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Glucogalactan: A polysaccharide isolated from the cell-wall of *Verticillium Lecanii*

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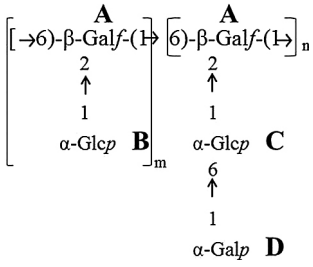
ABSTRACT

A water-soluble polysaccharide was extracted with alkali from the cell wall of *Verticillium lecanii* (also called *Lecanicillium lecanii*). After freezing and thawing, the water-soluble fraction was purified by gel filtration chromatography on Sepharose CL-6B and eluted as one peak by HPSEC/RID. Monosaccharide analysis showed galactose and glucose (1.1:1), with traces of mannose (<1%). The structural characteristics were determined by spectroscopic analysis, FT-IR and 1D and 2D ^{1}H and ^{13}C NMR, and methylation results. On the basis of the data obtained, the following structure of the polysaccharide (E_3S_{IV} fraction) was established:



where n ≈ 22 and m ≈ 22.

1. 1 Introduction

Verticillium lecanii is an entomopathogenic fungus, also known as *Lecanicillium lecanii* (Zare & Gams, 2001). It has been successfully used as a biological insecticide in horticulture and agriculture (Easwaramoorthy & Jayaraj, 1978; Ferron, 1978; Hall, 1981; Khalil, Bartos, & Landa, 1985) because it produces enzymes, such as proteases and chitinases that degrade the insect cuticle (Deshpande, 1999; Leger, Joshi, & Roberts, 1997). *V. lecanii* is also considered a mycoparasite of rust (Allen, 1982; Benhamou & Brodeur, 2001; Kuter, 1984; Sugimoto, Koike, Hiyama, & Nagao, 2003) and it was described for the first time in 1861, where the first isolates were obtained by scientists of the Glasshouse Crops Research Institute (now Warwick HRI, formerly Horticulture Research International) (Copping, 2009). Compounds produced by this fungus have been developed in some countries and applied in oilseeds, soy crops, ornamentals and vegetables (Copping, 2009). These compounds are marketed under the names Mycotal and Vertalec. While there is considerable literature about substances produced by *V. lecanii*, little or nothing is known about the biomass of this microorganism, especially in regard to polysaccharides that are responsible for about 80–90% of the dry matter present in the cell walls of fungi (Farkas, 1979).

Domenech et al. (2002) isolated an alkali-extractable and water-soluble polysaccharide from three strains of *Verticillium fungicola* var. *fungicola* cell wall. The polysaccharide had a main chain of mannopyranosyl α-(1 → 6) linkages substituted at O-4 with β-galatopyranosyl residues. Ahrazem et al. (2006) isolated from *Verticillium albo-atrum* and *Verticillium dahliae* a branched polysaccharide composed of galactose (54%), glucose (32%) and mannose (14%) with the main chain consisting of galactofuranosyl-β-(1 → 6) residues and glucose as branches. Both authors found that the alkali-extractable and water soluble fungal polysaccharides, which are minor components of the cell-wall (2–8%), differ in composition

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and structure among genera and, in certain cases, among groups of species of a genus. As a consequence of the diversity of polysaccharides, they can be used as chemotaxonomic markers at the genus or subgenus level. Also, these polysaccharides are antigenically relevant and they probably are involved in cell–cell and/or cell–host recognition mechanisms.

Since little or nothing is known about the glucidic constituents of *V. lecanii*, the goal of this study was the determination of the chemical structure of an alkali-extractable and water-soluble polysaccharide from the cell wall of *V. lecanii*. We expect that the polysaccharide can be useful as a chemotaxonomic marker and the microorganism a source of new polysaccharide structure.

2. Experimental

2.1. Microbial strain and culture conditions

The fungus *V. lecanii* was isolated from grass (*Brachiaria brizantha*) and we received it from URM Culture Collection, UFPE–Brazil (registration number 5286). It was maintained at 4 °C on potato-dextrose-agar. The conditions of fungal growth were according to Steluti et al. (2004), except that commercial sucrose (50 g/L) was used as the sole carbon source. The mycelium recovered by centrifugation was separated from the culture medium by exhaustive washing with distilled water until no sugars were detectable in the washings.

2.2. Analytical techniques

Total sugars were determined by the phenol-sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) with D-glucose as standard. Protein was measured by the Bradford method (Bradford, 1976) using bovine serum albumin as standard.

2.3. Extraction of cell wall polysaccharides

Mycelium from *V. lecanii* obtained after multiple water washings was freeze-dried, ground to a powder, and submitted to sequential extractions as follows (Scheme 1): 16.5 g of mycelial powder was extracted with ethanol on a Soxhlet system (1:20 w/v, 78 °C, 2 h). After centrifugation (8000 × *g*/15 min/4 °C), the resulting residue (R_1) was ground and submitted to aqueous extraction (1:20 w/v, 100 °C, reflux, 4 h). The last procedure was repeated three times and the supernatant solutions were combined (E_2) and concentrated, and the polysaccharides were precipitated by the addition of three volumes of EtOH. The residue from aqueous extraction (R_2) was treated with 2% (w/v) aqueous KOH (1:20 w/v, 100 °C, 2 h, 2×), containing traces of $NaBH_4$. The alkaline extracts were combined (E_3), neutralized with acetic acid (on ice bath), dialyzed against distilled water for 48 h, concentrated under reduced pressure and precipitated with three volumes of ethanol.

2.4. Purification of polysaccharide extract E_3

The alkaline extract (E_3) was frozen, and then subjected to repeated freeze-thaw cycles (six times) with thawing conducted at 20 ± 2 °C. This procedure resulted in two sub-fractions; one soluble, the other insoluble. The soluble sub-fractions were combined (E_3S), concentrated and the polysaccharides precipitated with EtOH (3:1). The insoluble material was combined and labeled E_3P. A 10-mg portion of E_3S was dissolved in water (2 mL) and fractionated by gel filtration chromatography on a Sepharose CL-6B column (2.5 × 45.0 cm) eluted with water (1.2 mL/min). Fractions (3.0 mL) were collected and analyzed for carbohydrate by the phenol-sulfuric acid method. Fractions corresponding to peaks were pooled and freeze-dried. Gel filtration chromatography was repeated until sufficient material was obtained to perform the structural characterization.

2.5. Monosaccharide composition

Each polysaccharide fraction and sub-fraction (0.05 mg total sugar) was hydrolysed with 4 M TFA (0.3 mL) in a sealed tube for 2 h at 100 °C (found to be optimal after testing several conditions for recovery and reproducibility). After hydrolysis, the solutions were dried under vacuum, and the residue dissolved in 0.5 mL water and dried again. The dissolution–evaporation cycle was repeated twice. Finally, the residue was dissolved in 0.2 mL water, and 0.025 mL aliquots analyzed by high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC/PAD) on a Dionex Chromatograph DX 500. Neutral monosaccharides were separated isocratically (0.014 M NaOH) using a CarboPac PA-10 (Dionex Chromatography) column (4 × 250 mm) equipped with a PA-10 guard column at a flow rate of 1.0 mL/min. Elution was performed using water (eluent 1) and 0.2 M NaOH (eluent 2). The column was regenerated after 20 min using 100% eluent 2 for 15 min, followed by a return to 0.014 M NaOH. Monosaccharide quantification was carried out from peak area measurements using response factors obtained with standard monosaccharides.

2.6. Determination of polysaccharide homogeneity

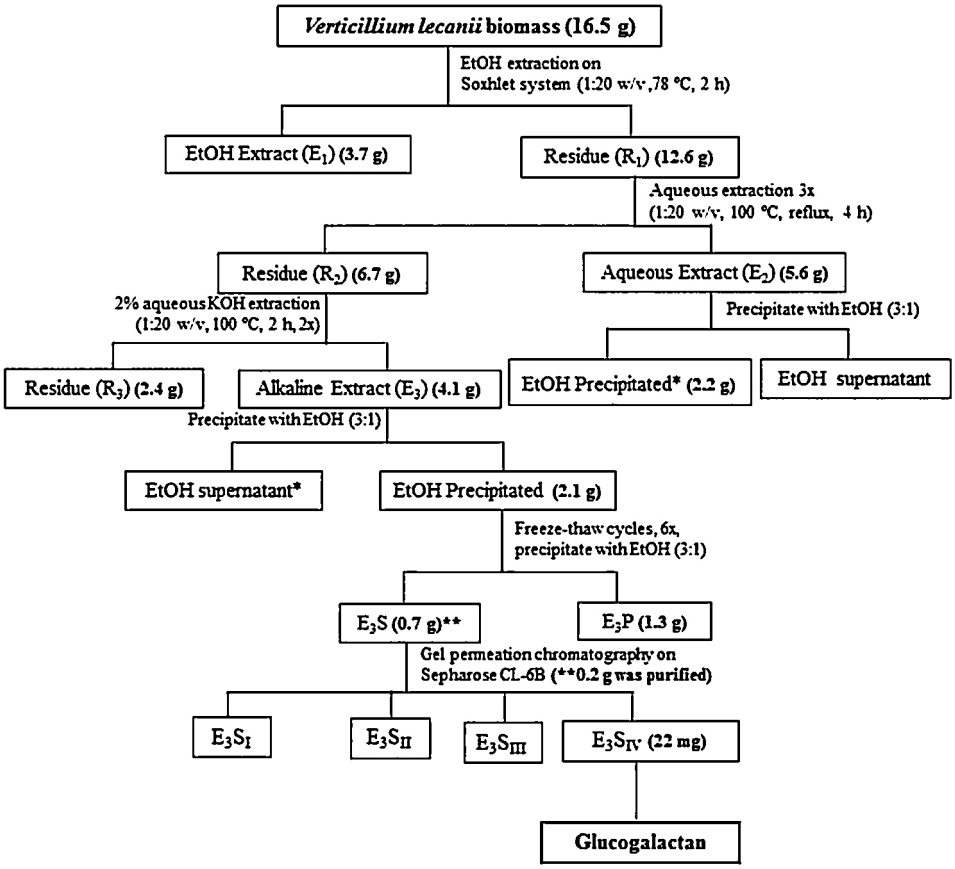
The homogeneity of water-soluble polysaccharides was determined by high performance steric exclusion chromatography (HPSEC) coupled to a refractive index (RI) detector model RID 10A. The chromatography system consisted of an HPLC pump (Model Shimadzu-10AD), a manual injection valve (Shimadzu) fitted with a 200-μL loop and an Ultrahydrogel column (7.8 × 300 mm) system (Waters) with exclusion limit of 7×10^6, 4×10^5, 8×10^4 and 5×10^3 Da arranged in series. The mobile phase was 0.1 M $NaNO_3$ with sodium azide (0.03%), and a flow rate 0.6 mL/min. Data analysis was performed using LC solution software (Shimadzu Corporation). To determine the average molecular weight of E_3S_{IV} the standard curve of dextran with molecular weights of 670, 410, 266, 150, 72.2, 60.0, 40.2, 22.8, and 9.4 kDa was made.

2.7. FT-IR and ^1H and ^{13}C NMR spectroscopy

FT-IR spectroscopy was performed using a Bruker Vector 22 spectrometer on 1 mg freeze-dried samples (E_2S and E_3S_{IV}) in 250-mg KBr discs, and scans conducted within 1800-600 cm^{-1} at a resolution of 1 cm^{-1}. 1D and 2D NMR spectra were obtained on a Bruker Avance DRX400 spectrometer equipped with 5-mm wide bore probe, operating at 400.13 and 100.63 MHZ for ^1H and ^{13}C, respectively. Samples were deuterium exchanged by successive lyophilization steps in D_2O and examined at 50 °C. The experiments were carried out using the pulse programs supplied with the Bruker manual. Proton chemical shifts refer to residual HDO at δ = 4.61 ppm (50 °C) and carbon chemical shifts to internal acetone at δ = 31.07 ppm (Ahrazem et al., 1997). The data were analyzed using the Bruker TopSpin 2.1 software.

2.8. Methylation analysis

The E_3S_{IV} polysaccharide from *V. lecanii* (5 mg) was methylated (3 times) using the procedure described by Haworth (1915) and outlined by Ciucanu and Kerek (1984). The methylated products were isolated by partitioning in a $CHCl_3$ and H_2O mixture, and the organic phase containing the methylated sugars was washed ten times with 4 mL water and dried. The methylated products were hydrolyzed using 45% formic acid (1 mL) at 100 °C for 15 h, then reduced with sodium borohydride and acetylated with acetic



Scheme 1. Diagrammatic scheme outlining the protocol for the extraction and purification of the mycelial polysaccharides from *Verticillium lecanii*. * Not purified.

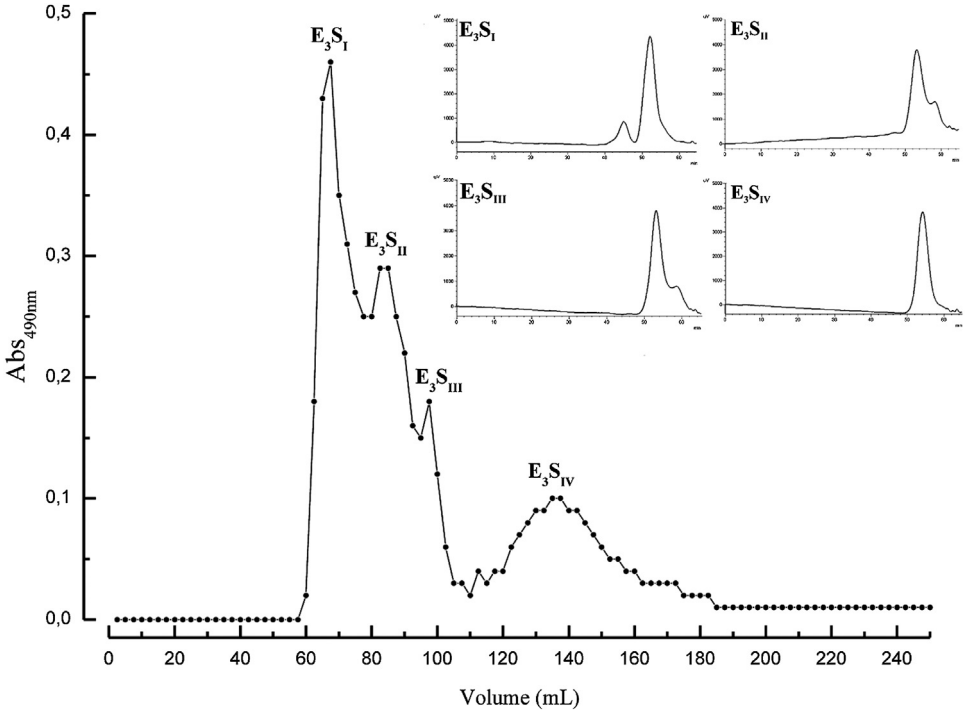


Fig. 1. Gel permeation chromatography of fraction E_3S on Sepharose CL-6B and HPSEC/RID (inset) of E_3S_I (a), E_3S_{II} (b), E_3S_{III} (c) and E_3S_{IV} (d).

anhydride-pyridine (1:1) as described by Corradi da Silva et al. (2005). The products were analyzed by gas chromatography-mass spectrometry (GC-MS) using a Varian 3800 gas chromatograph connected to a Varian Saturn 2000R ion-trap spectrometer, with He as the carrier gas at a flow rate of 1 mL/min. A DB-225 capillary column (30 m × 0.25 i.d.) was used applying a linear temperature gradient of 50 to 210 °C at 40 °C/min.

3. Results and discussion

Mycelium from *V. lecanii* was produced after cultivation under previously described conditions (Section 2.1). The mycelium was separated by centrifugation and washed with water until the washings were negative for sugars as judged by the phenol-sulfuric acid method, indicating that extracellular polysaccharide had been completely removed from the mycelium.

The alcohol extraction was used to remove fat compounds. Hot water (4×) and 2% aqueous KOH (2×) extractions were conducted to obtain water-soluble polysaccharides. The soluble fractions obtained from hot-water extraction (E_2) were combined and concentrated, and the polysaccharides precipitated in ethanol. That material contained carbohydrate (95%) and protein (5%). Monosaccharide analysis following acid hydrolysis showed glucose, galactose, mannose and fucose in the molar ratio 20:10:2:1. The chromatographic profile of E_2 on gel permeation chromatography with refractive index detection (HPSEC/RID) showed two peaks at 44.2 min and 51.0 min, suggesting the presence of at least two distinct polysaccharides (not shown). On the basis of these results, separation by gel permeation chromatography on Sepharose CL 6B, 4B and 2B was tried but was not successful under the conditions studied. Therefore, from this point, the E_2 fraction was not investigated in this work.

Since the storage of the alkaline extract (E_3) at low temperature precipitated an insoluble material, the first procedure for further purification of the fraction was submitting it to repeated freeze-thaw cycles until no further precipitate appeared (Scheme 1). After centrifugation, the soluble fraction was precipitated with ethanol (E_3S), resulting in a fraction containing carbohydrate (73%) and protein (27%). The high percentage of protein compared to the aqueous extract was probably due to the extraction method, since hot potassium hydroxide solution destroys the cell wall, saponifies the fat components of the cell membrane and exposes the cellular contents, rich in proteins and glycoproteins. After acid hydrolysis the monosaccharide composition of E_3S was glucose, galactose and mannose with a respective molar ratio of 19:10:1.

The homogeneity of E_3S was verified by HPSEC/RID. The chromatographic profile showed two peaks at 46.0 min (minor) and 52.7 min (larger) indicating the necessity for further purification (not shown). The E_3S fraction was then purified by gel permeation chromatography on Sepharose CL-6B. The chromatographic profile (Fig. 1) of E_3S showed four distinct fractions, designated in descending order of their apparent molecular weights E_3S_I, E_3S_{II}, E_3S_{III} and E_3S_{IV}, which eluted after blue dextran. The fraction E_3S_{IV} gave a single symmetrical peak on HPSEC/RID (Fig. 1 inset) with an average molecular weight of about 18.0 kDa. The carbohydrate content of E_3S_{IV} was 99.0% based on the phenol–H_2SO_4 method and 1% of protein according to the Bradford method. The other fractions behaved as heterogeneous materials. Acid hydrolysis of E_3S_{IV} showed glucose (47%) and galactose (53%) with traces of mannose (<1%).

The FT-IR spectrum (not shown) of E_3S_{IV} showed bands at 855 cm^{-1} and 890 cm^{-1}, characteristic of α and β glycosidic linkages. Other signals at 1158 and 1048 cm^{-1} indicate glucopyranosyl residues (Schmid et al., 2001). 1D and 2D NMR analyses were performed to characterize the polysaccharide structure of E_3S_{IV}. The ^1H NMR spectrum showed three anomeric signals at 5.19, 5.09 and 5.03 ppm in the proportion 2:2:1 (Fig. 2a). Four anomeric protons in minor intensity were also observed at 5.37, 5.22, 5.16 and 4.98 ppm (0.4:0.1:0.2:0.1). The anomeric proton (5.19 ppm, A unit) showed a correlation with the signal at 107.2 ppm in the HSQC spectrum (Fig. 3a), suggesting the presence of β-D-Gal*f* units. Assignment of the non-anomeric protons of A units was based on COSY and TOCSY spectra (Fig. 4a and b) and the corresponding ^{13}C chemical shifts were assigned in accordance with HSQC data (Table 1). The ^1H and ^{13}C chemical shifts of A units were very similar to those reported in the literature for 2-*O*- and 6-*O*-substitued β-D-Gal*f* units (Ahrazem et al., 1997; Guo, Mao, Li, Tian, & Xu, 2013). The substitutions of A units were confirmed on the basis of HMBC correlations (Fig. 3b). The spectrum showed cross peaks at 107.2/3.67 and 70.2/5.19 ppm, which were assigned to $C_{A1}/H_{A6'}$ and C_{A6}/H_{A1}, respectively. In addition, we also observed the correlation between C-2 (87.6 ppm) of A units and the anomeric proton at 5.09 ppm. Together, these data confirmed the β-(1 → 6) linkages and C-2 substitution at the furanosidic units (A). According to the literature, many alkali-extractable and water-soluble

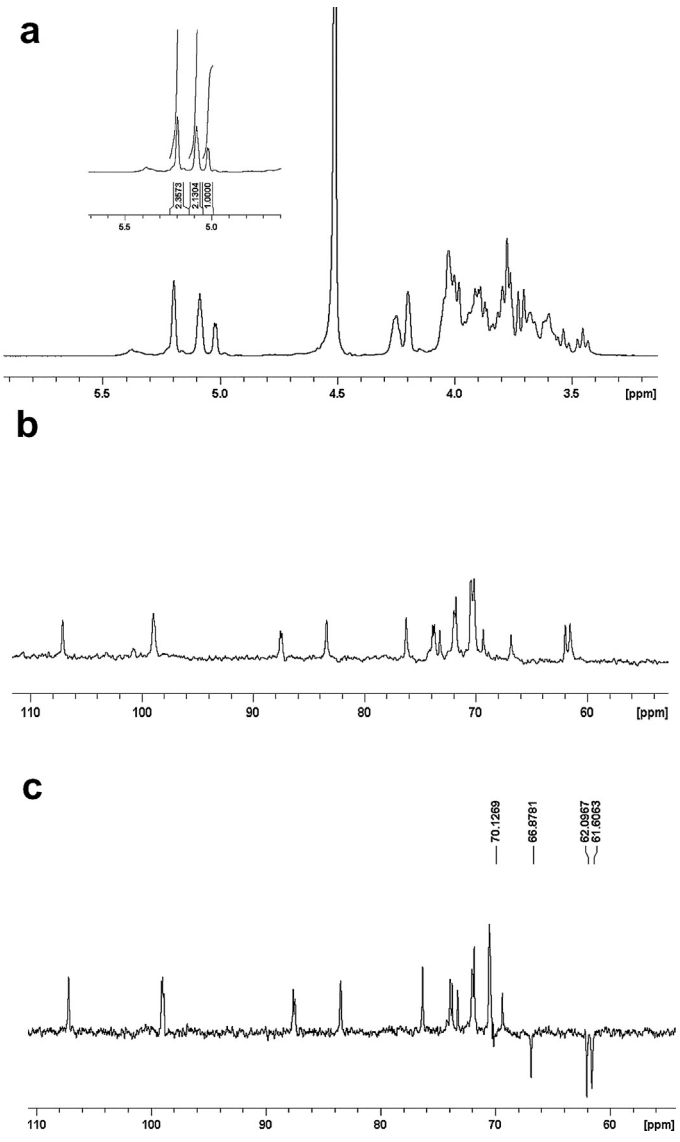


Fig. 2. Spectra of the E_3S_{IV} polysaccharide isolated from *Verticillium lecanii*. (a) ^1H NMR spectrum (400.13 MHz, D_2O, 50 °C), (b) ^{13}C NMR spectrum (100.63 MHz, D_2O, 50 °C) and (c) ^{13}C NMR spectrum (DEPT-135). On left side of the figure the ^1H NMR spectrum (a) is presented on a different scale to show the integration of the anomeric proton.

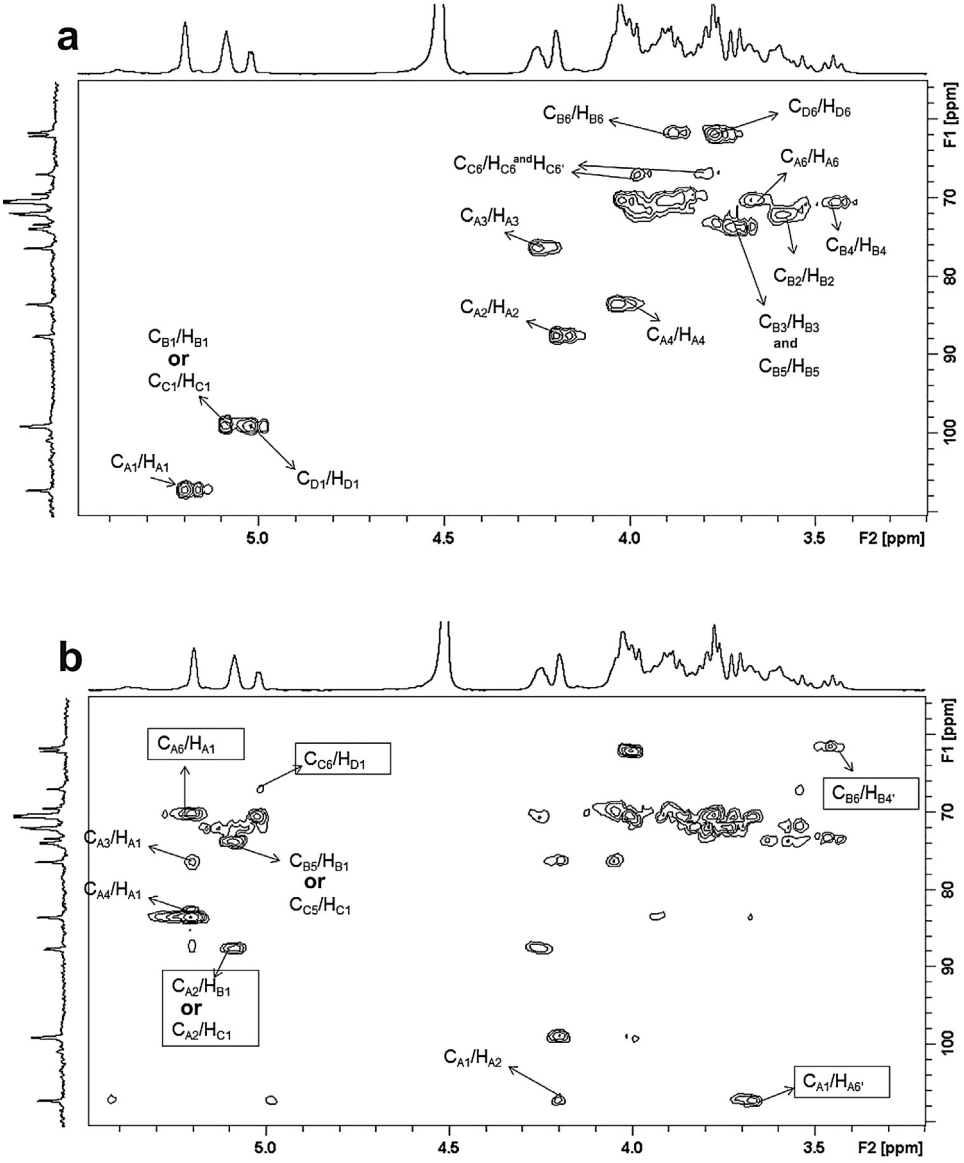


Fig. 3. 2D-NMR spectra for selected regions of the E_3S_{IV} polysaccharide from *Verticillium lecanii*: (a) HSQC and (b) HMBC. Significant crosspeaks have been labeled.

polysaccharides from fungal cell walls are made up of β-D-galactofuranose chains of different lengths and linkage types. The C-2 substitution of the β-D-galactofuranose residues by glucuronic acid or other sugars produces different polysaccharides that could be used as chemotaxonomic markers (Ahrazem et al., 1997; Ahrazem, Prieto, Gómez-Miranda, Bernabé, & Leal, 2001; Bernabé, Ahrazem, Prieto & Leal, 2002).

In the ^{13}C NMR spectrum (Fig. 2b), the signals corresponding to C-2 (87.6 ppm) and C-3 (76.3 ppm) of galactofuranosyl units (A) were split into two suggesting that the chemical shifts were affected by the different nature of substituents. These data together with integration results from ^{1}H NMR spectrum at 5.09 ppm indicated that two distinct units were linked to A residues. These units (B and C) were attributed to glucopyranose residues and the small

Table 1
^{1}H- and ^{13}C- NMR chemical shifts (δ)* for the alkali-extractable water–soluble polysaccharide E_3S_{IV} isolated from *Verticillium lecanii*.

Units	$^{13}C/^{1}H$[a]					
	1	2	4	5		6
→6)-β-D-Gal*f*-(1→	107.2	**87.6**[b]	76.3	83.4	70.4	**70.2**[b]
(A)	5.19	4.20	4.25	4.03	4.00	3.91/3.67
α-D-Glc*p*-(1→	100.0	71.8	73.9	70.4	73.3	61.6
(B)	5.09	3.59	3.75	3.44	3.76	3.88
→6)-α-D-Glc*p*-(1→	100.0	nd[c]	nd[c]	nd[c]	nd[c]	**66.8**[b] 3.98/3.80
(C)	5.09					
α-D-Gal*p*-(1→	100.0	69.3	nd[c]	nd[c]	nd[c]	62.0
(D)	5.03	3.83	3.91	4.02		3.77

[a] Chemical shifts from 2D NMR experiments.
[b] Underlined bold numbers represent glycosylation sites.
[c] nd = not determinated.

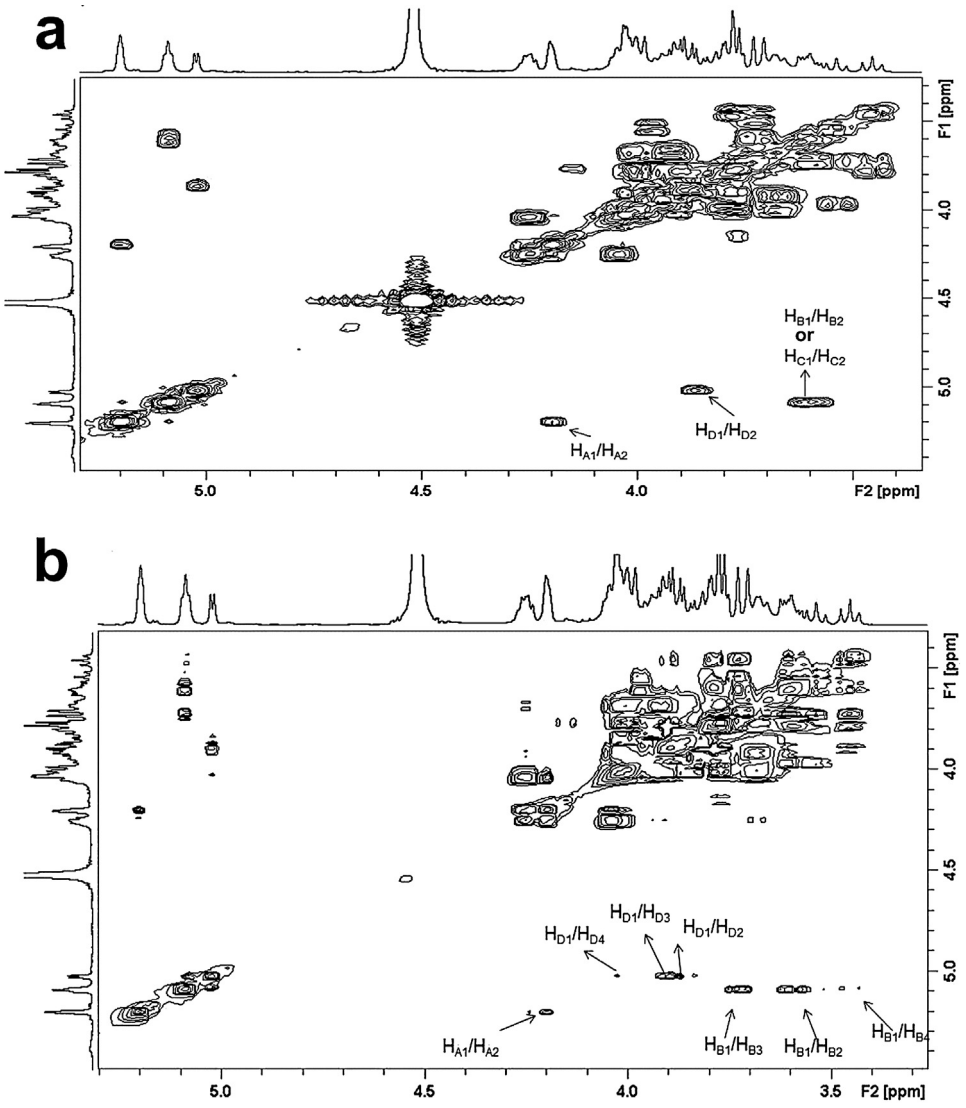


Fig. 4. Selected regions of 2D NMR spectra of the E_3S_{IV} polysaccharide from *Verticillium lecanii*, showing relationships among: (a) COSY and (b) TOCSY. Significant crosspeaks have been labeled.

coupling constant ($J_{1,2}$ < 4.0 Hz) suggested α-glycosidic linkages. The ^{13}C NMR (DEPT-135) spectrum (Fig. 2c) showed four inverted signals. Two of them at 61.6 and 62.0 ppm were assigned to non-substituted C-6 of the units designated B and D. The chemical shifts at 70.2 and 66.8 ppm were attributed to substituted C-6 of A and C units, respectively. ^{1}H and ^{13}C assignments of B units were according to COSY, TOCSY and HSQC spectra (Table 1), which were the same as those reported for non-reducing α-Glc*p* substituent at β-D-Gal*f* units (Ahrazem et al., 1997). In the HMBC data (Fig. 3b), the connection at 66.8/5.03 ppm was assigned to C_{C6}/H_{D1}, indicating that residue D is linked at carbon 6 of the C unit.

If D residues were glucose, one could expect the ^{1}H and ^{13}C chemical shifts to be similar to B units. In the COSY experiment (Fig. 4a), the anomeric signal of the D residue (5.03 ppm) was connected to H-2 at 3.83 ppm, indicating a significant difference from H-2 of α-Glc*p* (3.59 ppm) present in the B unit. In addition, there is a chemical shift at 69.3 ppm in the ^{13}C-NMR spectrum (Fig. 2b) that cannot be attributed to the α-Glc*p* units (B and C). However, this signal can be input to C-2 from α-D-galactopyranosyl units, since galactose is present in the E_3S_{IV} fraction and the chemical shifts are in accordance with the literature data (McIntyre & Vogel, 1989, Guo et al., 2012). The coupling constant observed for D units ($J_{1,2}$ = 3.5 Hz) indicated the α-configuration in the D-Gal*p*.

Methylation analysis of E_3S_{IV}, including GC-MS of the partially methylated alditol acetates, revealed derivatives of 2,3,4,6-tetra-*O*-Me-Glc*p*, 2,3,4,6-tetra-*O*-Me-Gal*p*, 2,3,4-tri-*O*-Me-Glc*p* and 3,5-di-*O*-Me-Gal*f* (Table 2). The non-stoichiometric ratio of methylated derivatives was probably due to steric hindrance of di-and tri-*O*-substituted units.The results from NMR data and methylation analysis suggest the structure below for the cell-wall polysaccharide from *V. lecanii.* The structure represents the proportion of side chains in the galactan backbone and not the real sequence in the polymer, since the distribution of branching points may be irregular. The NMR data in association with M.wt. estimation of fraction E_3S_{IV} permitted to calculate the sub-indices, which are around n ≈ 22 and m ≈ 22.

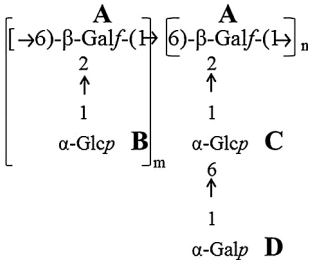


Table 2
Partially *O*-methylalditol acetates formed on methylation analysis of E_3S_{IV} polysaccharide from *Verticillium lecanii*.

Partially *O*-methylated alditol acetate	Linkage type	R_T (min)	Fraction (mol%)	Mass fragmentation (*m*/*z*)
2,3,4,6-Me_4-Glc	Glc*p*-(1→	7.98	40	87,101,117,129,145, 161,205
2,3,4,6-Me_4-Gal	Gal*p*-(1→	8.38	29	87,101,117,129,145, 161,205
2,3,4-Me_3-Glc	6→)-Glc*p*-(1→	10.78	21	87,101,117,129,161, 173,189,233
3,5-Me_2-Gal	2,6→)-Gal*f*-(1→	16.17	10	87,101,117,129,139, 159,173,189,233

The alkali-extractable and water–soluble polysaccharides from several fungi have a small α-(1 → 6)-mannan core and long chains of β-(1 → 6) galactofuranose, almost fully branched at O-2 by different side chains. Usually, one type of branch is neutral, containing a single glucopyranose residue, and the other is acidic, with single residues of glucuronic acid (Ahrazem et al., 1997), di- or tri-saccharides of glucuronic acid and β-mannopyranose (Ahrazem et al., 2001) or disaccharides of glucuronic acid and β-glucopyranose (Ahrazem, Gómez-Miranda, Prieto, Bernabé, & Leal, 2000). There are still terminal β-galactopyranose residues incorporated into half of the neutral chains and in all the acidic chains of the similar polysaccharide from *Plectosphaerella cucumerina* (Ahrazem et al., 2006). The polysaccharides from *V. dahliae* and *V. albo-atrum* lack the terminal galactopyranosyl residue in the acidic chain. According to the presented results the polysaccharide from *V. lecanii* does not have acidic residue but it has disaccharide branch composed by glucosyl and galactopyranosyl residues.

Fungal cell wall polysaccharides are implicated in the regulation of the primary contact events between parasites and host (Leal, Jiménez-Barbero, Bernabé & Prieto, 2008; San-Blas & San-Blas, 1982). Therefore, knowing the chemical structure of cell wall polysaccharides can be useful for understanding the mechanism of infectious process. In addition, the fungal cell wall can be a valuable source of new molecules.

Acknowledgements

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