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An α-glucan elicitor from the cell wall of a biocontrol binucleate *Rhizoctonia* isolate

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Abstract—Binucleate *Rhizoctonia* (BNR) isolate (232-C6) is an effective biocontrol agent for protection of potato from *Rhizoctonia* canker, a disease caused by *Rhizoctonia solani*. Production of hydrolytic enzymes is one of the best known inducible defense responses following microbial infection. We isolated and characterized a cell wall α-glucan from BNR, which induces β-1,3 glucanase activities in potato sprouts, the primary site of infection by *R. solani*. An autoclaving method, previously reported for isolation of oligosaccharide elicitors was used, and the glucan purified by chromatographic techniques. Maximal induction of β-1,3 glucanase activity in potato sprouts was obtained with 250 μg of the α-glucan elicitor after 6 days from inoculation time. Both, BNR mycelium and the α-glucan produced a similar kinetic response of β-1,3 glucanase. However, the α-glucan did not induce phytoalexin accumulation, previously correlated with the defense response. Uronic acids (~10% with respect to total neutral sugars) were determined and identified as glucuronic acid by high-pH anion-exchange chromatography. Methylation analysis showed that the glucan consists of (1→3) and (1→4)-linked glucose units with preponderance of the first ones. Some of the (1→4) linkages were branched at position 6. The glucan was partially degraded with amyloglucosidase. This, together with the NMR spectra data and the high optical rotation of the original (+195°) and degraded glucans (+175°) proved the α configuration. Further methylation of the amyloglucosidase degraded glucans indicated that they consist of (1→3)-linked glucoses. The present study is the first report on the isolation and characterization of an α-glucan from *Rhizoctonia*, that may be important as a biocontrol factor.

Keywords: Binucleate *Rhizoctonia*; α-glucan; β-1,3 Glucanase induction; Elicitor

1. Introduction

Rhizoctonia solani is a pathogenic fungus that infects many plant species.[1] Binucleated *Rhizoctonia* (BNR) spp. have been described as effective biocontrol agents against several plant diseases caused by *R. solani*.[2–4] In particular, potato could be protected from *Rhizoctonia* canker with BNR.[3] Understanding the mechanisms involved in protection of susceptible plants is essential for successful disease control. Oligosaccharides from fungal cell walls that induce defense responses in plants have been characterized.[5,6] Accumulation of phytoalexins by the plant[5] and induction of β-1,3 glucanase activity[7] have been correlated with the defense response.

Chitin and (1→3)-β-D-glucan are the main skeletal polysaccharides of fungal cell walls.[8] The presence of (1→3)-α-D-glucans is less frequently described. They are usually extracted with alkali and thus called alkali-soluble glucans. In *Aspergillus nidulans* they account for 25% of the dry weight of the cell wall.[9] In this way, an α-1,3 glucan was isolated from the well-known phytopathogenic fungus *Phytophtora infestans*.[10] However, it had been reported that a water-soluble

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(1→3)-β-glucan from *Phytophtora* was responsible for the inhibition of local lesion development by a potato virus.[11]

Protection properties described for BNR in potato,[3] together with the fact that elicitor-active oligosaccharides were obtained from mycelial walls, prompted us to study the glycans from BNR. In this article we report the isolation and characterization of an α-glucan solubilized by a heat treatment similar to that used to obtain elicitor active oligosaccharides from *Phytophtora*.[12–14] Phytoalexin production and induction of β-1,3 glucanase activity by the α-glucan, in potato sprouts, were also studied.

2. Results and discussion

In order to prove if β-1,3 glucanase activity was induced by the non-pathogenic isolate, as in bean plants,[7] potato sprouts of *Solanum tuberosum* cv Kennebec were inoculated with BNR. Enhancement in β-1,3 glucanase activity of 3-fold with respect to controls was observed between days 4 and 6, being maximal at day 6 (Fig. 1A). In contrast, potato sprouts inoculated with the pathogenic isolate *R. solani* AG3 did not show induction of β-1,3 glucanase activity during the experiment.

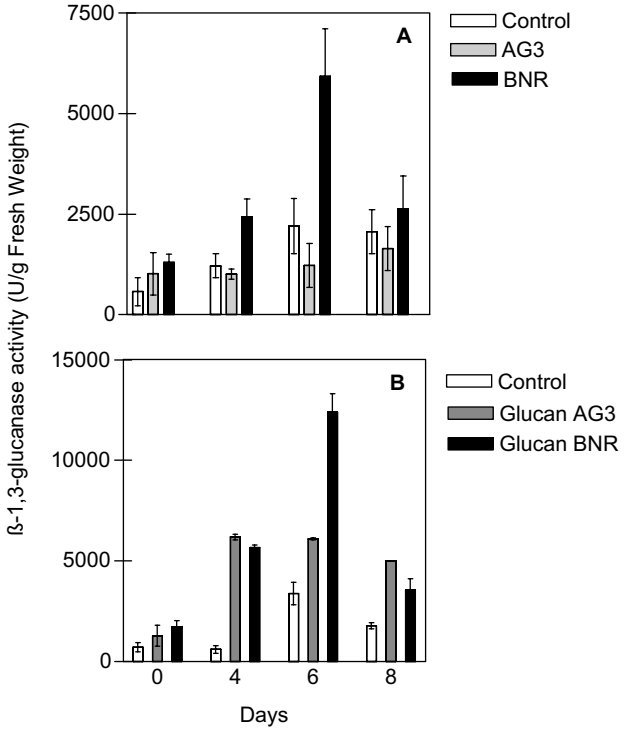


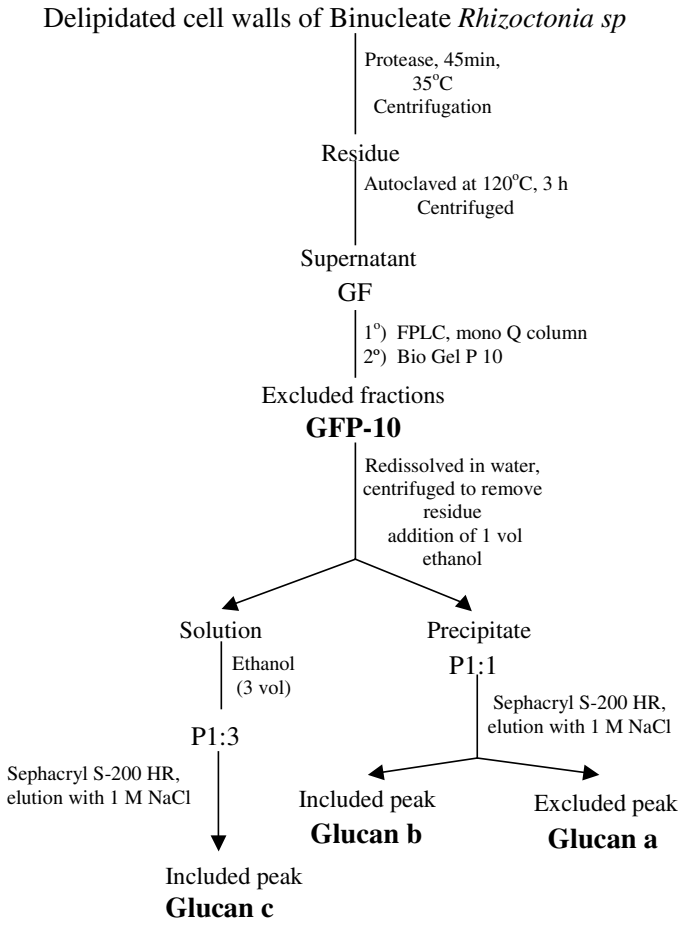
Figure 1. Induction of β-1,3 glucanase activity in potato sprouts. (A) Potato sprouts were inoculated with a disc of agar containing mycelium of BNR or *R. solani* (AG3) and were maintained at 18 °C in the dark. Controls were inoculated with a disc of agar without mycelia. (B) Sprouts were injected with 250 μg of the glycan fraction (GF) isolated from BNR or AG3. Controls were injected with distilled water. Glucanase activity was determined as described under Experimental. Bars represent the mean values ± SD of three independent experiments, with five replicates per experiment.

Different glucan fractions of the mycelial cell wall were obtained from BNR and purified as outlined in Scheme 1. The autoclaving method of isolation was chosen for extraction of the elicitor as this technique was previously used for the isolation of oligosaccharide elicitors.[12,14] The glycan fraction (GF), in the aqueous extract was assayed on potato sprouts for phytoalexin synthesis. Phytoalexin accumulation was determined 2, 3, 4, and 6 days after injection as described.[15] Control and treated sprouts showed no accumulation of phytoalexins (data not shown). To test if the glucan isolated from BNR was the elicitor of the β-glucanase activity, an experiment was performed with GF isolated from both, BNR and AG3 (Fig. 1B). Sprouts were inoculated with 250 μg of total sugar as this was the optimal dose found in a dose–response experiment (not shown).

The higher activity obtained with the isolated glucan would indicate that it is responsible for β-1,3 glucanase induction. Based on these results GF was purified and partially characterized. Charged and low molecular weight components of the extract were removed by ion exchange (Mono Q) followed by gel filtration (Bio Gel P10) chromatography. Activity was recovered in the excluded fractions (**GFP-10**) from both chromatographies. Analysis of monosaccharides in **GFP-10** as alditol acetates showed that glucose was a major component (78%, Table 1). A 3.2% content of uronic acids was found by colorimetric analysis.[16]

To prove the activity of **GFP-10**, an immunological assay was performed using an antibody raised against a basic isoform of β-1,3 glucanase (36 Kda) purified from intercellular fluid of potato leaves infected with *P. infestans*[17] (Fig. 2). Immunoblot analysis showed that a protein of 36 kDa was expressed at very low levels in a control of sprouts injected with water (Fig. 2A, lane 1). This level increased when the sprouts were challenged with **GFP-10** (Fig. 2A, lane 2). When the intensity of the immunological reaction was estimated by densitometry, the level of β-1,3 glucanase was 5-fold higher in sprouts treated with **GFP-10** relative to the control (Fig. 2B, lane 2).

The **GFP-10** fraction was purified by adding an equal volume of ethanol to the aqueous solution (**P1**:**1**). Another fraction was obtained from the mother liquors by adding 3 volumes of ethanol (**P1**:**3**). Both fractions showed induction of β-1,3 glucanase activity (Fig. 3A). However, since the highest value was obtained with P1:1, we further characterized this fraction. The total neutral sugar content of **P1**:**1** was 83% and the uronic acid analyzed was 4.8% with respect to total sugars. In order to identify the uronic acid, hydrolysis was performed under conditions that minimize destruction of uronic acids based on previous conversion to the corresponding methyl ester.[18] Analysis of the hydrolyzate was



Scheme 1.

Table 1. Compositional analysis of neutral sugars in the glycan fractions obtained from cell walls of binucleate non-pathogenic *Rhizoctonia* spp. isolate according to Scheme 1[a]

Monosaccharide	GFP-10	Fractions from Sephacryl		
		a	b	c
Rhamnose	1.25	0.22	0.17	1.44
Fucose	2.14	0.37	3.79	4.85
Xylose	4.60	2.36	0.56	2.35
Mannose	5.90	6.7	1.41	7.57
Galactose	7.49	3.2	11.65	16.42
Glucose	78.62	87.1	82.43	67.37

[a] Analyses were performed by capillary gas liquid chromatography after acid hydrolysis of each sample and derivatization as the alditol acetates. Abundance of sugars are given in molar proportions (%). Conditions are described under Experimental.

performed by HPAEC-PAD under conditions for the separation of glucuronic and galacturonic acids. Analysis (Fig. 4A) showed glucuronic acid (retention time 17.8 min) and another peak (II) of retention time (rt) 7.7 min. Peak II would correspond to a partially hydrolyzed oligosaccharide containing uronic acid. The rt for galacturonic acid was 13.3 min and neutral sugars are not retained under these conditions (maltose, rt 2.55 min). The monosaccharides in Peak I were analyzed by changing the conditions of the HPAEC-PAD. Isocratic elution with 15 mM NaOH gave the pattern of Figure 4B, showing glc:gal:man:fuc in the ratio 16:6:2:1. Remaining glucose would be in part combined with uronic acid in peak II of Figure 4A.

The high optical rotation, $[\alpha]_D$ +195.3 (*c* 0.5, water), suggested an α-glucan structure. In agreement, the ^{13}C NMR spectrum in DMSO-d_6 showed the characteristic signals for other (1→3), (1→4)-α-D-glucans (Fig. 5A).[19,20] Thus, signals in the anomeric region at 99.7 and 101.1 ppm correspond to C-1 of (1→3)- and (1→4)-linked α-glucan. Moreover, the signals at 82.4 and 78.8 ppm were in agreement with data[19] for C-3

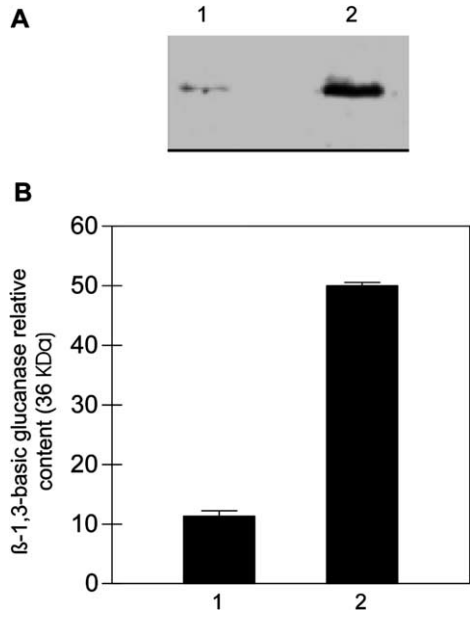


Figure 2. Immunoblot analysis of a basic β-1,3 glucanase in potato sprouts treated with the glycan fraction **GFP-10**. Proteins of potato sprouts injected with **GFP-10** (lane 2) or a control injected with distilled water (lane 1), were analyzed after 6 days by 12% SDS-PAGE. The proteins were transferred onto nitrocellulose and immunodetected with a monoclonal antibody raised against a basic β-1,3 glucanase isoform. (A) Western blot. (B) The relative content of β-1,3 basic glucanase was determined by densitometric scanning of the western blot. The value of the band intensity of a basic β-1,3 glucanase induced by **GFP-10** was calculated respect to the control. Equivalent amounts of protein (approximately 700 μg) were loaded on each lane. The results are representative of at least three identical experiments.

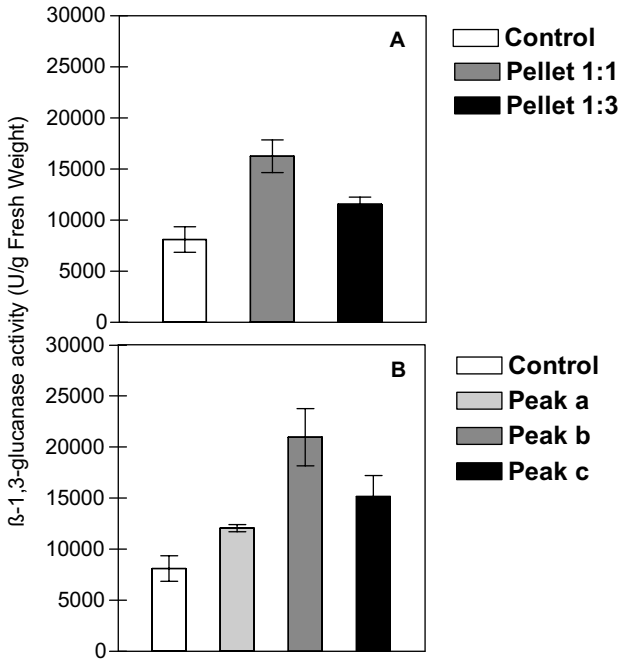


Figure 3. Effect of different glycan fractions on β-1,3 glucanase activity. Potato sprouts were injected with 20 μL (250 μg) of glycan fractions obtained according to Scheme 1. The tissue was processed after 6 days and β-1,3 glucanase activity was determined. Bars represent the mean values ± SD of three independent experiments, with five replicates per experiment.

and C-4 of the 3-*O*-substituted and 4-*O*-substituted glycosyl units, respectively. Due to the viscosity of the solution the ^{1}H NMR spectra showed broad signals above 5.05 ppm (not shown). The signal for H-1 in α (1→3)-glucans appeared at δ 5.14.[19] A value of 4.55 was given[20] for a (1→3)-β-glucan.

Fractions **P1**:**1** and **P1**:**3** were further purified on a Sephacryl S200 HR column eluting with 1 M NaCl (Fig. 6). An excluded (**a**) and a broad included peak (**b**) were obtained from **P1**:**1** (Fig. 6A) whereas only a broad included peak (**c**) was obtained from **P1**:**3**, (Fig. 6B). The wide peaks obtained for fractions **b** and **c** would indicate microheterogeneity probably caused by degradation during the extraction by autoclaving. All the peaks showed induction of β-1,3 glucanase activity, but peak **b** showed the highest activity, which increased with the last purification step (Fig. 3B). The separated fractions were analyzed for total neutral sugar and for uronic acid content. Fractions **a**, **b**, and **c** contained 9%, 10.3%, and 13.7%, respectively, of uronic acid with respect to total neutral sugars.

A compositional analysis of neutral monosaccharides was performed by GLC of all the fractions after acid hydrolysis and derivatization as the alditol acetates (Table 1). Analysis indicated that fractions **a** and **b** were

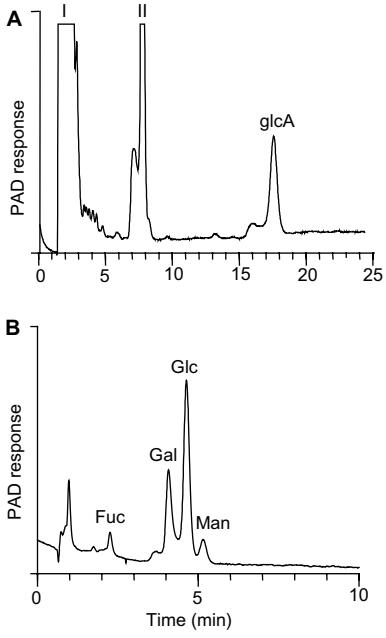


Figure 4. HPAEC-PAD of monosaccharides in glycan fraction from BNR. (A) Methanolysis and TFA hydrolysis (see Experimental) of a sample of **P1**:**1** was performed and analyzed on a Carbo-Pac PA-10 column under Condition 1. (B) Peak I of panel A was collected and rechromatographed on the same column under condition 2. The elution position of standards is shown: GlcA, glucuronic acid; Fuc, fucose; Gal, galactose; Glc, glucose; Man, mannose.

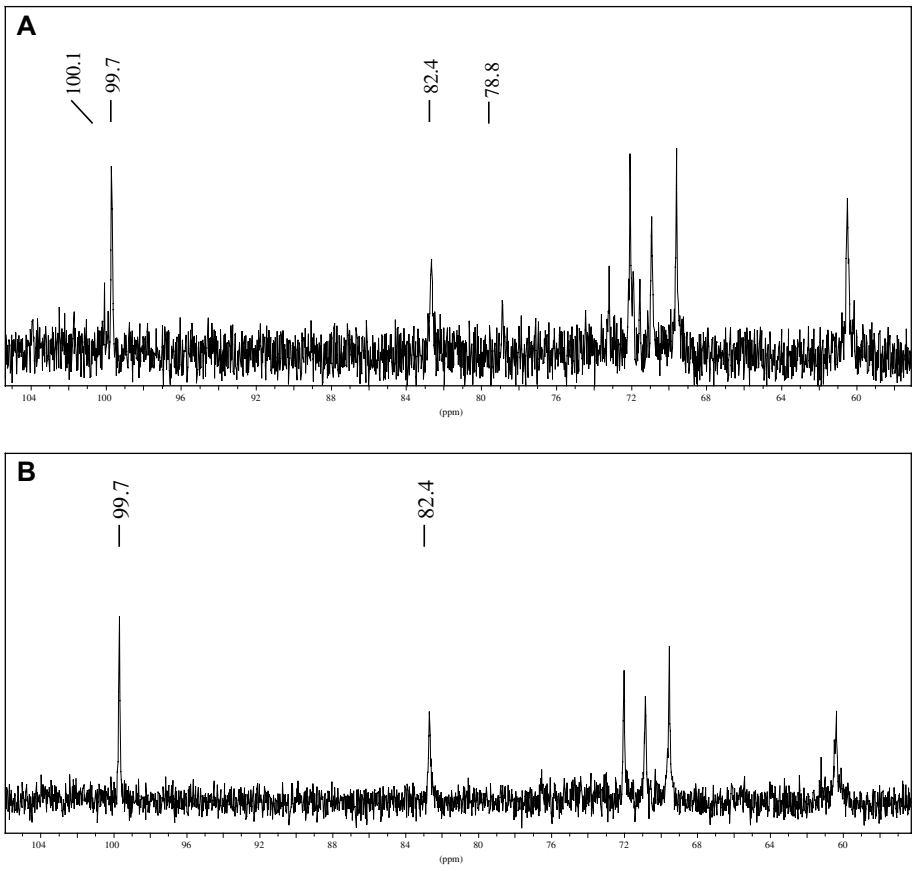


Figure 5. ^{13}C NMR spectra in Me_2SO-d_6 at 30 °C of the α-glucan obtained from binucleata *Rhizoctonia* isolate before (A) and after amyloglucosidase treatment, according to Experimental (B).

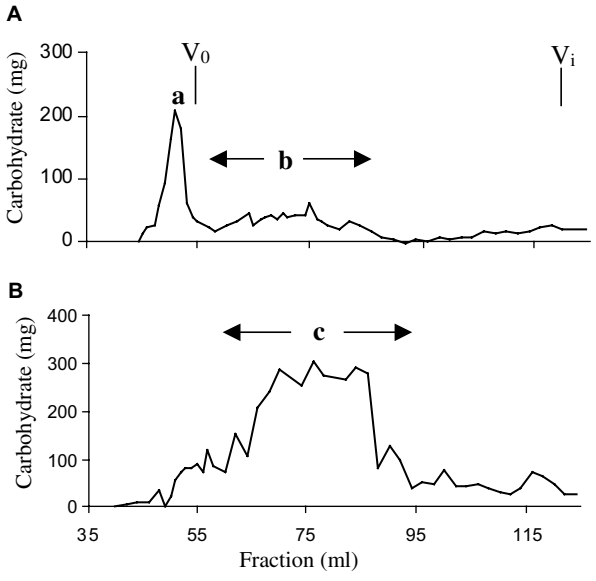


Figure 6. Gel filtration on Sephacryl S-200 HR of glycan fractions obtained from the cell wall of BNR according to Scheme 1. Elution was performed with 1 M NaCl (aq). (A) Glycan fraction (**P1**:**1**) precipitated with ethanol/water (1:1) from an aqueous solution of **GFP-10**. (B) Mother liquors of **P1**:**1** were further precipitate with 3 vol of ethanol (**P1**:**3**).

glucans (more than 80% glucose) with different amounts of galactose, mannose, xylose, and fucose. It is possible that fucose is underestimated since the 6-deoxy sugar is in part destroyed under the conditions of hydrolysis.

In order to characterize the linkages, both fractions (**a** and **b** from the Sephacryl column, Fig. 5A) were studied by methylation analysis (Table 2). GLC–MS showed in both fractions α-(1→3)- and α-(1→4)-linked glucose. The ratio was 3:2 for glucan **a** and 5:1 for glucan **b**. The results showed a branched structure mainly in O-6 of the (1→4)-linked glucose units for both glucans.

For obtaining further information on the fine structure of the glucan, fraction **P1**:**1** was treated with amyloglucosidase from *A. niger*. After treatment, only 37% of the polysaccharide (phenol–sulfuric acid method) was excluded from a PD-10 desalting column. The high optical rotation (α_D +170) of this fraction showed that after the enzymatic digestion, the remaining glucoses were also α-linked. A low specific rotation of +4 was reported for a (1→3)-β-glucan.[20] The enzyme is an exohydrolase highly specific for an α-D-glucopyranosyl disaccharide, in the order α-(1→4) > α-(1→3) > α-(1→6).[21] Hydrolysis of part of the (1→3)-linked glucose residues, besides the (1→4) linked, could explain the low percentage of glucan recovery after the treatment. In the ^{13}C NMR spectrum

Table 2. Linkage analysis of α-glucans and their amyloglucosidase degraded products from cell walls of BNR[a]

O-Methyl sugar	Linkage	**a**	**a**+Amyloglucosidase	**b**	**b**+Amyloglucosidase
2,3,6-Fuc	Fuc(1→	—	—	0.14	nd
2,3,4,6-Gal	Gal(1→	0.16	0.26	0.10	0.09
2,3,4,6-Glc	Glc(1→	1	1	1	1
2,3,4,6-Man	Man(1→	—	0.30	—	—
3,4-Rha	→2Rha(1→	0.2	—	0.34	nd
2,3-Xyl	→4Xyl(1→	0.29	0.31	—	—
2,4,6-Glc	→3Glc(1→	2.80	6.64	5.54	3.36
2,3,6-Glc	→4Glc(1→	2.05	0.10	1.20	0.29
2,4,6-Man	→3 Man(1→	0.24	0.38	—	—
3-Xyl	→2,4Xyl(1→	1.05	0.91	—	—
2,6-Gal?	→3,4Gal(1→	—	—	0.19	0.21
2,6-Glc	→3,4Glc(1→	0.20	0.40	0.29	0.29
2,3-Glc	→4,6Glc(1→	0.75	0.29	0.57	0.15

[a] Abundance of sugars are given in molar proportions (%). Conditions are described under Experimental.

of the amyloglucosidase degraded glucan (Fig. 5B), the signal assigned to C-1 of α-(1→4)-linked Glc disappeared and also the peak at 78.8. Only the signal at 99.7 ppm appeared in the anomeric region. No signals that could correspond to a β-glucan were observed at low fields. Carbonero et al.[20] reported δ 102.8 for C-1 and δ 86.0 ppm for C-3 of (1→3)-β-glucans. The results were confirmed by methylation analysis of the degraded glucans obtained by amyloglucosidase treatment of **a** and **b** (Table 2). Both were almost exclusively composed of (1→3) linkages although a small proportion of (1→4) linkages still remained. On the basis of the present results the most probable structure consists of an α-(1→3)-linked Glc*p* backbone with branches of (1→4)-linked glucoses, some of the latter would be branched at position 6. No evidences could be obtained at this stage as to whether the minor components are structural components of the glucan. Branching would be located in a region far from the non-reducing end since glucoamylase degraded 60% of the glucan. In all cases, the degraded glucans were less active than the original (60% of the activity).

This is the first report on the presence of a cell wall α-glucan in a *Rhizoctonia* species. Polysaccharides from *Rhizoctonia* hyphal walls have not been thoroughly investigated.

In a classification of fungi based on cell-wall chemistry,[8] *Rhizoctonia* were included in a category of fungi with chitin-glucan. However, it was reported that the walls of *R. solani* are not significantly degraded with β-glucanase/chitinase.[22]

Interestingly, a report on the binding of an antifungal exo α-1,3 glucanase to the cell wall of *R. solani*[23] suggested the presence of accessible (1→3)-α-glucan. The α-glucan structure for an oligosaccharide elicitor was unexpected. To our knowledge, only β-glucans with elicitor activity have been obtained by autoclaving fungal cell walls.[13,24] Similarly, (1→3)-β-glucans with antitumor activity have been characterized, however, also some α-glucans have been reported as antitumor polysaccharides.[25]

It is intriguing the mechanism by which the α-glucan triggers β-1,3 glucanase activity in potato.

3. Experimental

3.1. Material

Potato tubers (*S. tuberosum* cv. Kennebec) were obtained from the Experimental Station of INTA Balcarce, Argentina. This cultivar was selected because it is moderately susceptible to *Rhizoctonia* disease.

Binucleate non-pathogenic *Rhizoctonia* (BNR) isolate (232-CG) and pathogenic *R. solani* (AG3) were obtained from the Laboratorio de Fitopatología, INTA, Balcarce, Argentina. Stock cultures were maintained on potato–2% glucose agar, at 18 °C in the dark. For mycelial wall extraction, both isolates were grown on potato–2% glucose liquid culture at 18 °C in the dark for 15 days.

3.2. Isolation and purification of polysaccharides from binucleate *Rhizoctonia* cell walls

For the preparation of mycelial cell walls and polysaccharides the method employed by Ayers et al.[12] was adapted. Mycelium from BNR (4–5 g) was homogenized in 500 mM potassium phosphate buffer pH 7.2 and filtered through glass fiber filters (Millipore type 1, Sigma). The solid residue was washed with 1:1 $CHCl_3$–MeOH and dehydrated with acetone. The dried cell walls (2–3 g) were incubated with protease from *Streptomyces griseus* (Sigma) 50 μg/mL in Tris–HCl, 10 mM, pH 7.2 at 35 °C for 45 min. The incubation mixture was centrifuged and the solid residue remaining after protease treatment was resuspended in distilled water (100 mL/g) and autoclaved at 120 °C for 3 h. The suspension was centrifuged and 10 mL of the aqueous extract containing 50 mg neutral sugars was chromatographed by fast performance liquid

chromatography (FPLC, Pharmacia) using a MonoQ column, and eluting with 1 M sodium chloride in 50 mM Tris–HCl buffer pH 8, at 1 mL/min. Non-retained material was collected, the fractions concentrated to 2 mL and then chromatographed on a column of Bio Gel P-10 (1 × 80 cm) equilibrated and eluted with distilled water. The fractions eluting in the void volume were pooled and lyophilized. This fraction was called **GFP-10**.

The dry powder (100 mg) was dissolved in 10 mL of distilled water, 10 mL of EtOH was added and stored at 4 °C overnight and then centrifuged for 20 min at 8000*g* and 5 °C. The pellet (**P1**:**1**) was separated, the supernatant was concentrated under reduced pressure and the residue was dissolved in water, precipitated with three volumes of EtOH and centrifuged as already described (**P1**:**3**).

Gel permeation chromatography of **P1**:**1** (12–30 mg in sugar) was performed on a Sephacryl S-200 HR (Amersham Biosciences) column (1.8 × 95 cm) eluting with 1 M NaCl. Fractions (1 mL) were colleted and 50 μL of each were assayed for sugars by the phenol–H_2SO_4 method.

3.3. Analytical methods

Neutral sugars were determined by the phenol–H_2SO_4 method, with glucose as a standard.[26]

Uronic acids were determined by the *m*-hydroxydiphenyl–sodium tetraborate method.[16]

Optical rotations were determined in water with a Perkin–Elmer 343 polarimeter.

The NMR spectra were recorded with a Bruker AM 500 Spectrometer at 30 °C overnight. The samples (8–10 mg) were dissolved in 0.5 mL of Me_2SO-d_6 containing two drops of D_2O. Chemical shifts (δ) are expressed relative to the resonances of Me_4Si ($\delta = 0$) obtained in a separate measurement.

Analysis by high-pressure anion exchange chromatography with pulse amperometric detection (HPAEC-PAD) was performed using a Dionex DX 300 HPLC system. A CarboPac PA-10 anion exchange analytical column (4 × 250 mm), equipped with a guard column PA-10 (4 × 50 mm) was used with the following isocratic programs. Condition 1: Eluent was 50 mM NaOH, 100 mM NaAcO for 30 min at a flow rate of 1.0 mL min^{-1} at room temperature. Condition 2: Eluent was 15 mM NaOH for 30 min at a flow rate of 1.0 mL min^{-1} at room temperature. The pulsed amperometric detector sensitivity was set to 100 nA and the pulsed potentials were as follows: *E1* = +0.05 V; *E2* = +0.6 V and *E3* = −0.60 V.

Glucuronic acid and the monosaccharides peaks were identified by comparison with the retention time of standards and cochromatography. The data were analyzed using A-I-450 chromatography software (DIONEX).

3.4. Monosaccharide analysis

Sugar composition was analyzed after acid hydrolysis of glycan (2 mg) with 2 M trifluoroacetic acid (TFA) at 105 °C for 3 h. After centrifugation, the solution was evaporated to dryness, sugars were converted into the alditol acetates[27] and analyzed by gas–liquid chromatography (GLC). Capillary GLC was performed with a Hewlett–Packard 5890 gas chromatograph with nitrogen as the carrier gas and a SP-2340 column (0.20 mm × 30 m, Supelco). The following conditions were used: flow rate, 1.4 mL min^{-1}, column temperature 220 °C, injector temperature 230 °C, detector temperature 250 °C. The monosaccharides were identified by comparison with the retention time of standards and cochromatography.

For uronic acid determination, methanolysis was performed previous to TFA hydrolysis. The method of De Ruiter et al.,[18] with slight modification was used. Methanolysis was conducted in a 10 mL screw-cap (Teflon-lined) test tube at 80 °C using methanolic HCl. The reaction solution was prepared by the addition of acetyl chloride (0.1 mL) to cold anhydrous methanol (MeOH, 3.5 mL, −40 °C). The glycan (2 mg) was added to this solution, and the mixture was sealed and heated at 80 °C for 16 h. The mixture was evaporated under reduced pressure and the carbohydrates were hydrolyzed further with 2 M TFA for 3 h at 105 °C.

3.5. Amyloglucosidase treatment

A sample of glycan (3 mg) was dissolved in 1 mL 100 mM acetate buffer, pH 4.8–5 and incubated with 5 units of amyloglucosidase from *Aspergillus niger* (Merck) overnight at 60 °C. The reaction was stopped by heating 2 min at 80 °C. The mixture was centrifuged and the supernatant was passed through a PD-10 desalting column (Amersham Pharmacia Biotech) and the excluded and included volumes were analyzed by TLC and HPAEC. These two fractions were also tested for induction of β-1,3 glucanase activity.

3.6. Methylation analysis

Glycan samples containing 2 mg sugar were methylated by the method of Ciucanu and Kerek[28] with modifications.[29] Hydrolysis of methylated samples was performed with 2 N trifluoroacetic acid at 110 °C for 3 h. Partially methylated sugars were reduced with $NaBH_4$, acetylated with Ac_2O–pyridine and analyzed by GLC on a capillary column SP-2340 (0.25 × 30 m, Supelco) with a temperature program of 160–210 °C at 2 °C min^{-1}, and then from 210 to 240 °C at 5 °C min^{-1}. The injector temperature was 225 °C, and the detector temperature was 250 °C. Gas–liquid chromatography–mass spectrometry was performed using a TRIO-2VG

Masslab at 70 eV with SP-2340 column similar to that as described above. The temperature program was from 140 (2 min) to 235 °C at 8 °C min^{-1}; the injector temperature was 240 °C and the detector temperature, 250 °C.

3.7. Assay of elicitor activity

3.7.1. Extraction and determination of phytoalexins. Phytoalexins were extracted from potato sprout tissue by the method described by Shih et al.[30] and analyzed as described.[15]

3.7.2. β-1,3 glucanase assay. Potato sprouts were homogenized with a mortar in 50 mM sodium acetate buffer pH 5.2 containing 0.1% sodium metabisulfite. The homogenate was centrifuged for 20 min at 12,000*g*. A satd aq $(NH_4)_2SO_4$ (100%) was added to the supernatant. The precipitate was dissolved in 50 mM sodium acetate buffer pH 5.2 and dialyzed overnight against the same buffer. The samples were stored at −20 °C for β-1,3 glucanase assays. All operations were carried out at 0–4 °C.

Glucanase activity was assayed by measuring the rate of reducing sugar production with laminaran from *Laminaria digitata* (Sigma) as the substrate. The reaction mixture consisted of 0.06 mL of 50 mM sodium acetate buffer pH 5.2 containing 1% laminaran, 0.01 mL of enzyme extract, and 0.05 mL of sodium acetate buffer pH 5.2. After 2 h of incubation at 37 °C the enzyme reaction was heated in boiling water for 2 min and the reducing sugar released was measured using glucose as standard.[31] The activity was expressed as nmol glucose released per hour per ml enzyme extract.

3.7.3. Bioassay with BNR isolate and with pathogenic *R. solani* (AG3) isolate. Potato sprouts were inoculated with a disc of agar containing mycelium from BNR or AG3 on the base of potato sprout and were maintained at 18 °C in the dark for different times. Controls included potato sprouts inoculated with a disc of agar without mycelia.

3.7.4. Assays with glycan fractions. Elicitor activity was determined by measuring the effect of glycan (5 mg/mL) fractions from BNR cell walls on accumulation of phytoalexins and glucanase activities in potato sprouts. Controls were potato sprouts injected with distilled water, respectively.

The potato sprouts were injected with the fractions (250 μg) in sterile conditions in the dark and then incubated at 18 °C. The sprouts were processed at different times and β-1,3 glucanase activity was determined as described below.

3.7.5. Western blot analysis. For Western blot analyses of induced glucanases, proteins (700 μg) were separated on 12% SDS-PAGE and subsequently transferred electrophoretically (15 V, 20 min, 25 mM Tris–HCl, pH 8; 0.192 M glycine, 20% MeOH) to nitrocellulose membranes. Membranes were incubated with a monoclonal antibody (1:10.000 dilution) raised against a basic β-1,3 glucanase (36 kDa) purified from intercellular fluid of potato leaves infected with *P. infestans*.[17] Then the membranes were incubated with goat antirabbit IgG alkaline phosphatase conjugate (1:10.000 dilution). Immunodetection was performed using 5-bromo-4-chloro-indolyl-phosphate (BCIP) and nitrobluetetrazolium (NBT), method described by Turner.[32]

The intensity of the immunological signal was measured using densitometric analysis (TN-Image, Image Analysis Software, Compuserve, IBMAPP, Rockville, MD, USA).

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