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The structure of the exopolysaccharide fraction from *Pseudomonas savastanoi* strain ITM519 and the defence-response it induces in non-host plants

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Summary. The main exopolysaccharide (EPS) obtained from the phytopathogenic bacterium *Pseudomonas savastanoi* pv. *nerii*, strain ITM519, has a very complex highly branched structure consisting of fucose, galactose, N-acetylgalactosamine and N-acetylglucosamine. EPS triggers a defence response in non-host plant cells. This capability could be a consequence of the complex and heterogeneous structure of the molecule, part of which might mimic elicitors produced in the plant–pathogen interaction.

Key words: phytopathogenic bacteria; EPS; plant-defence response; PAL and APX activities.

Abbreviations used in the text: APX: Ascorbate Peroxidase; DeoxyHex: deoxyhexose; EPS: exopolysaccharide; FAB: Fast Atom Bombardment; FT: Fourier Transform; Fuc: fucose; Gal: galactose, GalNAc: 2-Deoxy-2-acetamidogalactose; D-Gal-ol: D-galactitol; Gal*p*: galactopyranose; GLC: Gas Liquid Chromatography; GlcNAc: 2-Deoxy-2-acetamidoglucose; Glc*p*: Glucopyranose; Hex: hexose; HexNAc: N-acetylhexosamine; MS: Mass Spectrometry; NMR: Nuclear Magnetic Resonance; PAL: Phenyl Alanine Ammonia-Lyase; TFA: Trifluoro Acetic Acid; TLC: Thin Layer Chromatography; TMS: TriMethyl Silyl.

Introduction

It is well known that exopolysaccharides (EPSs) from phytopathogenic bacteria have multiple and important biological functions, such as protection from desiccation or toxic molecules, adhesion to biological surfaces, promotion of bacterial infection, and colonisation of host tissues (Jahr *et al.*, 1999).

In a recent study (Cimino *et al.*, 1998b) some preliminary results were reported on the chemical composition of the EPS fractions, produced *in vitro* in liquid cultures, of two strains of *Pseudomonas syringae* subsp. *savastanoi* (now re-named *Pseudomonas savastanoi*): strain ITM519 from oleander (*P. savastanoi* pv. *nerii*) and ITM317 from olive (*P. savastanoi* pv. *savastanoi*). *P. savastanoi* is

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the causal agent of olive (*Olea europea* L.) knot, a disease which also occurs on oleander, privet and ash, and which is characterised by the formation of galls on young stems and, less frequently, on other plant organs (Surico and Iacobellis, 1992). In that study a strict dependence was found between the chemical structure of the EPSs and the three culture media on which the two strains were grown.

The present paper reports further structural features of the main EPS from *P. savastanoi* pv. *nerii* strain ITM519, grown in King's B liquid medium and presents evidence that this EPS in a non-host system induces metabolic changes involved in the plant-pathogen interaction.

Materials and methods

General methods

FAB mass spectra were recorded in positive mode with a VG ZAB HF instrument (Manchester, UK) equipped with a FAB source. TLC was carried out on silica gel F_{254} plates (Merck, Darmstadt, Germany). All compounds were revealed by spraying plates with a saturated solution of chromic oxide in concentrated H_2SO_4, followed by heating at 120°C for 15 min. UV absorbance was determined on a Perkin-Elmer (Norwalk, CT, USA) Lambda 7 instrument. GLC was performed with a Carlo Erba (Milan, Italy) EL 490 instrument equipped with a flame-ionisation detector. TMS ethers of methyl glycosides were analysed by GLC on a SPB-1 capillary column (Supelco, Belleforte, Pa, 30 m x 0.25 mm i.d., flow rate 1 ml min^{-1}, N_2 as carrier gas), with the temperature programme: 150°C for 8 min, 150°C→200°C at 2°C min^{-1}, 200°C→260°C at 6°C min^{-1}, 260°C for 15 min. The quantitative evaluation was made using mannitol as internal standard, and appropriate response factors. Partially methylated alditol acetates were analysed by GLC-MS on a Hewlett-Packard (Amsterdam, The Netherlands) 5890 instrument, in the following conditions: SPB-1 capillary column (Supelco 30 m x0.25 mm i.d., flow rate 0.8 ml min^{-1}, He as carrier gas), with the temperature programme: 100°C for 2 min, 100°C→240°C at 2°C min^{-1}. The GC quantitative evaluation was made on the same column. Oligosaccharide fractions recovered after HF hydrolysis were reduced with $NaBD_4$ for 1 hour at room temperature, and analysed both as acetylated (Corsaro *et al.*, 1999) and as permethylated alditol derivatives. The latter were obtained according to the Ciucanu and Kerek (1984) procedure. Sugar composition, glycosyl linkage analyses and absolute configuration analyses were performed as previously described (Cimino *et al.,* 1998a; Cimino *et al.*, 1998b).

All NMR spectra were recorded in D_2O at 30°C in FT mode on a Bruker (Karlsruhe, Germany) DRX400 spectrometer equipped with a dual probe. The ^{13}C and ^{1}H chemical shifts were measured using 1,4-dioxane (67.4 ppm) and TSP (sodium 3-trimethylsilylpropionate-2,2,3,3-d_4: 0 ppm) respectively as internal standards. Two-dimensional homonuclear shift correlation (COSY) and gradient heteronuclear single quantum coherence (HSQC) spectra were performed using standard pulse sequences available in the Bruker software**.** Chromatographic purifications were carried out on supports purchased from Bio-Rad (Hercules, CA, USA) and Pharmacia (Uppsala, Sweden).

Isolation and purification of EPS

Strain ITM519 was isolated from knots on oleander plants growing in southern Italy and cultured on King's B medium (Cimino *et al.*, 1998b). The lyophilised culture filtrate (37 g per 4.6 l) was treated as previously described (Cimino *et al.*, 1998b), giving crude EPS (1.82 g), which was purified as described in the text. The chromatographic purification was performed on Q-Sepharose (Pharmacia) as described in Cimino *et al.* (1998b). Fraction A (260 mg) was chromatographed on a Bio-Gel A-1.5m column (Bio-Rad, 98x1.5 cm, 50 mM NaOAc, pH 5.2) (Fig. 1). The pooled fractions 43–70 (121 mg) and 71–93 (73 mg) were collected. Only the first fraction (EP) was chromatographed on Bio-Gel P-100 (Bio-Rad, 48 x 2.5 cm, NaOAc 50 mM, pH 5.2) obtaining a single peak.

Smith degradation

The EP (100 mg) was dissolved in 2.4 ml of water and treated with 2.5 ml 0.1 M $NaIO_4$ at 4°C for 140 h, as previously described (Corsaro *et al.*, 1999). After the usual reaction work- up, the sample was treated with 4 ml 0.5 M TFA at room temperature for 6 days. After TFA evaporation 74 mg of the crude mixture was purified on a Bio-Gel P-2 (Bio-Rad, 90x1.5 cm) column, using ultrapure

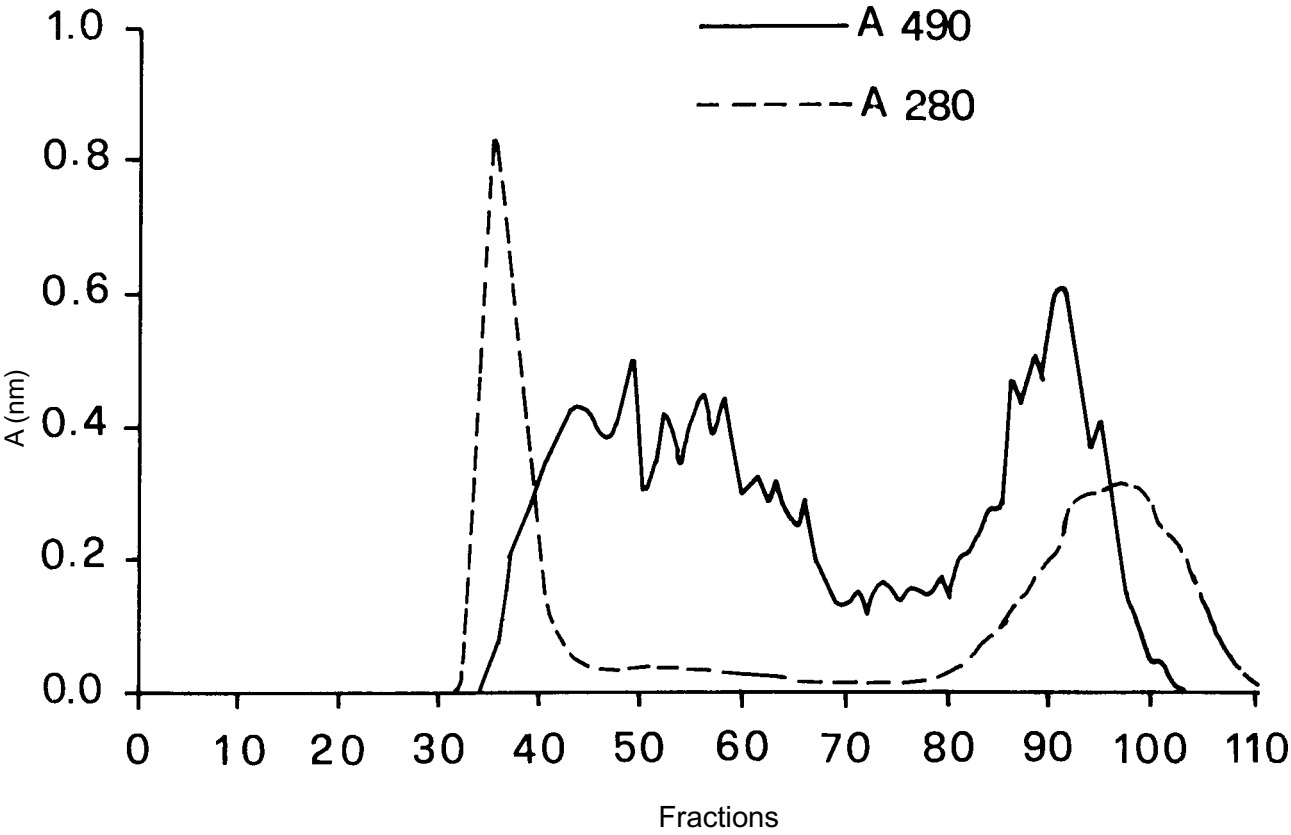

Fig. 1. Bio-Gel A1.5m of fraction A. Column conditions: 98x1.5 cm, 50 mM NaOAc, pH 5.2.

Milli-Q water as effluent. Fractions were pooled in four main groups: SM1 (46 mg) eluted in the void volume, SM1A (2 mg), SM1B (5 mg) and SM1C (20 mg). Fraction SM1 was further chromatographed on a Sephacryl S-300 HR column (Pharmacia, 68x1.5 cm, NH_4HCO_3 50 mM), obtaining a single peak.

HF solvolysis

A dried EP sample (20 mg) was treated with 2 ml of liquid HF for 20 min at -50°C under stirring (Knirel *et al.*, 1989). After the usual reaction work-up (Corsaro *et al.*, 1999) the sample was chromatographed on Bio-Gel P-2, obtaining only two fractions, one of which was eluted in the void volume (15 mg), while the other was retained (3 mg). Another EP sample (40 mg) was treated as above at 4°C giving fractions A' (23 mg), B' (4 mg), C' (3 mg) and D' (6 mg) after chromatography in the same way.

Biological activity

The biological activity of purified EP from strain ITM 519 was determined utilising a cell culture of *Nicotiana tabacum* Bright Yellow 2 and measuring the effect that EP addition to the culture medium had on electrolite leakage, phenylalanine ammonia-lyase (PAL) and ascorbate peroxidase (APX), two enzymes whose role in plant pathogen interaction is well known (Lamb and Dixon, 1997; Mittler *et al.*, 1998). Cell growth conditions were as described in de Pinto *et al.* (1999). EP (0.05 mg ml^{-1}) was added to the culture medium of 3-day-old cultured cells (beginning of the exponential phase). After 20 hours, the cells were collected and the changes in PAL and APX activity were determined according to established procedures (De Gara *et al.*, 1997; Sarna and Sarna, 1999). Protein determination was analysed as in Bradford (1976).

Electrolyte leakage was measured according to established procedure (Iacobellis *et al.*, 1992) on 1

ml of cells pelletted at 250 x *g*, washed twice with 3% sucrose and then re-suspended in 1 ml of 3% sucrose.

Results and discussion

Chemical composition

Our preliminary results, reported in Cimino *et al.* (1998b), indicated that the EPS fraction (King's B medium) of *P. savastanoi* ITM519 has a very complex chemical composition, suggesting that it was not chemically homogeneous. In this study, therefore, the EPS batch used was larger than the previous one, and several purification steps were performed. The EPS fraction was chromatographed on a Q-Sepharose column giving the fractions A and B, the latter of which was discarded due to its high protein content and because it was not active in the biological assay described below. Fraction A was chromatographed on Bio-Gel A-1.5m (Fig. 1) and the fractions from 43 to 70 were pooled, since they were less protein-rich. The chemical homogeneity of the pooled fraction was suggested by the appearance of a single peak on a Bio-Gel P-100 column. This fraction, named EP, consisted of Fuc, Gal, GalNAc and GlcNAc in a ratio of 1.0: 2.0: 1.2: 2.2.

The EP ^{13}C NMR spectrum (Fig. 2) showed many anomeric signals in the range 106–99 ppm, and nitrogen-bearing C-2 signals at δ 49.2, 54.8 and 56.1, which, according to the literature (Lipkind *et al.*, 1988), suggests the presence of α-Gal*p*NAc and of both α and β-Glc*p*NAc residues respectively. In addition, methyl and carbonyl signals of the *N*-acetyl groups were present at about 23 and 175 ppm respectively. Heterocorrelated 2-D NMR spectra established scalar correlations among the main proton anomeric signals at δ 5.32 and 4.89, with carbon signals at δ 100.1 and 99.0 respectively. Methylation analysis showed a wide variety in linkage positions for all sugars except fucose, which was only terminal (Table 1).

In the ^{1}H NMR spectrum the Smith degraded polysaccharide (SM1) showed four anomeric broad signals at δ 4.91, 4.73, 4.56 and 4.47, which were

Table 1. Interglycosidic linkages and molar ratios among residues for EP and SM1 Smith degraded polysaccharides.

Monosaccharidic residue	EP	SM1
t-Fuc*p*	0.8	-
t-Gal*p*	0.8	0.5
2-Gal*p*	0.6	-
3-Gal*p*	1.1	1.1
4-Gal*p*	2.1	-
6-Gal*p*	-	0.1
2,3-Gal*p*	0.5	-
3,6-Gal*p*	1.1	0.9
2,3,4,6-Gal*p*	1	1
t-Glc*p*NAc	2	1.1
3-Glc*p*NAc	2.8	0.8
4-Glc*p*NAc	1.2	0.8
4,6-Glc*p*NAc	0.2	0.2
t-Gal*p*NAc	0.7	0.4
3-Gal*p*NAc	1.7	0.7
6-Gal*p*NAc	-	0.4
3,6-Gal*p*NAc	1.4	0.1

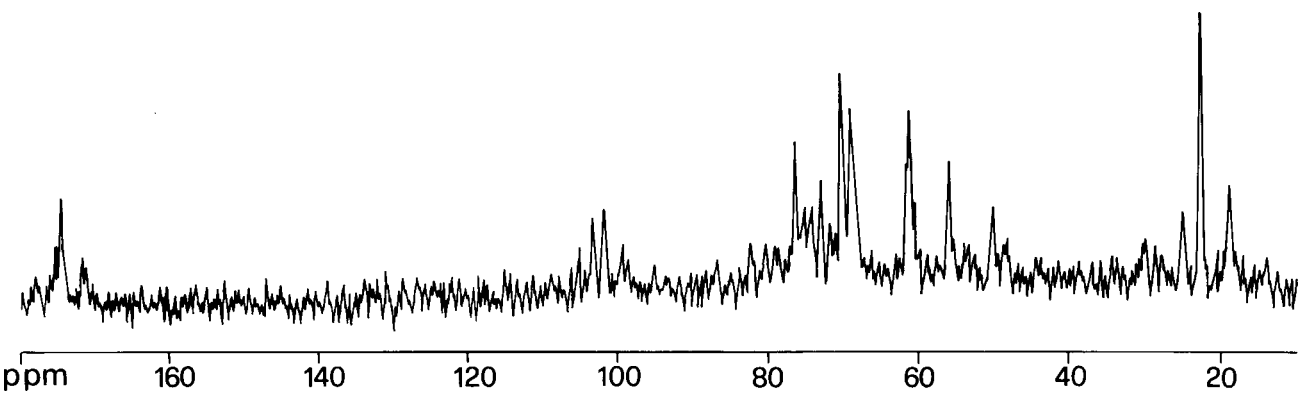

Fig. 2. ^{13}C-NMR spectrum of EP in D_2O at 62.5 MHz.

correlated with carbon signals at δ 99.4, 103.1, 102.2 and 103.4 respectively. These signals were assigned after identification of H-2 signals by a COSY, and of C-2 correlated carbon signals by an HSQC experiment. The signal at δ 4.91 was assigned to α-Gal*p*NAc, as its C-2 signal occurred at δ 50.5, the signals at δ 4.73 and 4.56 at β-Glc*p*NAc, as both their C-2 signals occurred at δ 56.3 (Lipkind *et al*., 1988). The last signal, at δ 4.47, which was correlated with carbon at δ 103.4, was assigned to β-Gal*p*, on the basis of proton and carbon chemical shift values, and because its H-2 was not correlated with any nitrogen-bearing carbon signals.

In the SM1 ^{13}C NMR spectrum (Fig. 3) the disappearance of the signal at δ 54.8 suggested that the α-Glc*p*NAc residues were terminal in the EP, the t-Glc*p*NAc units being the only residues of the Glc*p*NAc units in the EP, degradable from the periodate. Other signals which disappeared in the NMR spectra of SM1 were that of the proton at δ 5.32 and that of the correlated carbon at δ 100.1 (see below).

Further structural information emerged from the solvolyses of EP with HF. An initial solvolysis at -50°C gave a slightly degraded polysaccharide where terminal Fuc residues were almost the only residues lost. The ^{1}H and ^{13}C NMR of this product showed the disappearance of the signal at 5.32 ppm, which was correlated with the carbon signal at 100.1 ppm. These signals assigned to terminal Fuc units indicated an α configuration on the basis of the chemical shift values. A second HF solvolysis run at 4°C yielded mono- and oligosaccharide fractions. The oligosaccharide mixture was purified on Bio-Gel P-2, giving four main fractions, named A'-D', on the basis of TLC analysis. These fractions were analysed by FAB-MS as both acetylated and permethylated alditol derivatives.

Fraction D' contained only monosaccharides. Permethylated fraction B' showed $[M+H]^+$ signals at *m/z* 717, 687 and 513, and fragment ions at *m/z* 464, 434, 260 and 189. These signals indicated a mixture of trisaccharides and disaccharides with the structure HexNAc-Hex-Hex, DeoxyHex-HexNAc-Hex and HexNAc-Hex. When this fraction was injected into the GC-MS, only subfractions consistent with the three disaccharide products were revealed. The mass spectrum of peak 1 was characteristic (Lönngren and Svensson, 1974) of the disaccharide glycosyl alditol consisting of a HexNAc unit linked at position 3 of a hexose alditol unit. The fragments at *m/z* 260 and 228 indicated the presence of t-HexNAc. The fragments at *m/z* 296, 236 and 45, 46, 89, 90 and 133 indicated a terminal reducing 3-Hex. From the mass spectrum of peak 2, which showed the same signals as peak 1, the presence of two disaccharides HexNAc-(1→3)-Gal*p* was deduced in fraction B'. The alditol fragmentation of the third peak was similar to those described above except for the lack of an ion at *m/z* 133, diagnostic of 3-linked alditol, and the presence of a fragment at *m/z* 134, which was diagnostic of 4-linked alditol. Thus the presence of a disaccharide HexNAc-(1→4)-Gal*p* was also deduced.

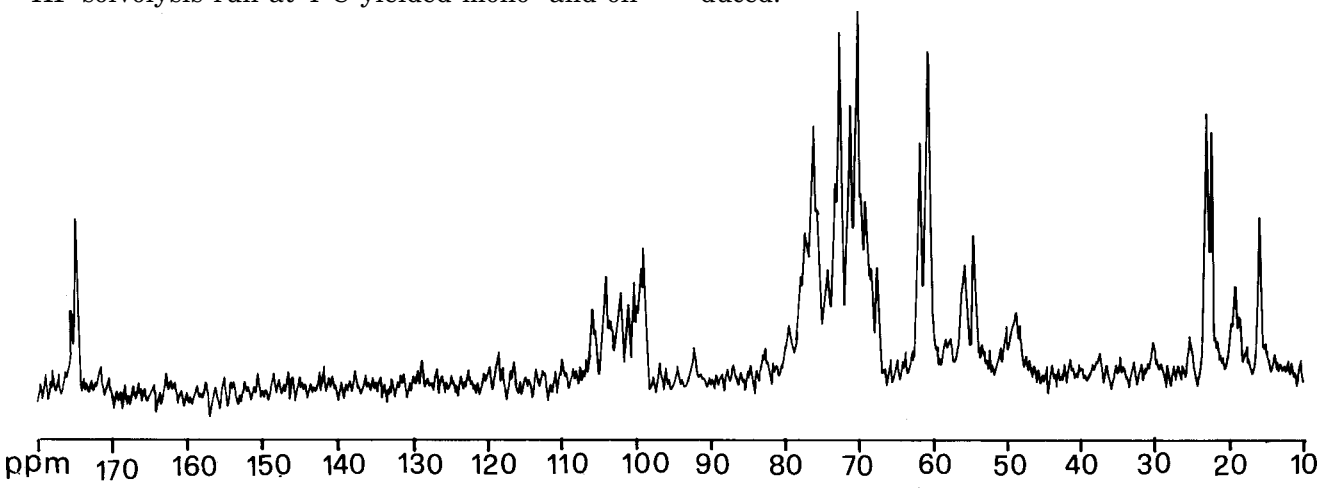

Fig. 3. ^{13}C-NMR spectrum of SM1 in D_2O at 62.5 MHz.

^{1}H NMR analysis of fraction B' showed three main anomeric signals at δ 5.10 (3.6 Hz), 5.03 (3.6 Hz) and 4.58 (7.5 Hz), which were correlated with carbon signals at δ 97.9, 98.5 and δ 102.3 and, by a COSY spectrum, with three H-2 signals. These H-2 signals were in turn correlated with carbon signals at 49.6 (α-Gal*p*NAc), 53.7 (α-Glc*p*NAc) and 55.5 (β-Glc*p*NAc) ppm respectively. These data, together with the MS data, and on the basis of a comparison with NMR data of strictly related oligosaccharide structures (Bradbury and Jenkins, 1984; Hounsell *et al.*, 1988; Strecker *et al.*, 1995) suggested the presence in the mixture of the following alditol disaccharides: α-D-Gal*p*NAc-(1→3)-D-Gal-ol, β-D-Glc*p*NAc-(1→3)-D-Gal-ol and α-D-Glc*p*NAc-(1→4)-D-Gal-ol.

Peak C' contained, in addition to the same compounds as in fraction B', the trisaccharide Fuc-Gal-Gal as shown by the pseudomolecular ion of its peracetylated derivative at *m*/*z* 977 $[M+Na]^+$ and by fragmentations at *m*/*z* 561 and 273. Lastly fraction A' appeared to be a high molecular mass fraction eluted in the void volume on Bio-Gel P-100.

On the basis of the above results the structures of the oligosaccharides listed in Fig. 4 were identified.

Biological activity

In a previous work it was found that EPSs produced by strains of *P. savastanoi* from olive (*P. savastanoi* pv. *savastanoi*) did not interfere significantly with knot induction by homologous strains on oleander (Cimino *et al.*, 1998b). In the present study, the biological activity of EPS from *P. savastanoi* strain ITM519 was tested on cultured cells of the non-host plant *Nicotiana tabacum*.

Treatment with EP did not induce electrolyte leakage in tobacco cells (data not shown), suggesting that EPS did not directly cause damage at plant plasmalemma level, at least in the first 20 hours of treatment. However, EPS in the culture medium seemed to be recognised by the tobacco cells and to trigger some of the metabolic changes that typically occur when a phytopathogenic micro-organism attacks a non-host plant. EPS thus induced a considerable change in PAL and APX activities. Since PAL is involved in phytoalexin biosynthesis, PAL levels are well known to increase in plant tissues responding to fungal elicitors or under phytopathogen attacks. EPS from *P. savastanoi* strain ITM519 induced in tobacco cells a three-fold increase in PAL activity compared with the control (Table 2). By contrast, APX, which is reported to be post-transcriptionally suppressed at the sites of phytopathogen infection during the hypersensitive reaction (Mittler *et al.*, 1998), has a 30% lower activity in cells treated with EPS than in control cells (Table 2). Although the changes induced by EPS in cell metabolism indicate that tobacco cells recognise the EPS molecule (or part of it) as coming from a micro-organism against which they must activate their defences, EPS alone did not induce

Table 2. Changes in phenil alanine ammonia-lyase (PAL) and ascorbate peroxidase (APX) activity induced by EPS.

Treatment	PAL[a]	APX[b]
Control	19 ± 2	1085 ± 56
EPS 0.05 mg/ml	56 ± 9 **	798 ± 31*

Results are the means of 5 independent experiments ± SD.
[a] Nanomoles *trans*-cinnamic acid produced min^{-1} mg $proteins^{-1}$.
[b] Nanomoles ascorbate oxidised min^{-1} mg $proteins^{-1}$.
* and ** indicate values that differ significantly (P>0.05 and 0.01) from controls by Student's T test.

α-L-Fuc*p*-D-Hex*p*NAc-D-Gal,
α-L-Fuc*p*-β-D-Gal*p*-D-Gal,
D-Hex*p*NAc-β-D-Gal*p*-D-Gal,
α-D-Gal*p*NAc-(1→3)-D-Gal,
α- D-Glc*p*NAc-(1→4)- D-Gal,
β- D-Glc*p*NAc-(1→3)- D-Gal.

Fig. 4. Oligosaccharide structures.

a typical hypersensitive reaction, since its infiltration into tobacco leaves induced necrotic symptoms only after 4–5 days (Lavermicocca P., and De Gara L., personal communication).

Conclusions

In conclusion, although the complexity of the *P. savastanoi* EPS fraction prevented us from obtaining a complete arrangement, the structural features described above suggest a very interesting polysaccharide structure. Also, the fact that induces a defence response in non-host plant cells is an interesting new finding that may be due to the complex and heterogeneous structure of this molecule, all or part of which could be recognised as alien at plant-surface level, or mimic elicitors produced in the plant–pathogen interaction. Further studies are in progress to ascertain whether part of the molecule is recognised by plant cells, or whether, in the interaction between the EPS and the plant cell wall, new molecules triggering biological activity are produced, as has already been reported for plant elicitors of a saccharide nature (Darvill and Albersheim, 1984).

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