less interactive with cellulose than XLXG for both Xg sources.

The distribution of galactose residues on either side of the backbone of Xg was estimated according to Buckeridge et al. (1992). The data presented in Table 3 indicate that the uneven distribution of galactose apparently favours a higher binding capacity. This probably occurs due to the presence of a higher proportion of galactosyl residues at one side of the polysaccharide molecule, which exposes the glucose residues from the other side for interaction with cellulose by hydrogen bonding. On the other hand, a tendency towards uniform distribution of galactoses would lead to a more twisted backbone structure, resulting in a lower binding capacity (Levy et al., 1991, 1997).

Dea and Clark (1986) reported that the distribution of galactosyl units along the main chain can have a significant effect on the interactive properties of galactomannans. They showed that galactomannans from different sources with significant differences in the content of galactose (40 and 29%) could show similar interaction.

Although the galactosyl distribution index is higher for *C. langsdorffii* (0.92) than for *T. majus* (0.75), their binding capacities were not statistically different. This suggests that at high levels of uniformity (possibly above 0.7) of the galactosyl distribution, no change in the binding capacity occurs. However if this high uniformity was lost, both the binding capacity and the capacity for self-association will be increased. Removal of approximately 50% of galactoses results in formation of a gel (Reid, Edwards & Dea, 1988; Suisha et al., 1998). This factor could be important for storage cell wall Xg because during seed maturation and storage mobilisation some hydrolytic enzymes (e.g. β-galactosidases) could alter the degree of galactosylation of the polysaccharides, resulting in alteration of rheological properties.

Our results showed that the galactosyl residues seem to influence the interaction between Xg and cellulose. However, it is not well understood which factor related to the fine structure could be really affecting the binding

capacity, the degree of galactosylation or the distribution of galactosyl residues.

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