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Glucuronoarabinoxylans as major cell walls polymers from young shoots of the woody bamboo *Phyllostachys aurea*

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ABSTRACT

Young shoots of *Phyllostachys aurea* showed glucuronoarabinoxylans (GAX) as the major hemicellulosic components, being extracted in major amounts with 1 M KOH (ratio Xyl:Ara:GlcA, 100:67:8), but also with water, showing a broad structural variability. Mixed linkage glucans were also present, but in minor amounts, mostly concentrated in the 4 M KOH extracts, while pectin polymers were very scarce. Arabinogalactan proteins were an important part of water extracts, determined by the presence of the typical arabinogalactan structures (3- and 6-linked Gal *p*; terminal and 5-linked Ara *f*), in addition to small amounts of hydroxyproline (2–3% of total protein) and positive reaction to Yariv's reagent. Morphological and anatomical characteristics of young shoots are described, as well as localization of some cell wall components, and related with chemical analysis. A method for determination of uronic acids as their N-propylaldonamide acetates and separation and quantification by GC/MS was adapted for its use with grass cell wall fractions.

1. Introduction

Woody bamboos are perennial evergreen plants belonging to the family Poaceae, with very high rates of growth, and comprising aerial and underground parts (culms, and rhizome and roots, respectively; Shanmughavel & Francis, 1997). Bamboo shoots are the new culms that arise from the rhizomes, containing nodes and internodes in a vertically miniaturized form (Choudhury, Sahu, & Sharma, 2012; Nirmala, Bisht, & Haorongbam, 2011). They emerge mostly in spring and grow quickly, usually up to 20–30 cm long, getting lignified in a few days (Park & Jhon, 2010). Woody bamboos comprise 120 genera that include 1641 species, nearly 200 of them are commonly utilized for their edible and palatable shoots (Lobovikov, Paudel, Piazza, Ren, & Wu, 2007; Soreng et al., 2015), which are consumed in raw, canned, boiled, fermented, frozen, liquid and medicinal forms (Choudhury et al., 2012), especially in Asia. With up to ~90% content of water, bamboo shoots are tender structures appreciated by their nutritional properties: high dietary fiber, low fat and rich mineral content (Chung, Cheng, Lin, & Chang, 2012), as well as phenolic compounds with antioxidant capacity and phytosterols with cholesterol-lowing activity. Besides consumption of the whole shoots, bamboo shoot fiber, mainly composed by hemicelluloses and cellulose, is a common ingredient in many food products in some countries (Nirmala et al., 2011). Nevertheless, no systematic study on its nutritional significance is available (Singhal, Bal, Satya, Sudhakar, & Naik, 2013).

As in the case of all plant resources, the composition and organization of the cell wall is an important aspect to address, since it is a crucial multifunctional structure that represents an important proportion of plant biomass. Some studies have been performed on the cell walls of different bamboo species, but information is still

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scarce, taking into account the potential of bamboo as a sustainable resource with multiple applications.

The genus *Phyllostachys* Siebold & Zucc. has its center of distribution in China, where its species have been the principal source of paper pulp, timber and handcraft materials, as well as edible bamboo shoots (McClure, 1957). One of the species, *P. aurea* is extensively cultivated not only in Asia, but also in Europe and America (Lessard & Chouinard, 1980; Rúgolo, 2016). This species shows an invasive growth based on the development of leptomorph rhizomes with a monopodial branching pattern and many aerial culms, consequently the removal of young elongating shoots constitutes an effective method of growth control.

Regarding cell walls from bamboo shoots, *Phyllostachys* is the genus that comprises the most studied species. The early studies carried out on specimens of this genus reported a xylan, an arabinogalactan and an α-glucan as major structural polysaccharides soluble in water (Maekawa & Kitao, 1973; Maekawa 1975a, 1975b). Later, it was found that shoots from chinese Moso bamboo, *P.edulis*, which constitutes the basis of major industrial development in this country, are composed of xyloglucans, arabinoxylans (AX) and mixed-linkage glucans. The structure of these polysaccharides was studied in detail (Edashige & Ishii, 1998; Ishii & Hiroi, 1990a, 1990b). Besides, a boron-rhamnogalacturonan-II complex was isolated from the same species (Kaneko, Ishii, & Matsunaga, 1997). More recently, water soluble polysaccharides from the shoots of *P. praecox* were isolated, and their prebiotic activity was evaluated (He et al., 2016). In addition, some aspects of the cell walls from the shoots of *P. bambusoides* and *P. pubescens* were analyzed as potential bioethanol producing sources (Shimokawa, Ishida, Yoshida, & Nojiri, 2009).

In the present work, sequential extraction was performed on the cell walls of young bamboo shoots from *Phyllostachys aurea*, and the structure of their major components was determined. Additionally, a method for determination of uronic acids was tested for its use with grass cell walls, which allowed to detect and quantify galacturonic, glucuronic, and 4-*O*-methylglucuronic acids by GC/MS. Besides, anatomical studies were performed on young shoots in relation to their chemical properties, and histochemical tests using Toluidine Blue *O* were used to differentially detect charged polysaccharides and lignin at cell and tissue levels.

2. Experimental

2.1. Chemicals

Safranin, fast green, toluidine Blue *O*, Na_2CO_3, DMSO, D_2O, amylase, *myo*-inositol and D-(+)- glucuronic acid γ-lactone were from Sigma-Aldrich (Saint Louis, USA). KOH, H_2SO_4, pyridine, acetic anhydride, phenol and NaOH were from Anedra (Buenos Aires, Argentina). HCl and gum arabic were from Biopack (Buenos Aires, Argentina). CDTA, Folin-Ciocalteu reagent, TFA, $NaBH_4$, N-propylamine and gallic acid were from Merck (Darmstadt, Germany). LiCl was from Mallinckrodt (New York, USA), acetone from Sintorgan (Buenos Aires, Argentina), and D-(+)-galacturonic acid from Eastman Kodak (Nueva York, USA). For detail, see Suppl. Table 1.

2.2. Plant material

2.2.1. Plant species

Studies were carried out on clumps of *Phyllostachys aurea* Carrière ex Rivière & C. Rivière (Poaceae, Bambusoideae, Bambuseae), cultivated at the Botanical Garden of the School of Agriculture (University of Buenos Aires). The species has been cultivated here since 1915. Flowering occurred once after ca. 15 years of vegetative growth and extended during two years without clump death (Parodi, 1936), after that, vegetative growth continued so far. A voucher specimen is deposited at Gaspar Xuarez Herbarium (BAA), according to the following specification: ARGENTINA. Buenos Aires: Capital Federal, Cult. Jardín Botánico de la Facultad de Agronomía, 1 Sep 1934, fl., *A. Burkart 6692* (BAA).

2.2.2. Sampling

A pool of young shoots, of similar size (25–30 cm long), were collected during spring of 2014 and 2015. Emerging shoots were clothed by culm leaves, which were removed before selection of culm material used in anatomical and chemical studies.

2.2.3. Anatomical and histochemical studies

Segments of ca. 1 cm^2 were taken from the middle portion of third and sixth internodes from young culms; internodes were numbered from the base to the top of the culms. The sixth internodes were situated at 6–10 cm from the base of the culm. Materials were fixed in ethanol 70% and embedded in paraffin according to traditional techniques (DíAmbrogio de Argüeso, 1986). Tranverse (TS) and longitudinal (LS) sections 10 μm thick were cut with a rotary microtome, dehydrated in an ethanol series, and double stained with safranin-fast green.

Cross sections prepared from fresh culms were used in staining techniques. Sections were treated with Toluidine Blue *O* (0.05% w/v in 0.1 M HCl) for 4 min, gently washed with distilled water, and observed to differentially stain carboxylated polysaccharides and polyaromatic substances, such as lignin and tannins.

Observations and photographs were made with a light microscope Zeiss Axioplan (Oberkochen, Germany).

2.3. Chemical analysis of cell wall polysaccharides

2.3.1. Extraction of cell wall polysaccharides

Sequential extraction of the polysaccharides was carried out on a pool of shoots following the standard procedures (Fry, 1988). After discarding the culm leaves, the fresh culms were cut in small pieces, dried in an oven at 50 °C and then milled. The milled material was extracted with 70% EtOH (100 g/L) for 4 h at room temperature, giving a residue (AIR: alcohol insoluble residue). AIR was treated with α-amylase (type VI-B from bovine pancreas; Sigma-Aldrich, USA); a solution of the sample (40 mg/mL) in phosphate buffer 0.1 M at pH 6.9 was kept 24 h at room temperature with constant agitation. During this time, three aliquots of enzyme were added to give each time a concentration of 4 μg/mL of fresh enzyme. The suspension was centrifuged giving a product, which was recovered from the supernatant and a residue. This procedure was repeated twice, giving extracts W-A and W-B. The residue obtained (AIR/amylase) was then sequentially extracted with 0.05 M CDTA at pH = 6, 0.05 M Na_2CO_3, and 1 M and 4 M KOH solutions in the same way (twice with each solvent), obtaining 8 extracts (CDTA-A, CDTA-B, Na_2CO_3-A, Na_2CO_3-B, KOH1M-A, KOH1M-B, KOH4M-A, and KOH4M-B) and a final residue (FR). All the extracts and residues were dialyzed (MWCO 6-8000) against tap water for 48 h, then against distilled water for further 24 h, and finally freeze dried.

2.3.2. General methods of analysis

Total carbohydrates content was analyzed by PhOH—H_2SO_4 method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956); for these determinations, samples were prepared considering them as insoluble material (Ahmed & Labavitch, 1977). Total phenolics (cell wall phenolic esters, conjugated phenolic acids and free phenolics) were determined following Bunzel, Ralph, Marita, and Stainhart (2000). After saponification, the phenolic content was evaluated in the supernatant using the Folin–Ciocalteu technique (Shui & Leong, 2006). Gallic acid (Merck, Germany) was used as standard and

Table 1
Yields and analysis of the extracts and residues obtained from the shoots of *P. aurea*.

Fraction	Yield[a] (%)	TC (%)[b]	Total protein (%)	Monosaccharide composition (mol%)[c]							
				Rha	Ara	Xyl	Gal	Glc	GalA	GlcA	4-*O*-Me GlcA
AIR	73.9	57.6	nd[d]	tr[e]	23.9	19.0	9.7	39.7	5.1	2.6	–
W-A	8.9	38.9	28.8	–	32.3	25.5	24.3	12.8	2.2	1.6	1.3
W-B	5.0	37.2	nd	3.0	30.2	27.8	21.7	9.9	2.5	3.1	1.9
CDTA-A	1.9	47.8	13.7	2.5	41.1	35.7	10.6	3.1	5.8	1.2	tr
CDTA-B	2.3	47.2	nd	1.7	33.6	40.2	10.8	8.4	3.5	1.8	tr
Na_2CO_3-A	1.2	40.1	48.6	1.9	32.6	16.2	22.4	17.2	5.1	2.2	2.4
Na_2CO_3-B	0.8	36.4	nd	2.4	26.8	18.4	22.5	14.2	9.8	3.7	2.3
KOH1M-A	12.1	48.6	19.4	–	27.6	41.3	4.8	20.5	2.5	1.3	1.9
KOH1M-B	5.4	59.1	nd	–	19.6	56.9	4.5	8.9	1.3	5.2	3.6
KOH4M-A	4.0	60.6	<2.5	tr	22.4	24.1	8.7	40.7	1.5	2.4	tr
KOH4M-B	2.7	59.2	nd	1.0	7.9	21.8	2.7	64.6	tr	1.3	–
FR	17.5	50.1	3.0	–	–	tr	tr	98.0	1.0	1.0	–

[a] For AIR, yield is expressed as percentage of dry matter. For the extracts and FR, values express yield as percentage of cell wall (AIR/α-amylase + W-A and W-B).
[b] TC: total carbohydrate content.
[c] Small amounts of mannose (1–3%) were detected in extracts obtained with water and CDTA.
[d] nd = not determined.
[e] Percentages lower than 1% are given as traces (tr).

results were expressed as mg of gallic acid equivalent (GAE) per 100 g of sample. The soluble protein content of the water-soluble extracts was determined colorimetrically (Lowry, Rosenbrough, & Farr, 1951), while for the other extracts, total nitrogen was estimated, after total degradation of the sample, by conversion to N_2. Then, the gas mixtures were separated by gas chromatography (GC) with a Porapak column in Carbo Erba EA 1108 chromatograph (Milan, Italy), using a thermic detector, and the total protein content was expressed using a factor of 6.25. Hydroxyproline content was determined according to Leach (1960), after hydrolysis of the samples with HCl 6 M (24 h, 105 °C). Dot-blot assay was performed with b-Glc Yariv phenylglycoside reagent to localize AGPs due to its specificity, and α-Man Yariv phenylglycoside reagent was used as a negative control, while gum arabic was used as standard. The monosaccharide composition was determined by hydrolysis and conversion of the monosaccharides to the corresponding alditol acetates, by dissolving the samples in TFA 13 M (37 °C, 1 h), followed by dilution of the acid to 11.5 M heating at 100 °C for 1 h, and further dilution to 2 M to achieve the regular hydrolysis conditions for insoluble polysaccharides (Morrison, 1988); the hydrolysate was derivatized to the corresponding alditol acetates. In some cases, the method comprising reductive hydrolysis and acetylation was also carried out (Stevenson & Furneaux, 1991).

2.3.3. Determination of uronic acids

First, uronic acids content was estimated by the method of Filisetti-Cozzi and Carpita (1991), the samples were prepared considering them as insoluble material (Ahmed & Labavitch, 1977). However, important interferences occurred, as the samples gave a brownish colour before addition of 3-phenylphenol. A blank was prepared for each of the samples and concentrations used, and deduced from the reading of the fully prepared sample. In spite of this, the values obtained were considered not reliable.

Then, galacturonic, glucuronic and 4-*O*-methylglucuronic acids were quantified according to Lehrfeld (1987) and Walters and Hedges (1988). Hydrolysis of the samples was carried out with 0.5 M TFA (135 °C, 2 h). After elimination of the acid, they were treated with 0.5 M Na_2CO_3, then 4% $NaBH_4$ was added to reduce aldoses to alditols and alduronates to aldolactones, which were treated with pyridine and N-propylamine (in ratio 1:1), 30 min at 55 °C and the mixture was evaporated to dryness. Finally, acetic anhydride and pyridine (1:1) were added and the samples were heated at 95 °C for 45 mi to give the corresponding alditol acetates and N-propylaldonamide acetates. It was necessary to use 200 μl of 0.5 M Na_2CO_3 instead of 75 μl, in the original protocol, to obtain full conversion of alduronates from alduronic acids. *Myo*-inositol was used as internal standard. The relative response of the uronic acids derivatives was determined, using D-**(+)**-glucuronic acid γ-lactone (Sigma) and D-(+)-galacturonic acid (Fluka) as standards. Then a factor of recovery was estimated (1.57 for GlcA and 4-*O*-Me-GlcA, and 2.29 for GalA). In these conditions, it was not possible to quantify neutral sugars and uronic acids at the same time. Consequently, the temperature program for GC of the N-propylaldonamide acetates arising from the uronic acids was modified in order to save time (see 2.2.5).

2.3.4. Methylation analysis

The polysaccharides (10 mg) were dissolved in DMSO and methylation was carried out using finely powdered NaOH as base (Ciucanu & Kerek, 1984). An 8.4% LiCl solution in DMSO was used in the case of insoluble extracts (Petruš, Gray, & BeMiller, 1995). The methylated samples were dialyzed (MWCO 3500), freeze dried and then derivatized to the alditol acetates as described for the polysaccharides. Two or three methylation sequences were carried out to achieve permethylation of the samples.

2.3.5. Analysis of the monosaccharide composition by GC–MS

GC of the derivatized samples, as well as those of the partially methylated alditol acetates were carried out on an Agilent 7890A gas-liquid chromatograph (Avondale PA, USA) equipped with a flame ionization detector and fitted with a fused silica column (0.25 mm id × 30 m) WCOT-coated with a 0.20 μm film of SP-2330 (Supelco, Bellefonte PA, USA). Chromatography was performed as follows: (a) from 200 °C to 240 °C at 2 °C min^{-1} followed by a 15-min hold for alditol acetates; (b) from 235 °C to 248 °C at 2 °C min^{-1} followed by a 30-min hold for uronic acids quantitation; (c) from 160 °C to 210 °C at 1 °C min^{-1} and then, from 210 °C to 230 °C at 2 °C min^{-1} followed by a 30 min hold, for partially methylated alditol acetates. N_2 was used as the carrier gas at a flow rate of 1 ml min^{-1} and the split ratio was 80:1. The injector and detector temperature was 300 °C.

GC–MS of the alditol acetates, N-propylaldonamide acetates, and partially methylated alditol acetates was performed on an Agilent 7890A gas-liquid chromatograph interfaced to a GCMSQP 5977A mass spectrometer. Chromatography was performed on the SP-2330 capillary column as described above, but in this case He was used as carrier gas at a flow rate of 1.3 ml/min. Mass spectra was recorded over a mass range of 30–500 amu.

2.3.6. Nuclear magnetic resonance spectroscopy

The sample (20 mg), previously exchanged with deuterium by repeated evaporations in D_2O, was dissolved in D_2O (0.5 ml) into 5-mm tubes. Spectra were recorded at room temperature on a Bruker Avance II 500 spectrometer (Karlsruhe, Germany). For ^1H NMR experiments the parameters were: a spectral width of 6.25 kHz, a 76° pulse angle, an acquisition time of 3 s, a relaxation delay of 3 s, for 32 scans. For 125 MHz proton decoupled ^{13}C NMR experiments the parameters were: a spectral width of 29.4 kHz, a 51.4° pulse angle, an acquisition time of 0.56 s, a relaxation delay of 0.6 s, for 25000 scans. Signals were referenced to internal acetone at 2.21 ppm for ^1H NMR and 31.1 ppm for ^{13}C NMR experiments, respectively. Pulse sequences for ^1H-^1H COSY and TOCSY, and ^1H-^{13}C HSQC spectra were supplied by the spectrometer manufacturer; spectra were recorded at room temperature and were obtained at a base frequency of 500 MHz for ^1H and 125 MHz for ^{13}C.

3. Results and discussion

3.1. Morphological and anatomical characterization. Localization of some cell wall components

Young shoots of *P. aurea* are clothed and protected by culm leaves with a conspicuous leaf-sheath and a scarcely developed leaf-blade (Fig. 1A). After removal of culm leaves, their culms show an incipient development of adventitious roots and contiguous basal nodes distant from each other in 1–2 cm long, while the upper ones are more spaced and separated by 2–3 cm long internodes (Fig. 1B). Culms are hollow and in complete transverse sections, the epidermis is formed by a layer of cells, the hypodermis by 8–9 layers, and peripheral, transitional and central vascular bundles are immersed in parenchymatic tissue (Fig. 1C and D). Collateral vascular bundles are surrounded by fibers of sclerenchyma tissue, polygonal in shape, with non-lignified primary cell walls (Fig. 1E). Peripheral, transitional and central vascular bundles are mainly composed of cells with primary walls, and only show secondary walls in the annular thickenings of protoxylem elements (Fig. 1C–E). Protoxylem lacuna is observed in transitional and central vascular bundles (Fig. 1E). Third and sixth internodia anatomical structure is similar, only differing in wall thickness (1–1.1 and 0.5–0.7 cm width, respectively) and number of vascular bundle cycles from the periphery to the inner zone (16–18 and 12–13 cycles, respectively).

Toluidine Blue *O* staining reveals an uniform purple color due to the presence of carboxylated polysaccharides, and a bright blue color in protoxylem elements which develop a lignified secondary cell wall (Fig. 1F). Parenchyma cells exhibit numerous amyloplasts (Fig. 1G).

3.2. Cell wall polysaccharides

The alcohol insoluble residue (AIR) is usually considered as equivalent to cell wall material. In the present study, it was found that after enzymatic treatment with α-amylase a major portion of AIR was lost, suggesting that it contained important amounts of reserve substances. Reserve α-glucans are present in amyloplasts found in parenchyma cells. However, as bamboo shoots are considered low-starch and high-fiber materials, also other components with low molecular weight would be solubilized and lost during the enzymatic treatment and further dialysis. On that basis, the residue obtained from AIR after this treatment (AIR/α-amylase, which represents 19.9% of the milled material) plus the product isolated from the extracts after dialysis (W-A and W-B) were considered as a more appropriate reference point to express yields (Table 1). Thereby, cell wall polysaccharides represent 23% of the shoots dry weight of *P. aurea*.

Sequential extraction was performed obtaining 10 extracts and a final residue (FR) that was mainly composed of glucose (98%), being cellulose the major carbohydrate component, as shown by results from methylation analysis (see below, Table 2).

As expected for Poaceae, the most important yields were observed for extracts rich in hemicelluloses obtained in strong alkaline conditions. In particular, KOH1M-A gave the highest yield, showing xylose, arabinose and glucose as major monosaccharides. On the other hand, yields and composition of the extracts obtained with water in the presence of α-amylase, CDTA and Na_2CO_3 confirm the low content of pectins that characterizes grasses. Moreover, it has been proposed that GAX could play, in grass cell wall matrices, some of the roles of pectins in Type I cell walls (Cornuault et al., 2015). These extracts gave a relatively low content of total carbohydrates (36–48% w/w), and they are a mixture of pectic polysaccharides and/or arabinogalactan proteins (AGP), and water soluble arabinoxylans (Table 1). Extracts obtained with water and solutions of CDTA also have small amounts of phenolic compounds (2.8–6.8 mg of GAE/100 g) from which ~20-25% was released by saponification of the samples in case of W-B, CDTA-A, and CDTA-B, but only 2% for W-A (Suppl. Table 2).

W-A is the most important of these extracts, since it represents ~9% of the whole cell wall material. Methylation analysis (Table 2) and NMR spectra (Table 3, Suppl. Fig. I) show the presence of (1 → 3)(1 → 6)-β-D-galactan structures, and α-L-arabinans with 5-linked α-L-arabinofuranose as major units, and also terminal α-L-arabinofuranose units. These arabinogalactan (AG) structures, along with the content of total protein (29% w/w, one third of which are soluble proteins), and the presence of small amounts of hydroxyproline (2.3% w/w of total protein content) suggest that these AG derive from arabinogalactan proteins (AGP). In fact, positive reaction with Yariv β-glucosyl reagent was observed in a dot blot assay. In the NMR spectra, two different types of terminal α-L-arabinofuranose units were found, with anomeric signals at 108.7/5.01 and 108.9/5.31, the first one corresponds to the arabinogalactan structure, while the second one derives from soluble arabinoxylans (molar ratio 1.2:1.0, calculated from the ^1H NMR spectrum) (Fig. 2). Besides, the presence of AXs in W-A is well established from methylation analysis (Table 2) and the small signals corresponding to 4-linked β-D-xylose units, partially substituted with terminal α-L-arabinofuranose units on C3, which were clear in the HSQC spectrum. In addition, the terminal Xyl *p* units detected by methylation analysis could derive from low molecular weight xylans, or could be part of other complex polysaccharides. Regards the degree of ramification, about 47% of the xylose units are mono or disubstituted. The signal of the methyl group of 4-*O*-methylglucuronic acid is clear in the spectrum (at δ 60.8/3.38), in agreement with the small amounts of this unit (Table 1).

W-A and W-B have very similar monosaccharide composition, however, methylation analysis of W-B shows some structural differences, as the presence of higher quantities of 4-linked galactose units and rhamnose. These results indicate that a small amount of pectins is present in this extract. On the other hand, AXs in W-B are more substituted, as deduced by the higher degree of substitution of the xylose units (Table 2) and higher quantities of (4-*O*-methyl)-glucuronic acid (Table 1).

Extracts CDTA-A and B and Na_2CO_3-A and B gave lower yields (together, they represent ~6% of the cell wall) and also contain a mixture of different polysaccharides. Methylation analyses show the presence of pectic polysaccharides and arabinoxylans in different proportion. NMR spectra of CDTA-A showed signals corresponding to low molecular weight glucuronoarabinoxylans, partially acetylated on C2 (signal at δ 21.3/2.10 was assigned to CH_3 of this group) (Mansfield, Kim, Lu, & Ralph, 2012) of some of

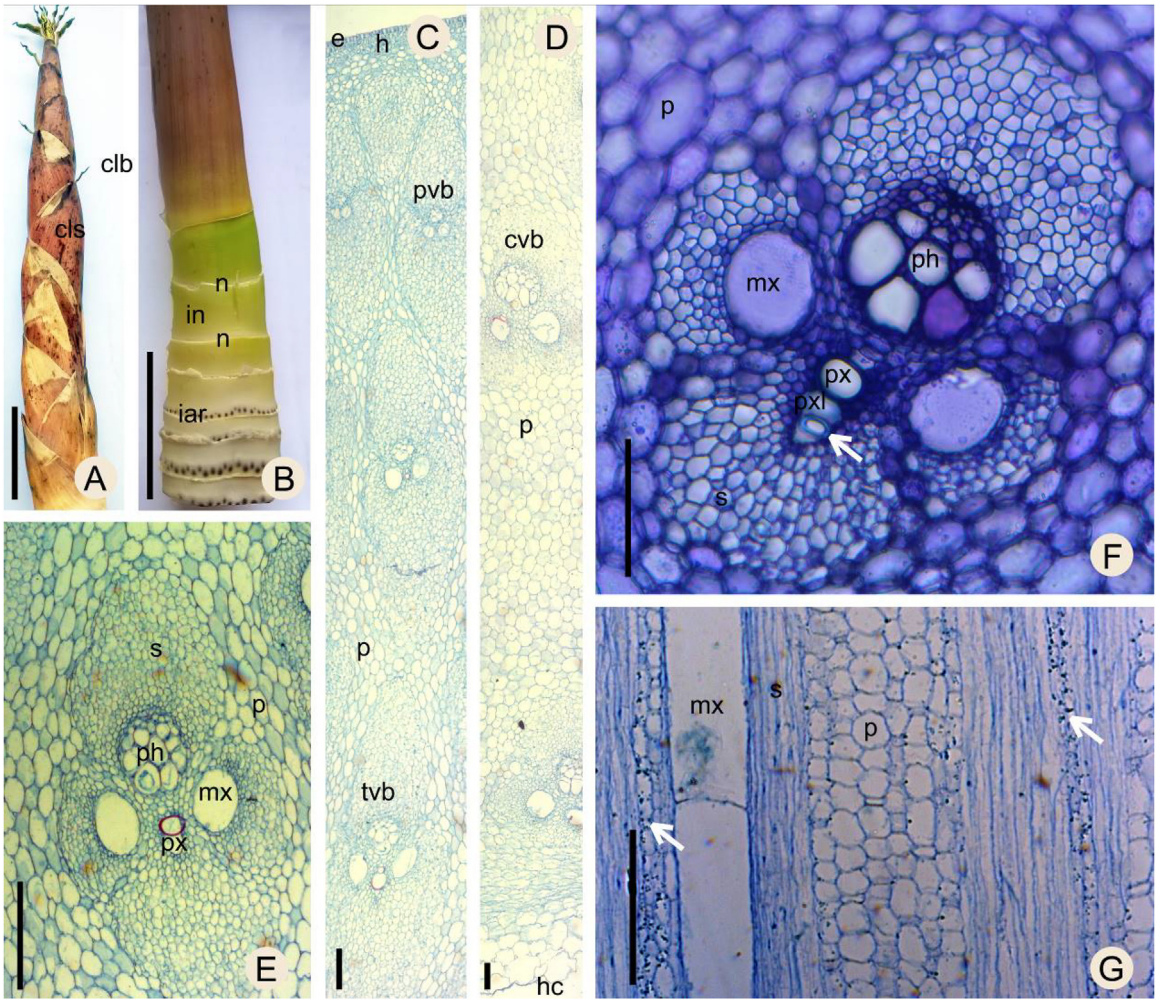

Fig. 1. Morphology and anatomy of young shoots from *P. aurea*. A. Young shoot, external view. B. Young shoot devoid of culm leaves. C-D. Culm in transverse section: C. Epidermis, hypodermis, with peripheral and transitional vascular bundles surrounded by parenchyma tissue. D. Central vascular bundles surrounded by parenchyma tissue and hollow medule. E. Transitional vascular bundle, detail. F. Transitional vascular bundle stained with Toluidine Blue *O*, showing a brigh blue color in the annular thickenings of protoxylem elements (arrow). G. Culm longitudinal section showing abundant amyloplasts in parenchyma cells (arrows). References: clb, culm leaf blade; cls, culm leaf sheath; cvb, central vascular bundle; e, epidermis; h, hypodermis; hc, hollow cavity; iar, incipient adventitious roots; in, internode; mx, metaxylem; n, node; p, parenchyma; ph, phloem; pvb, peripheral vascular bundle; px, protoxylem; pxl, protoxylem lacuna; s, sclerenchyma; tvb, transitional vascular bundle. Scale bars: A-B, 5 cm; C-D, F, 100 μm; E, G, 200 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the xylose units, as major structures (Table 3, Suppl. Fig. II). Also, signals corresponding to terminal and 5-linked α-L-arabinofuranose units are clear. On the other hand a signal at δ 109.1/5.25 was attributed to 3-linked α-L-arabinofuranose units (Shakhmatov, Toukach, Michailowa, & Makarova 2014). The anomeric signals at δ 99.0/5.27 and 98.7/5.22 were assigned tentatively to α-galacturonic acid from pectins and α-glucuronic acid from AX side chains, respectively, a signal at δ 174.8 was clear in the ^{13}C NMR spectrum, which corresponds to the carboxyl group. Only a small signal at δ 17.8/1.23 was detected and assigned to C6 of the rhamnose units, in agreement with the low content of this sugar. It was not possible to confirm the presence of the 4-linked galactan, suggested from data of methylation analysis (Table 2). Extract CDTA-B is very similar to CDTA-A, being the most important difference detected by methylation analysis, the absence of 4-linked galactans. On the other hand, the AX in this extract has higher molecular weight and higher degree of substitution with arabinose.

Extract Na_2CO_3-B is the richest in galacturonic acid; it also comprises small amounts of rhamnose (Table 1). In spite of that, structural units corresponding to these sugars are not clear in the corresponding spectra (Suppl. Fig. III). Although a signal at δ 99.6/5.37 was tentatively assigned to C1/H1 of galacturonic acid units, the diagnostic peaks corresponding to C4/H4 and C5/H5 were not detected (Petersen, Meier, Duus, & Clausen 2008). A wide variety of partially methylated galactose and arabinose derivatives appeared by methylation analysis of this fraction, in agreement with the presence of complex pectin molecules (Table 2). Accordingly, NMR spectra were also complex (Table 3, Suppl. Figure III), comprising also the signal at δ 109.1/5.25 which was detected in the spectra of CDTA-A, and attributed to 3-linked α- L-arabinofuranose units. In addition, low molecular weight xylans, less substituted with arabinose, were also detected in important amounts. In this case, the substitution of some of the 4-linked β-D-xylose units with (4-*O*-methyl)-α-D-glucuronic acid is clearly detected in the HSQC spectrum by the presence of C1/H1 and C2/H2 of the 2-substituted units and C1/H1, C4/H4, and C5/H5 of 4MeGlcA (Hromádková, Koštálová, Vrchotová, & Ebringerová 2014). The peak corresponding to the methoxyl group is also evident (Table 3).

The major alkaline extract (KOH 1M-A) is constituted mainly by glucuronoarabinoxylans, with a backbone of 4-linked β-D-xylopyranose with significant degree of ramification (43% of the xylose units are substituted, giving a ratio of 1:0.8 between 4-linked unsubstituted units and 3-, 2-substituted plus 2,3-disubstituted units) (Table 2). KOH 1M-A also shows terminal and 5-linked ara-

Table 2
Methylation analysis of the extracts and final residue obtained from the shoots of *P. aurea*.

Monosaccharide[a]	Unit	W		CDTA		Na_2CO_3		KOH1M		KOH4M		FR
		A	B	A	B	A	B	A	B	A	B	
2,3,4 Rha	Rha *p*(1→	–	–	–	–	1.0[b]	1.9	–	–	–	0.6	–
4 Rha	→2,3)Rha *p*(1→	–	–	–	–	1.1	0.9	–	–	–	–	–
Rha	→2,3,4)Rha *p*(1→	–	3.2	2.8	1.9	–	–	–	–	–	0.4	–
2,3,5 Ara	Ara *f*(1→	19.9	19.3	22.0	19.5	16.5	11.2	17.1	16.4	12.8	3.0	–
3,5 Ara	→2)Ara *f*(1→	1.9	2.2	3.2	2.4	5.1	4.2	–	–	2.8	1.1	–
2,5 Ara	→3)Ara *f*(1→	2.2	1.1	7.5	3.5	4.6	3.0	–	–	2.1	1.1	–
2,3 Ara	→5)Ara *f*(1→	10.0	5.2	10.1	8.5	7.6	9.3	10.8	4.3	3.7	2.0	–
2 Ara	→3,5)Ara *f*(1→	tr	tr	1.1	1.3	1.3	1.0	1.4	1.3	–	–	–
Ara	→2,3,5)Ara *f*(1→	–	4.8	–	–	1.0	3.0	–	–	2.1	1.0	–
2,3,4 Xyl	Xyl *p*(1→	5.3	4.3	2.9	3.4	2.5	3.6	1.6	2.1	1.6	1.4	–
2,3 Xyl	→4)Xyl *p*(1→	9.0	7.8	17.4	10.2	8.7	8.5	23.4	29.3	16.9	11.7	–
2 Xyl	→3,4)Xyl *p*(1→	9.7	13.8	16.4	24.3	5.5	5.0	14.6	25.6	2.4	2.8	–
3 Xyl	→2,4)Xyl *p*(1→	1.3	1.0	1.5	1.3	1.2	1.0	3.0	4.2	1.0	1.0	
Xyl	→2,3,4)Xyl *p*(1→	1.7	3.2	–	3.4	–	3.8	1.2	1.8	3.2	5.3	–
2,3,4,6 Gal	Gal *p*(1→	1.7	1.7	1.7	3.3	18.4	5.0	4.7	4.0	7.3	2.8	–
2,4,6 Gal	→3)Gal *p*(1→	3.4	2.7	1.0	1.9	–	3.8	–	–	–	–	–
2,3,6 Gal	→4)Gal *p*(1→	2.7	4.3	7.3	1.0	–	6.6	–	–	–	–	–
2,3,4 Gal	→6)Gal *p*(1→	2.2	–	1.0	tr	3.5	7.8	0.4	–	–	–	–
2,4 Gal	→3,6)Gal *p*(1→	11.0	8.5	1.0	3.2	2.1	2.0	–	–	1.8	–	–
Gal	→2,3,4,6)Gal *p*(1→	4.6	6.3	–	1.8	0.7	1.4	–	1.0	–	–	
2,3,4,6 Glc	Glc *p*(1→	6.8	2.9	1.0	7.1	3.0	5.1	2.8	1.0	4.1	5.1	–
2,4,6 Glc	→3)Glc *p*(1→	3.5	2.7	–	tr	–	2.7	–	–	22.1	17.6	–
2,3,6 Glc	→4)Glc *p*(1→	3.2	–	1.2	1	9.8	7.2	17.0	6.0	14.1	42.1	88.8
2,3 Glc	→4,6)Glc *p*(1→	–	2.1	–	–	2.1	–	–	–	–	–	7.2
Glc	→2,3,4,6)Glc *p*(1→	–	3.1	1.9	1	4.2	2.0	2.0	3.0	2.1	1.0	4.0

[a] Methylated at the positions indicated.
[b] Mol% of the individual extracts or residues, considering only neutral sugars.

binofuranose units. Terminal α-D-galactose, α-D-glucuronic and 4-*O*-methyl-α-D-glucuronic acids, which are very clear in the NMR spectra, (Fig. 2) were also present, constituting, along with arabinose, the ramifications of the xylan backbone. Besides, considering the ratio between total xylose and terminal xylose, these arabinoxylans have DP = 27 (Coelho, Rocha, Moreira, Domingues, & Coimbra, 2016). The degree of substitution of the GAX in KOH 1M-B, is still higher, with a DP = 29, and a ratio of 1:1.1 between unsubstituted units and mono- plus di-substituted units. In anomeric region of the spectra of KOH1M-B, two small but well defined signals at δ 103.5/4.47 and 103.9/4.40 were attributed to mixed linkage glucans (Cui, Wood Blackwell, & Nikiforuk, 2000). It is important to note that these major extracts have well defined and simple GAX structures.

Finally, the extracts obtained with 4 M KOH present less quantities of GAX (with DP ~16) and predominance of glucans. Interestingly, methylation analysis of 4 M KOH-A showed predominance of 3-linked- over 4-linked-glucose units. This result suggests that in this extract, although there is a considerable amount of mixed linkage glucans (MLG), also callose is present. This is in agreement with the fact that callose was detected by *in situ* observation of the phloem. The solubility behavior reported for this polysaccharide is in agreement with these results (Kohler, Schwindling, & Conrath, 2000). On the other hand, in KOH4M-B, MLG predominate, with a ratio between 4-linked and 3-linked glucose units of 2.4:1. MLG are typical polysaccharides of elongating cells in grasses, where they are thought to play a role in cell expansion. The amount of MLG is growth-stage dependent and apparently they are deposited during cell expansion to be later degraded and replaced with GAX once expansion stops (Scheller & Ulvskov, 2010).

3.3. Determination of uronic acids

In nature, uronic acids are much more common than other types of acidic sugars (Ruiz-Matute, Hernández-Hernández, Rodríguez-Sánchez, Sanz & Martínez-Castro, 2011), and this is especially true for cell wall polysaccharides. Moreover, GAX from vegetative parts of grasses usually contain important amounts of glucuronic and 4-*O*-methylglucuronic acids, unlike cereal endosperm arabinoxylan (Scheller & Ulvskov, 2010). Uronic acids content is usually determined colorimetrically using *m*-hydroxydiphenyl as reagent (Blumenkrantz & Asboe-Hansen, 1973; Filisetti-Cozzi & Carpita, 1991), but this methodology can result inappropriate because of the interference of different components, as neutral sugars or proteins (Murado, Vázquez, Montemayor, López Cabo, González, 2005). This was the case of the samples studied in this paper, so another methodology was adopted. Analysis of uronic acids is difficult, since their linkages resist the usual hydrolysis conditions, and, if obtained, the resulting monomers are not easy to derivatize (Walters & Hedges, 1988). Lehrfeld (1987) initially proposed a method based in the conversion of lactones into N-(1-alkyl)-aldonamides by treatment with a primary amine in pyridine, followed by acetylation. These heat-stable aldonamides give only one peak in GC analyses and preserve the symmetry of the molecule, which would be lost by other procedures (Ruiz-Matute et al., 2011). Later, Walters and Hedges (1988) adapted this method proposing a hydrolysis with TFA 0.5 M at 135 °C for natural samples containing acidic polysaccharides (kelp, marine sediment, plankton and wood). As an attempt to contribute to the analysis of these components, in this paper, the method was applied to grass cell walls. The results obtained are included in Table 1 (as mol% along with neutral sugars), and, in addition, a gas chromatogram of a sample (obtained from extract Na_2CO_3-A) showing peaks of the three uronic acids, and the mass spectrum of the N-propylaldonamide acetate of 4-*O*-methylglucuronic acid are presented (Fig. 3).

When standards of galacturonic and glucuronic acids were treated as described above, mass losses were observed. Then, a factor of recovery was determined for each sugar, and applied to the values obtained in the samples. For 4-*O*-methylglucuronic acid, as it was not available as standard, the same factor of recovery as for GlcA was applied. On the other hand, not reliable results were obtained for neutral sugar composition with this methodology. Comparison of data obtained in these conditions with results from

Table 3
Signal assignments (ppm) of the major structures detected in the NMR spectra of W-A, CDTA-A, and Na_2CO_3-B from *Phyllostachys aurea*.

Structural units[a]	Chemical shifts, ppm[b]					
	C-1/H-1	C-2/H-2	C-3/H-3	C-4/H-4	C-5/H-5,5′	C-6/H-6,6′
W-A						
AGPs						
5-linked α-l –Ara *f*	110.5/5.17	82.4/4.14	77.5/3.87	81.8/4.05	67.7/3.81,3.72	
t α-l –Ara *f* arabinogalactan	108.7/5.01	81.9/4.05	77.8/3.93	84.8/4.05	62.1/3.75,3.63	
6-linked β-D-Gal *p*	104.5/4.45	71.8/3.45	73.6/3.59	69.4/4.07	74.5/3.87	70.3/3.97,3.85
3,6-linked β-D-Gal *p*	104.4/4.45	70.9/3.58	81.2/3.67	69.4/4.07	74.5/3.87	70.3/3.97,3.85
CDTA-A						
Glucuronoarabinoxylan						
4-linked β-D-Xyl *p*	102.2/4.47(a)	73.6/3.20	74.6/3.45	77.3/3.70	63.8/4.04,3.28	
4-linked β-D-Xyl *p* substituted on C3 with tAra *f*	102.3/4.41(a)	74.5/3.34(b)	78.2/3.63	74.6/3.72	63.7/4.04,3.28	
T α-L-Ara *f*	108.6/5.32	81.7/4.09(c)	78.2/3.84	85.7/4.19	62.2/3.80,3.73	
4-linked β-D-Xyl *p* substituted on C2 with Ac[5]	102.0/4.64	74.2/4.70	72.6/3.75			
T α-D-GlcA*p*	98.7/5.22					
4-linked D-Xyl Red α	93.1/5.10					
4-linked D-Xyl Red β	97.5/4.50					
Tβ-D-Xyl *p*	103.6/4.38	74.2/3.30(b)	76.6/3.36	70.2/3.57	66.0/3.88,3.60	
Pectins						
T α-L-Ara *f*	108.4/5.01	81.7/4.09(c)	77.5/3.90	84.8/4.05	62.2/3.80,3.73	
5-linked α-L-Ara *f*	110.3/5.16	82.2/4.14	77.5/3.90	82.0/3.96	67.3/3.80,3.60	
4-linked α-D-GalA*p*	99.0/5.27(c)			81.9/4.36	74.9/4.99	
Na_2CO_3-B						
Glucuronoarabinoxylan						
4-linked β-D-Xyl *p*	102.6/4.40(a)	73.8/3.19	74.7/3.47	77.3/3.70	63.8/4.01,3.38	
4-linked β-D-Xyl *p* substituted on C3 with tAra *f*	102.6/4.41(a)	74.1/3.35(b)	78.2/3.63	74.6/3.72	63.7/4.01,3.38	
T α-L-Ara *f*	108.6/5.32	81.8/4.08(c)	78.7/3.84	85.7/4.20	62.2/3.80,3.73	
4-linked β-D-Xyl *p* substituted on C2 with 4MeGlcA	102.7/4.53	77.4/3.41				
T 4MeGlcA	98.6/5.28			83.4/3.15	73.2/4.23	174.8
TGlcA	98.5/5.23					174.8
4-linked D-Xyl Red α	93.1/5.10					
4-linked D-Xyl Red β	97.5/4.50				63.4/3.95,3.56	
Tβ-D-Xyl *p*	104.0/4.45	74.2/3.30(b)	76.7/3.34	70.2/3.54	66.0/3.88,3,60	
T α-D-Gal	100.6/4.97					
Pectins						
T α-L-Ara *f*	108.4/5.01	81.7/4.09(c)	77.5/3.90	84.8/4.05	62.2/3.80,3.73	
5-linked α-L-Ara *f*	110.3/5.16	82.2/4.14	77.5/3.90	82.0/3.96	67.3/3.80,3.60	
4-linked α-D-GalA*p*	99.6/5.37					
β-Glucans						
4-linked β-D-Glc *p*	103.5/4.49					62.1/3.71

[a] Assignment based on data from Bock and Pedersen (1983), Bock, Pedersen, & Pedersen (1984), Ebringerová, Hromádková, Alfodia, & Hribalová (1998), Marques, Gutierrez, del Río, & Evtuguin (2010), Mazumder & York (2010), and Verbruggen et al. (1998).

[b] With reference to internal acetone at d_{CH3} 31.2/2.15. Signal corresponding to methoxyl group of 4-*O*-methylglucuronic acid at 60.8/3.38 ppm was present in all the samples. Peaks at δ 116.8/6.81, 130.1/7.34, 131.8/7.10, 130.3/7.24, 128.7/7.30, and 57.0/3.99 detected only in the spectra of W-A, were tentatively attributed to feruloyl residues, possibly linked to C5 of some Ara units of the AX (Schendel, Becker, Tyl, & Bunzel 2015). C1/H1 of acetyl group present at 21.3/2.06 detected in the spectra of CDTA-A, while C6 of the rhamnose units at 17.7/1.23 was found in the spectra of CDTA-A and Na_2CO_3-B. For (a)–(c), assignments could be interchanged.

the standard procedure showed losses of arabinose and xylose, so it is not appropriate to use it for determining both neutral and acidic sugars at the same time, at least for this type of material. The chromatographic conditions were then adapted, resulting in running times 15 min shorter than those obtained using the original conditions. Consequently, in this work neutral sugars were determined and quantified in the usual conditions (Morrison 1988), and the method described was applied only for determination of the uronic acids composition, and estimation of their content, using *myo*-inositol as internal standard.

4. Conclusions

Glucuronoarabinoxylans are the major hemicellulosic component of this material. Although they appear in major quantities in extracts obtained with 1 M KOH, which gave the highest yields, they did not show a unique and homogeneous type, but a wide range of structures with differences in several aspects: molecular weight, composition, and patterns and degree of ramification. Hence, they were obtained dispersed in almost all the extracts of the classic sequential extraction used in this work, instead of being concentrated in one or a few alkaline extracts. These results fit with the common distinction made between water soluble arabinoxylans and water insoluble arabinoxylans (Comino, Collins, Lahnstein, Beahan, & Gidley, 2014), although actually, they would comprise a continuum from low molecular weight/water extractable, to high molecular weight/alkali extractable GAX. However, the reasons behind AX extractability are not completely clear, since it seems to be determined by a complex combination of molecular weight, degree and pattern of substitution and phenolic acid-mediated cross-links. This heterogeneity is also in agreement with the idea of Scheller & Ulvskov (2010), that definitions based on extractability are not useful for hemicelluloses. These authors proposed a structural definition of hemicelluloses as a group of cell wall polysaccharides, neither cellulose nor pectin, with β-(1 → 4)-linked backbones and the same equatorial configuration at C1 and C4 (thus, formed by xylose, glucose or mannose).

Analysis of uronic acids is complex due to difficulties related to their hydrolysis, isolation and derivatization. The results of this

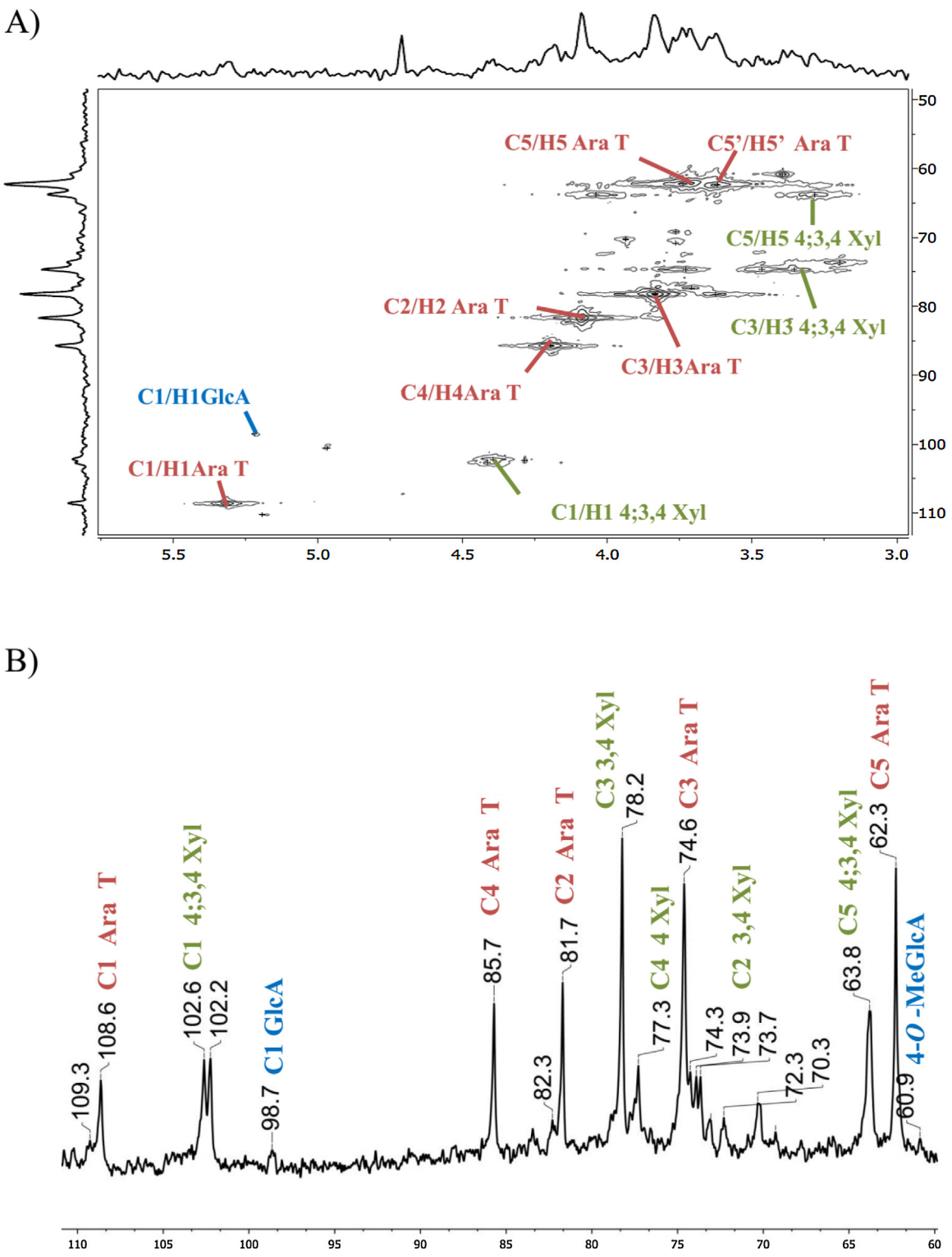

Fig. 2. A) HSQC NMR spectrum of KOH1M-A. B) ^{13}C NMR spectrum of KOH1M-A. References: Ara: arabinofuranose, Xyl: xylopyranose, GlcA: glucuronic acid, and 4-*O*-MeGlA:4-*O*-methyl-D-glucuronic acid. Cn or Cn/Hn refers to the position of the corresponding C or pair C/H in the sugar ring. T: terminal unit. Numbers before sugar indicate linkages of the structural unit (*i.e.*C1/H1 4;3,4 Xyl corresponds to the anomeric pair C/H of 4-linked and 3,4-linked xylopyranose units).

work represent a contribution to the characterization of these important components of the grass cell walls polymers.

In this work, anatomical and histochemical studies of culm material were included as a complementary aspect contributing to its understanding, which could help in further industrial applications of shoots of *P. aurea*.

The structures of the cell wall components isolated from shoots of *P. aurea* are those expected for grasses. However, important structural differences exist between species, organs, stages of growth and development, etc. Since these specificities could affect the properties of these materials and are with certainty essential

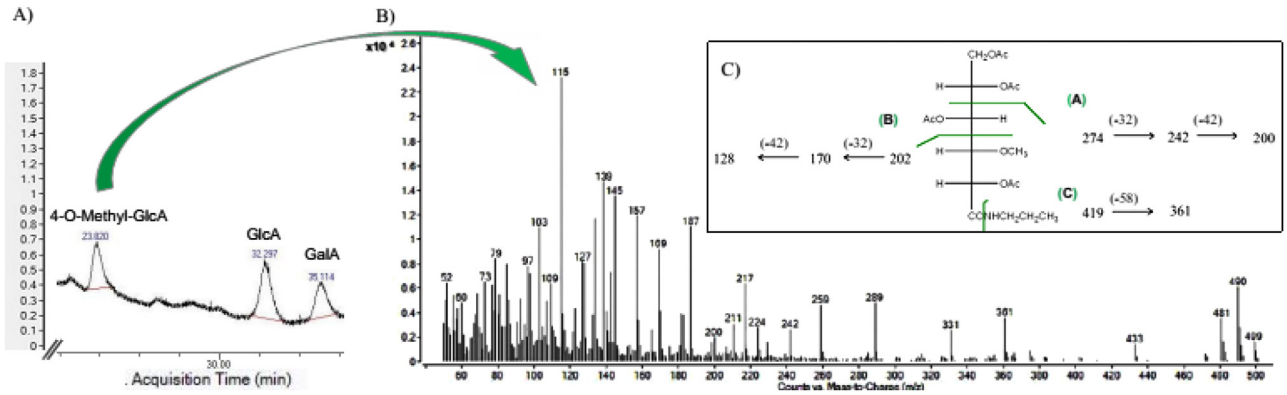

Fig. 3. A) Gas chromatogram of extract Na_2CO_3-A, showing peaks corresponding to uronic acids. B) Mass spectrum of 4-*O*-methylglucuronic acid. C) Fragmentation of 4-*O*-methyl-D-glucuronic acid during electron ionization MS. Primary fragmentations occur between two acetoxylated carbons (A: $m/z = 274$) and between a methoxylated carbon and an acetoxylated carbon (B: $m/z = 202$). Secondary fragmentation of (A) results in fragments with $m/z = 242$ and $m/z = 200$, after loss of methanol (CH_3OH; $m/z = 32$) and ketene (CH_2CO; $m/z = 42$), respectively. Secondary fragmentation of (B) results in fragments with $m/z = 170$ and $m/z = 128$, after loss of methanol (CH_3OH; $m/z = 32$) and ketene (CH_2CO; $m/z = 42$), respectively. (C) corresponds to a fragment with $m/z = 361$, resulting from the loss of $NHCH_2CH_2CH_3$ ($m/z = 58$). Additionally, the spectrum shows other fragments characteristic of peracetylated hexitols ($m/z = 97, 103, 115, 127, 139, 157, 217, 259, 289$).

for determining their possible applications, a detailed knowledge about them is necessary.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2017.03.015.

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