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Extracellular polysaccharide from *Ralstonia solanacearum*; A strong inducer of eggplant defense against bacterial wilt

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ABSTRACT

Ralstonia solanacearum a phytopathogenic bacterium produces virulence factor via production of extracellular polysaccharides (EPS). We have isolated EPS from *R. solanacearum* and characterized this by AFM, SEM, FTIR, LC-MS and NMR spectroscopy. Interestingly, the result showed pore size distribution, topology, rheological and pseudoplastic behaviour of EPS and three major monosaccharides, glucuronic acid, *N*-acetylglucosamine and glucose associated with EPS. Eggplant treated with intact crude EPS showed a significant 51% decrease in bacterial wilt incidence for both *in vitro* and greenhouse conditions. In eggplant cultivars EPS induces significant up-regulation of guaiacol peroxidase and ascorbate peroxidase defense genes, providing screen resistance against bacterial wilt.

1. Introduction

Microorganisms can infect both plants and human beings; however these differ in defense mechanisms, pathogenesis, virulence and antimicrobial resistance. Biofilm formation is a particularly common characteristic among microorganisms (Bales et al., 2013). The biofilms matrix is often composed of EPS, metal ions, nucleic acids, proteins and humic substances that helps bacterial adhesion, communication and protects microbes from environmental stress (Yildiz et al., 2014). Specifically, EPS plays a crucial role as an extracellular polymeric substance which serves as cement for adherence of individual bacterial cells. Composition of EPS has been differentiated into homo- and hetero-polysaccharides in bacterial cells. Hetero-polysaccharides are recognised as the major EPS component, having several monosaccharide associated with lactic, succinic, acetic and pyruvic acids, which can complicate the structural analysis of EPS (Maalej et al., 2014). In the last decade, EPS has been utilized by industry for sustainable production of fuel, emulsifiers, immune stimulating agents, anticoagulants and food ingredients (Maalej et al., 2014; Siddiqui et al., 2014). In addition, EPS isolated from microorganisms has been used as a potential bioactive agent for human and plant care (Fu et al., 2011; Shibuya and Minami, 2001).

Oligogalacturonides involvement in the induction of biosynthesis of phytoalexins in the cotyledons of soybean, acting as proteinase inhibitors in tomato leaves, inducing lignifications of cucumber cotyledons and activating resistance in cultured castor bean cells, have been reviewed (Shibuya and Minami, 2001). Bacterial plant pathogens consist of EPS and lipopolysaccharides, which are major virulence factors that increase disease severity in host organisms (Loucks et al., 2013; Orgambide et al., 1991; Park et al., 2008; Valepyn et al., 2014). EPS are also known to be pathogenic markers, which can act as elicitors of induced resistance thereby suppressing plant disease (de Pinto et al., 2003). The role of EPS in pathogenesis has been studied in many phytopathogens. For example a hetero-polymer (fucose (Fuc), galactose (Gal), *N*-acetylgalactosamine (Gal*N*Ac), *N*-acetylglucosamine (Glc*N*Ac)) produced by *Pseudomonas syringae* pv. *ciccaronei, P. savastanoi* pv. *neri* and *P. caryophylli* triggered a hypersensitivity response in *Nicotiana tabacum*. EPS extracted from *Pantoea agglomerans* (Ortmann et al., 2006) and *Xanthomonas campestris* pv. *vesicatoria* (Romeiro and Kimura, 1997) induced phytoalexin, a reactive oxygen species in plants. β-(1–4)-D-Glucuronic acid extracted from *Syncephalastrum racemosum* fungal pathogens reportedly activated the defense system in *A. thaliana*

Abbreviations: ANOVA, analysis of variance; APX, ascorbate peroxidase; EPS, extracellular polysaccharides; Gal*N*Ac, *N*-acetylgalactosamine; Glc*N*Ac, *N*-acetylglucosamine; H_2O_2, hydrogen peroxide; HPI, hours after pathogen inoculation; HS, highly susceptible; R, resistance; ROS, reactive oxygen species

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(Valepyn et al., 2014). β-(1–3)-Glucan and chitosan oligosaccharides have been recognized as elicitors of plant resistance against diseases which have enlivened the hopes of a green bio-pesticide (Fu et al., 2011).

Ralstonia solanacearum is a Gram-negative soil-borne phytobacterium which is known to secrete EPS. *R. solanacearum* is now known to be responsible for wilt disease in ~450 plant species, representing more than 50 families of botanical flora. However, the specific virulence factor in EPS inducing bacterial wilt is unknown. It has been hypothesized that EPS directly blocks the water flow in the xylem vessels that are densely-colonized with the help of biofilms. The EPS gene is highly conserved across diverse *R. solanacearum* pathogens. Interestingly, mutant EPS strains are unable to colonize in plant vessels and act as avirulent pathogens to bacterial wilt (Milling et al., 2011). In our previous reports we targeted abiotic-inducers which provide strong induction of disease-resistance in tomato against *R. solanacearum* and *X. perforans*. Breeding of resistant cultivars is a long-term task and expensive. An alternate might be to use suitable management strategies employing EPS which can induce host resistance to wilt disease, acting as an elicitor of virulence defense.

Structural elucidation of EPS has been reported in the GM1000 strain of *Pseudomonas solanacearum (R. solanacearum)*, whereby Gal*N*Ac, 2-*N*-acetyl-2-deoxy-galacturonic acid and 2-*N*-acetyl-4-*N*-(3-hydroxybutanoyl)-2,4,6-tri-deoxy-D-glucose were identified as constituent sugars (Orgambide et al., 1991). Similarly, EPS characterization in *Pseudomonas aeruginosa* reported constituent sugar residues of D-mannuronic and L-guluronic acid in the EPS (Govan and Deretic, 1996). Recently, the intact EPS was hydrolysed in hydrochloric acid to degrade the polysaccharides into its constituent sugars, whereby Gal*N*Ac and galacturonic acid were identified in the EPS of *Enterococcus faecalis, Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species (Bales et al., 2013). Few reports are available on EPS secreted by *R. solanacearum* where it acts as a defense elicitor against bacterial wilt in tomato (Milling et al., 2011; Orgambide et al., 1991; Schacht et al., 2011). An exhaustive literature search found a report on the NMR characterization of EPS of *R. solanacearum* (Orgambide et al., 1991) however, no reports exist on LC-MS, FTIR and HPLC, characterization of EPS from *R. solanacearum* in eggplant. Hence, the primary objective of our study is to investigate the structural characterization, surface topology and rheological properties of EPS from *R. solanacearum* (DOB R1) isolate. Further, to also evaluate the competence of EPS mediated resistance in eggplant against bacterial wilt disease.

2. Materials and methods

2.1. Bacterial source and growth conditions

Ralstonia solanacearum was isolated from a soil source in the agricultural fields of Karnataka state, India. The bacteria were isolated and maintained on a semi-selective Kelman's TTC medium (Kelman, 1954). The isolated pathogen was confirmed by biochemical/physiological, pathogenicity tests and molecular identification methods were performed by PCR amplification using both 16S rRNA and specific primers (*speI* and *egl*) (Avinash and Umesha, 2013; Umesha and Avinash, 2014). Amplified PCR products were purified using QIAquick gel extraction kit (Qiagen, Hilden, Germany) following the manufacturer's instructions and sequenced commercially (Eurofins, Bangalore, India) and sequences were compared with other *R. solanacearum* sequence from data base using multiple sequence alignment software. Sequences of both 16S rRNA and specific primers were deposited in the GenBank database. The glycerol stocks of bacterial cultures were prepared and submitted to the Stock Collection Centre (Department of Studies in Biotechnology, University of Mysore, Mysore, India) and were used for all further experiments. Bacteria were grown in fresh TZC broth (Kelman, 1954) by providing a large surface to afford biofilms without shaking the bacteria culture for 4 days at 28 °C, with appropriate control and used for further extraction of EPS from biofilms.

2.2. Isolation of extracellular polysaccharide (EPS)

EPS was extracted from *R. solanacearum* following the protocol of Milling et al. (2011) with slight modification. The developed biofilms were filter sterilized and centrifuged at 10,000 rpm for 10 min. The cell free supernatant was lyophilized for 10 days. The EPS filtrate was precipitated over night with water and acetone (1:4; v/v) at −20 °C. The precipitate was re-dissolved in 20 mM NaCl, further treated with DNaseI in a final concentration of 0.05 mg/mL and incubated at 37 °C for 1 h to inhibit DNA-associated with EPS. The EPS were extracted initially with phenol and further extracted successively with chloroform, until there was no interphase visible. The yellow mucoid upper layer was successively dialysed in distilled water. The crude EPS was precipitated from dialysate with 3 volumes of ethanol at −20 °C overnight and centrifuged at 12,000 rpm for 15 min. Cross contamination of protein in crude EPS were determined by the Bradford assay, using BSA as a standard (Sigma, Bangalore, India). Nucleic acid contamination was analysed by spectrophotometry using Nanodrop 2000/2000 (1 μL from 100 mg/mL) and further confirmed by 1% agarose gel electrophoresis with ethidium bromide staining (50 μL was loaded from 100 mg/mL). The EPS concentration was determined by the Elson-Morgan assay, using known different concentrations of Glc*N*Ac as a standard. The crude EPS was dried and yield was determined for 1 L of culture medium and stored at −80 °C for further purification and structural characterization.

2.3. Scanning electron microscopy

Microstructure and surface morphology of the freeze-dried crude EPS were determined using Scanning Electron Microscopy (SEM). A piece of dried polymer sample (EPS) was attached to the stub of double sided tape, and then coated with a layer of gold particle. The coated samples were analysed using a Carl Zeiss scanning electron microscope (EVOLS 15, Germany) operated at different accelerated voltages (15.0, 20.0 and 25.0 kV) and the images of EPS were observed using different magnification power of SEM.

2.4. Atomic force microscopy

A stock solution (1 mg/mL) was prepared by adding crude EPS to HPLC grade water. The aqueous EPS solution was diluted to the final concentration of 0.1 mg/mL and about 10 μL of diluted EPS solution was added to freshly cleaved mica surface and dried at room temperature. Atomic force microscopic images were visualized under ambient conditions using an A.P.E. Research System (A-100, Basovizza, Italy) operating with a tapping mode regime.

2.5. Analytical methods

2.5.1. Fourier transform infrared (FTIR) spectroscopy

Infrared spectra was recorded with a FTIR spectrometer (Perkin Elmer, System two 94012, Waltham, MA, USA) with direct infusion of 10 mg sample of isolated crude EPS. The spectrum was scanned in the range of 500–4000 cm^{-1} (Siddiqui et al., 2014).

2.5.2. HPLC and LC-MS analysis

The crude EPS was fractionated on a Sephadex® G-100 column (mesh size 200–270 Å). EPS sample (100 mg/mL) was loaded on to the column (5 cm × 20 cm) and eluted with 0.1 M sodium acetate buffer (pH 4.5) at flow rate 15 mL/h. EPS fractions of 10 mL were collected and analysed for carbohydrates using the Elson-Morgan assay. The monomer composition of the EPS was determined by partial acid hydrolysis in 1 M TFA (Capek et al., 2011). The hydrolyzed mixture