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Biochemical Characterization of Lipopolysaccharide of *Ralstonia solanacearum* and its Relation to Virulence

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This work was planned to study biochemical characters for *R. solanacearum* lipopolysaccharide (LPS) and its relation to virulence. All tuber isolates were highly virulence compared with soil and water isolates. These isolates were divided into two groups according to disease incidence and disease severity; the first group contained virulent isolates which isolated from tuber samples and the second group contained avirulent isolates which isolated from soil and water samples. Virulent isolates showed high content of either lipopolysaccharide or total sugars in LPS compared with avirulent isolates. Total sugars in LPS were determined using gas liquid chromatography (GLC). The glucose was the major content of *R. solanacearum* lipopolysaccharide in all examined isolates. Lipopolysaccharide for virulent isolates of *R. solanacearum* showed high amount of arabinose, xylose, mannose and galactose compared with the avirulent isolates, while the amount of mannitol was the highest in Lipopolysaccharide for avirulent isolates compared with virulent isolates. There is no significant difference for the amount of rhamnose, fructose and sorbitol in lipopolysaccharide for all isolates.

Keywords: Potato, *Solanum tuberosum*, bacterial wilt, *Ralstonia solanacearum*, Virulent, Avirulent isolates, monosaccharide, Lipopolysaccharide.

Ralstonia solanacearum (formerly *Burkholderia* or *Pseudomonas solanacearum*) Yabuuchi *et al*., (1995) is the causative agent of bacterial wilt, which affects numerous host plants of economic importance worldwide (Kelman, 1953; Buddenhagen and Kelman, 1964 and Hayward, 1964). The bacterium is found worldwide, mainly in tropical and subtropical areas, but also in warm-temperate countries and even in some cool-temperate regions (Hayward *et al*, 1998). Strains of *R. solanacearum* are differentiated into five races according to host range and six biovars according to the ability to oxidize three disaccharides and three hexose alcohols (Buddenhagen and Kelman, 1964; Hayward, 1964 and Williamson et al., 2002).

The lipopolysaccharide (LPS) protects the bacterial cell from hostile environments and in pathogenic bacteria, they represent important virulence factors through their direct interaction with eukaryotic host cells. Mutants of phytopathogenic bacterial strains with defects in their LPS biosynthesis, frequently

show reduced virulence and the number of viable bacteria in plant tissues declines rapidly (Esposito *et al.*, 2008). LPS are ubiquitous, indispensable components of the cell surface of gram-negative bacteria and their taxonomy that apparently have diverse roles in bacterial pathogensis of plant (Erbs and Newman, 2003 and Varbanets *et al.*, 2004). Abundant Exopolysaccharide (EPS) produced by pathogenic strains of *Ralstonia solanacearum* on solid medium and *in planta* has long been correlated with its pathogenicity (Araud-Razou *et al*., 1998 and Esposito *et al.*, 2008).

The aim of this work was to study the biochemical characters of the lipopolysaccharide (LPS) produced by *R. solanacearum* isolates in relation to its virulence.

Materials and Methods

Bacterial isolates:

Ten isolates of *R. solanacearum* (TD14, TG6, TK8, TM7, TK16, SG1, SK3, SM4, WK2 and WM3) were isolated from different samples (potato tubers, soil, and water) collected from Dakahlia, Gharbiya, Kalubiya and Menoufiya governorates. One isolate (Rs) was obtained from potato brown rot project, Dokki, Giza, Egypt and used as a reference isolate. These isolates were identified using traditional and standard tests according to Dhital *et al.*, 2001.

Plant growth and pathogen inoculation:

Healthy potato tubers (Nicola, cv.) were obtained from Potato Brown Rot Project, Dokki, Giza, Egypt. It was stored at 4°C for 20 days and placed at room temperature to stimulate germination. Germinated tubers were planted in clay pots (25cm, diameter) containing sterilized soil-sand mixture (1:1, v:v) under greenhouse conditions. Plants were allowed to grow to a height of 20-25 cm and inoculated with *Ralstonia solanacearum* isolates, using soil drench method.

The bacterial isolates were streaked on triphenyl tetrazolium chloride agar medium (TTC) and incubated at 28°C for 48h. Bacterial cells were suspended with sterile distilled water (SDW) and adjusted to A600 = 0.1 (about 10^8 cells/ ml). Bacterial suspension was poured around the base of each plant, and an alcohol-flamed knife was inserted 4-5 cm into the soil to cut the root along one side (Kempe and Sequeira, 1983 and Adhikari, 1993).

Disease assessment:

Disease incidence (DI) was recorded after 60 days from inoculation with *R. solanacearum* isolates. The disease was estimated as percentage of wilted shoots (No. of wilted shoots/total number of shoots × 100) and disease severity according to 0-4 rating scale, where 0= no symptoms, 1= up to 25% of the foliage wilted, 2= 25-50% of the foliage wilted, 3= 50-75% of the foliage wilted and 4= 75-100% of the foliage wilted (Kempe and Sequeira, 1983). Percentage of disease severity (DS) was calculated as following:

$$\mathbf{DS= (\Sigma\ R.T\ /\ N \times 4) \times 100}$$

T= Number of plants per degree rating (R 0-4)

N= Total number of tested plants

Also, mean disease rating was calculated and used to determine virulence of isolates.

Extraction of R. solanacearum lipopolysaccharide (LPS):

Isolates of *R. solanacearum* were grown at 28°C under shaking (240 rpm) in 500 ml flasks filled with 200 ml of a synthetic medium contained, in grams per liter: ($MgSO_4$.7H20, 0.2; KH_2PO_4, 3.0; Na_2HPO_4, 6.0, and glucose, 5.0). Approximately 0.1 ml of bacterial suspension (10^8 cfu/ml) was inoculated into the liquid medium and incubated for 48h at 28°C. Cells were centrifuged at 8000g at 4°C for 30 min. The wet cells were suspended with acetone and diethyl ether (Varbanets *et al*., 2004), and weighed sample of cell was extracted three times at 68°C with a 45% solution of phenol. After each extraction, the sample was cooled to 10 °C and centrifuged at 1,000 g for 45 min. The water phases from the three extractions were combined and washed three times with ether to remove phenol. Crude LPS was sedimented by ultracentrifugation (Beckman ModelL.2) at 100,000 g for 3 h at 4°C (Westphal and Jann, 1965).

Phenol sulfuric method was used to determine total sugars in LPS. A known weight of lipopolysaccharide was dissolved in one ml of 0.05N HCl in a screw capped test tube. The tube was then heated in a water bath at 70˚C for 30min. Total sugars in this LPS solution was determined as described by Dubois *et al*. (1956) as follows: 0.5 ml of the LPS solution was added in a clean test tube followed by 0.5 ml of 5% phenol and shaked well. Concentrated sulfuric acid (2.5 ml) was slowly added at the wall of the test tube. The tubes shaked well and allowed to cool. The absorbance was measured at 490nm against a blank tube. Standard curve was made from glucose in a concentration range from 20µg to 80µg/ ml.

Analysis of R. solanacearum lipopolysaccharide (LPS):

A known weight of the lipopolysaccharide (about 10 mg) was added in a screw-capped test tube. Two ml of 1N HCl were added, and the solution was hydrolyzed at 100°C for 6h in an oven. The solution was evaporated to dryness at 40°C under stream of nitrogen. After that, 0.5 ml of isopropanol (HPLC grade) was added to remove any residue of water, shaked gently, and evaporated to dryness under a stream of nitrogen at 40°C. Approximately 250 µl of oximation solution (2.5 % of hydroxylamine hydrochloride in anhydrous pyridine) was added and put in an oven at 80°C for 30 min. After cooling, 0.5 ml of sialylation reagent (trimethylchlorosilane: N,N-O bis-(trimethylsilyl) acetamide, 1:5 v:v) was added and put in an oven at 8°C for 30 min (Kirk and Sawyer 1991). Two µl of this final product was injected in gas liquid chromatography (GLC) instrument (Hewlett-Packard 6890) on a capillary column of HP-l stationary phase. The absolute configurations of the monosaccharides were determined by GLC of the acetylate glycoside with (-)-2-octahol.

Results and Discussion

Determination of virulence of Ralstonia solanacearum isolates:

Ten isolates of *R. solanacearum* were examined to determine their virulence on potato plants, using soil drench method. These isolates were obtained from tuber (TD14, TG6, TK8, TM7 and TK16), soil (SG1, SK3 and SM4) and water (WK2 and WM3) samples. The results shown in Fig. (1) indicate that all tuber isolates were highly virulent compared with soil and water isolates. Isolates from the soil and irrigation water didn't cause apparent infection and/or symptoms on potato plants. Therefore, these isolates were divided into two groups according to disease incidence. The first group contained virulent isolates (TD14, TG6, TK8, TM7 and TK16) which isolated from tuber samples where the disease incidence ranged from 71.9-75.6% with disease severity of 62.7-66.8%. The second group contained avirulent isolates (SG1, SK3, SM4, WK2 and WM3) which were isolated from soil and water samples, where no disease incidence was recorded.

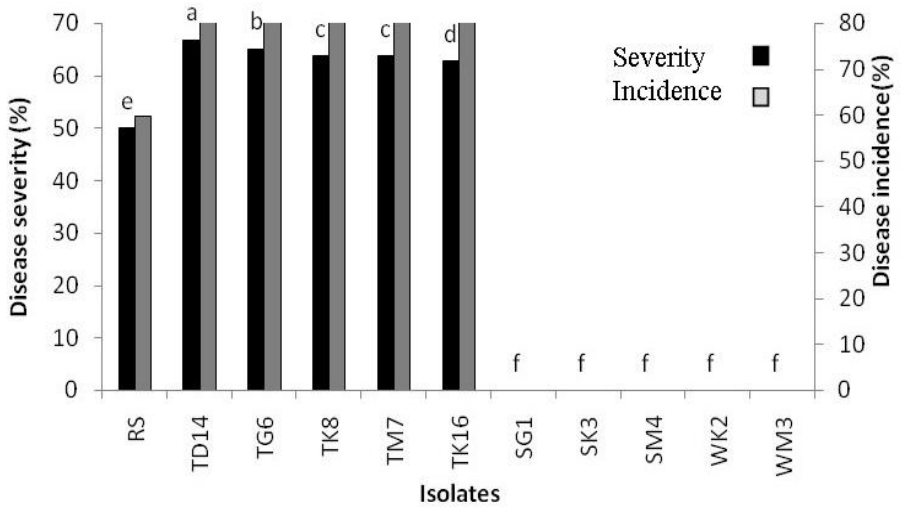

Fig (1): Determination of virulent and avirulent isolates of *R. solanacearum* according to disease incidence and severity, using soil drench method, under artificial inoculation conditions.

Biochemical analysis of R. solanacearum lipopolysaccharide (LPS):

Data in Fig. (2) indicate that all the virulent isolates could produce high amount of lipopolysaccharide compared with the avirulent ones. Percentage of LPS ranged from 6.64 to 9.64% for the virulent isolates and 0.61 to 2.71% for the avirulent isolates. TD14, TK16 and TM7 Isolates from tubers showed the highest content of LPS, where percentage of LPS was 9.64, 8.70 and 8.28% respectively. Meantime, WM3, SM4 and SG1 isolates from water and soil showed the lowest content of LPS, where percentage of LPS was 0.61, 1.52 and 1.85% respectively.

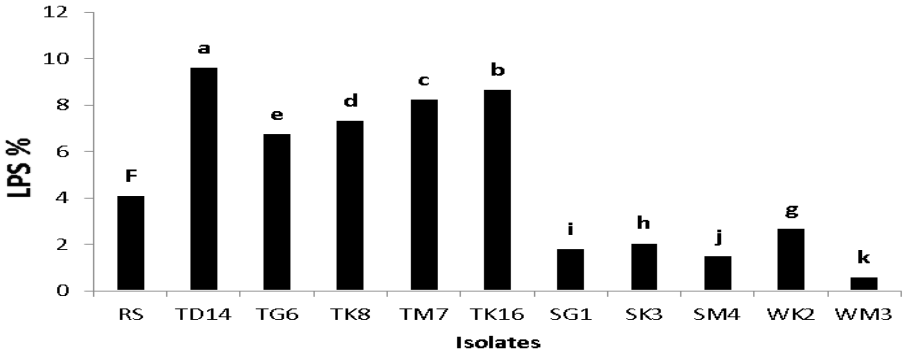

Fig. (2): Percentage of lipopolysaccharide (LPS) in isolates of *Ralstonia solanacearum obtained* from different sources (tuber, soil, irrigation water) (Columns followed by the different letter are significantly different at P= 0.05 according to Duncan's multiple range test) and 0.0-1.88%, respectively.

Virulent isolates (TD14, TG6, TK8, TM7 and TK16) appeared high content of sugar compared with avirulent isolates (SG1, SK3, SM4, WK2 and WM3), where percentage of sugar was 6.41-13.03% of LPS for virulent isolates and 1.3-3.3% of LPS for avirulent isolates. Isolate of TK16 had the highest content of sugar, where percentage of sugar was13.02% of LPS, but isolates of TD14, TG6, TM7 and TK8 had average amount of sugar, where percentage of sugar was 7.84, 7.18, 6.67 and 6.41% of LPS, respectively. Meanwhile, isolates of SK3, SM4, WK2, WM3 and SG1 had the least content of LPS, where percentage of sugar was 1.36,2.48,2.93,3.11 and 3.30% of LPS, respectively (Fig.3).

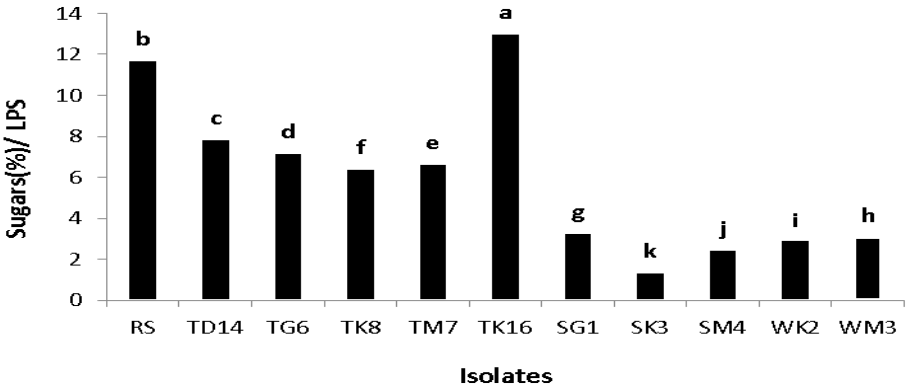

Fig. (3): Percentage of sugar content in lipopolysaccharide (LPS) for different virulent and avirulent isolates of *Ralstonia solanacearum (*Columns followed by the different letter are significantly different at P= 0.05 according to Duncan's multiple range test).

Data shown in Table (1) prove that glucose was the major content of *R. solanacearum* lipopolysaccharide, where its percentage was 66.3-90.85%. However, percentage of arabinose, rhamnose, mannose and galactose were 1.03-7.89, 2.27-

5.33, 1.03-8.62 and 1.00-9.09 % of lipopolysaccharide, respectively. Meanwhile, xylose, sorbitol and fructose were less frequent in *R. solanacearum* lipopolysaccharide, where their percentages were 0.0-3.05, 0.0-1.39, 0.0-3.31. (Columns followed by the different letter are significantly different at P= 0.05 according to Duncan's multiple range test).

However, arabinose, xylose, rhamnose, mannose and galactose were the major components in lipopolysaccharide of virulent isolates (TD14, TG6, TK8, TM7 and TK16). On the other hand, mannitol was an exclusive component in lipopolysaccharide of the avirulent isolates than the virulent ones (Table,1).

Table (1): Sugar analysis in lipopolysaccharides for *Ralstonia solanacearum* isolates, using Gas chromatography.

Isolate	Sugars								
	Arabinose	Xylose	Rhamnose	Sorbitol	Mannitol	Fructose	Mannose	Galactose	Glucose
RS	4.66	3.65	3.65	4.56	0.00	0.00	12.16	12.66	58.66
TD14	7.89	2.72	5.33	1.39	0.00	1.66	3.23	2.87	74.92
TG6	6.58	2.51	5.02	0.00	0.00	1.88	8.62	9.09	66.30
TM7	3.43	3.05	3.86	0.00	0.91	0.64	5.73	2.78	79.60
TK16	4.38	2.38	4.29	0.00	0.87	0.38	2.43	2.29	82.98
SG1	1.30	0.70	2.87	0.00	1.48	1.04	4.70	1.39	86.52
SK3	2.37	0.00	2.93	0.34	2.44	0.00	2.34	1.95	87.63
SM4	1.03	0.00	2.27	0.00	3.31	0.27	1.03	1.24	90.85
WK2	2.53	0.00	3.32	0.00	3.00	0.26	3.83	1.95	85.12
WM3	1.29	0.00	4.58	0.00	3.17	0.00	3.60	1.00	86.35

It is well known that *R. solanacearum* produces a variety of extracellular products that contribute to its ability to colonize host plants and cause disease symptoms. One of the most important of these is an acidic, high molecular mass extracellular polysaccharide (EPS1) (Genin and Bocher, 2002). Denny and Baek (1991) and Kao *et al*. (1992) suggested that EPS1 is the cause of wilting in infected plants, as it blocks the vascular system and thereby alters water movement. Although none of these EPS1-eficient mutants was totally non-pathogenic due to that these mutants poorly colonize the stem of infected plants suggest that EPS1 may contribute to minimizing or avoiding the recognition of bacterial surface structures by plant defense mechanisms (Aroud-Razou *et al.,* 1998 and Saile *et al*., 1997).

Our results agree with several authors who indicated that mutant of *R. solanacearum*, with defects in their LPS biosynthesis, frequently show reduced virulence (Esposito *et al.,* 2008). It is known from early reports that an absolute

correlation between EPS production and virulence in *R. solanacearum* is questionable. The results of the present study demonstrate that variation in most external polysaccharides is correlated with pathogen virulence.

In rich medium the wild type of *R. solanacearum* produces three EPSs (Orgambide *et al*.,1991 and Trigalet-Demery *et al*., 1993). The high-molecular-weight acidic polymer EPS1, which account for up to 90% of the dry weight of the EPSs and two minor polymers, namely a N-acetyl-glucosaminorhamnan (EPS3) and a glucan (EPS4). The structure of the trisaccharidic repeating unit of EPS1, composed of N-acetyl-galactoseamine, N-acetyl-galactoseaminuronic acid and

bacillosamine derivative in an equimolar ratio has been established (Orgambide *et al*.,1991). The EPS produced *in planta* by *R. solanacearum* increases the viscosity of the sap and may thus block the vascular stream within xylem vessels (Husain and Kelman, 1958). Microscopic studies confirmed the accumulation of large amounts of bacterial EPS within infected vessels (Wallis and Truter, 1978 and Aroud-Razou *et al*., 1998) and demonstrated that spontaneous EPS-deficient mutant strain of *R. solanacearum* was unable to invade pro-toxylem vessels (Vasse *et al*.,1995).

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التوصيف البيوكيماوى لـ Lipopolysaccharide فى خلايا بكتيريا *Ralstonia solanacearum* وعلاقته بالشدة الإمراضية

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يهدف هذا البحث إلى دراسة الكمية والتركيب الكيماوى لـ Lipopolysaccharide لخلايا بكتريا *Ralstonia solanacearum* وعلاقته بشدتها المراضية. كانت جميع العزلات المتحصل عليها من الدرنات أعلى فى شدتها المراضية مقارنة مع تلك المعزولة من التربة ومياه الرى. قُسمت هذه العزلات إلى مجموعتين حسب معدل الإصابة وشدة المرض. احتوت المجموعة الأولى على عزلات ممرضة معزولة من عينات الدرنات والمجموعة الثانية احتوت على عزلات عديمة القدرة المرضيه معزولة من عينات التربة والمياه. تم تقدير السكريات الكلية المكونة لـ Lipopolysaccharide للعزلات المختلفة ووجد أن نسبتها فى العزلات شديدة القدرة ألمرضية أعلى منها فى العزلات الفاقدة لشدتها الإمراضية. تم تحديد السكريات الكلية في الـ Lipopolysaccharide باستخدام التحليل الكروماتوجرافى (Gas Liquid Chromatography). وجد أن سكر الجليكوز هو المكون الاساسي لـ Lipopolysaccharide لكل عزلات *R. solanacearum* المختبرة. وجد في الـ Lipopolysaccharide أن نسبة كل من سكر الأرابينوز ، الزيلوز ، المانوز ،و الالجليكوز كانت أعلى في حالة عزلات *R. solanacearum* الشديدة المرضية عنها في العزلات الفاقدة لشدتها المرضية. وعلى العكس، كانت نسبة المانيتول في الـ Lipopolysaccharide فى العزلات الشديدة الإمراضية أقل منها فى العزلات الفاقدة لشدتها الإمراضية. بينما لا يوجد فرق معنوي لكمية سكر الرامنوز ، الفراكتوز أوالسوربيتول في الـ Lipopolysaccharide لجميع العزلات.