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Research Article

Polysaccharide Profiling of *Xanthomonas axonopodis*pv. *punicae* Causing Bacterial Blight of Pomegranate

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ABSTRACT

Lipopolysaccharides (LPS) of Xanthomonas axonopodispv. punicae constitute one of the main component of the outer membrane of thisbacteria which play an essential role for bacterial growth and survival.Similarly, the capacity of this bacteria to induce persistentwater-soaking in leaves plays a crucial role during pathogenesis that seems to be accomplished by asynergistic interaction between bacterial Exopolysaccharides(EPS) and plant polymers. We have studiedLipopolysaccharides (LPSs)and Exopolysaccharides(EPS)profiling of 23 different isolates of Xanthomonas axonopodispv. punicae in present investigation.Exopolysaccharides profiling of isolates showed that the amount of exopolysaccharides in the wall of different X. axonopodis pv. punicae isolates varied from 98.94 to 183.90 µg/mg. Higher exopolysaccharides was found in isolate KD-11 whereas lowest exopolysaccharides containing isolate was GBV-21.Whereas, Lipopolysaccharide profiling of these isolates showed that isolate KD-11 contained higher amount of lipopolysaccharides i.e. 139.90 µg/mg. while, less amount of lipopolysaccharides was observed in isolate RH-22 i.e. 48.86 µg/mg, others isolates contained varied amount of LPS.

Key words: *Xanthomonas axonopodispv. punicae, Bacterial blight, Exopolysaccharides, Lipopolysaccharide, Pomegranate.*

INTRODUCTION

Pomegranate (*Punica granatam* L.) an ancient fruit, is a native of Iran, where it was cultivated in about 2000 BC. At the global level, Iran is the world's largest producer and exporter of pomegranates with an estimated annual production of 670,000 tons besides Iran, other countries like India, Turkey, Spain, Tunisia, Morocco, Afghanistan, China, Greece, Japan, France, Armenia, Cyprus, Egypt, Italy and Palestine also cultivate this crop. India occupies an area of about 107.00 thousand ha under pomegranate and production is around 743.00 thousand tons. Bacterial blight (also known as oily spot) caused by *Xanthomonas axonopodis* pv. *punicae* has become increasingly serious threat for Pomegranate growers of India.

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Bacterial exopolysaccharides (EPS) play a major role in the induction of susceptible water soaking reaction[1]. The capacity of these bacteria to induce persistent water-soaking in leaves plays a crucial role during pathogenesis that seems to be accomplished by a synergistic interaction between bacterial EPS and plant polymers. Similarly, Lipopolysaccharides (LPSs) constitute one of the main component of the outer membrane of almost all Gram negative bacteria where they play an essential role for bacterial growth and survival. In particular, their external location enables them to communicate with the environment which, in the case of plant bacteria, is mainly constituted by a plant host. The main roles played by LPSs in the bacteria–plant interaction are to provide a structural barrier to plant-derived antimicrobial compounds, plant recognition, plant adhesion, induction of defence-related responses and infection in plant. Considering importance of exopolysaccharide and lipopolysaccharide in pathogenesis, present study was conducted to know the amount of exopolysaccharide and lipopolysaccharide present in different isolates of *Xanthomonas axonopodis* pv. *punicae.*

MATERIAL AND METHODS

Collection, isolation and maintenance of bacterial strains

Infected plant parts showing typical symptoms of bacterial blight of pomegranate were collected from different geographical regions of India particularly Maharashtra, Karnataka, Tamil Nadu, Andhra Pradesh, Gujrat, Rajasthan and Madhya Pradesh. The collected samples from different localities were subjected for bacterial isolation by following the method given by Schaad[9]. Twenty three isolates of *Xanthomonas axonopodis* pv. *punicae* were isolated from these samples. Out of 23 isolates nine were from Maharashtra, six from Karnataka, two from Tamil Nadu, Andhra Pradesh, and Gujrat each, one from Rajasthan and Madhya Pradesh each. Isolation was done by employing the streak plate method using Yeast glucose chalk agar (YGCA) medium (Yeast extract: 5.0 g, Glucose: 5.0 g, Calcium carbonate: 20.0 g, Agar: 15.0 g, Distilled water: 1000 ml).A typical pale yellow, glistening colonies of the bacterium were purified and maintained on Master plates containing Nutrient sucrose agar (NSA) medium (Peptone: 5.0 g, Beef extract: 3.0 g, Sucrose: 20.0 g, Agar: 15.0 g, Distilled water: 1000 ml). Cultures so obtained were stored in the refrigerator at 5^0 C, which served as a stock culture for further studies.

Lipopolysaccharide (LPS) profiling of *Xanthomonas axonopodis*pv. *punicae*

Lipopolysaccharide profiling of *Xanthomonas axonopodis*pv. *punicae* was done according to method suggested by Neeraj *et al*[7].

Cell wall isolation

The cells of *X. axonopodis*pv. *punicae* inoculated in Nutrient sucrose broth flask and kept on incubator shaker at 200 rpm at 27^0 C ± 2^0 C for incubation upto 72 hrs. The cells harvested during by centrifuging the cultures at 5000 X g for 10 minutes at 4^0 C. The supernatant discarded and pellet washed three times with Tris HCI buffer (pH 7.2). The cells crushed in pre-chilled mortar pestle with glass powder in homogenization buffer (4.0 ml).

Sucrose	: 0.25 M
Tris- HCl pH (8.5)	: 50.0 ml
EDTA	: 10.0 mM
Mercaptoethanol	: 5.0 mM
MgSO4	: 10.0 mM
KCl	: 10.0 mM

The obtained slurry centrifuged at 500 rpm for 5 min at 4^0 C to remove glass particles. The supernatant was centrifuged at 6000 rpm for 20 minute at 4^0 C with 40 per cent sucrose cushion and the pellet thus obtained was used as crude cell wall fraction. The supernatant was concentrated by filtration against polyethylene glycol/ 1.0 M sucrose solution in dialysis bags.

Estimation of Lipopolysaccharides (LPS)

LPS of cell walls was measured in terms of total heptose and glycoprotein because heptose is major constituent of the LPS. For this, essentially the method of Osborne (1963) using D- glycero monogluco heptose as standard was followed. Wall fraction of X. *axonopodis* pv. *punicae* made in 0.5 ml of

distilled water was cooled in an ice bath. 2.25 ml of reagent (conc. H_2SO_4 6.0 volume and 1 volume D.W.) was slowly added into it and mixed by shaking in the cold. After 3 min tubes were transferred to a water bath at temperature of 60^0 C for another 10 min. After cooling 0.05 ml of 3 per cent solution of freshly prepared cystein HCl was added in to each sample. Heptose produced a purple colour with absorbance maxima at 545 nm. Exactly two hours after adding the cystein HCl, the samples were read against the blank at 545 and 505 nm. The difference between two absorbance values is linear function of the heptose concentration. The concentration of LPS was expressed µg/mg fresh weight of cell wall.

Exopolysaccharides (EPS) profiling of *Xanthomonas axonopodis*pv. *punicae*

The amount of EPS was determined according to the method followed by Damery and Alexander, The *X. axonopodis*pv. *punicae* grown in NS broth at 27^0 C keep on a gyrorotary shaker at 200 rpm. At the exponential phase of growth (after 96 hrs) the supernatant was concentrated at the 60^0 C, and mixed with two volumes of acetone in the cold and stored for overnight at 4^0 C. The polysaccharide removed from solution by centrifugation at 5000 X g for 30 minutes. The weight of polysaccharide was determined. The amount of EPS production represented as mg/100 ml culture.

RESULTS AND DISCUSSION

Isolation of the pathogen and maintenance of pure culture

The causal organism was isolated from the infected leaf, bark of the stem and fruit pericarp showing typical symptoms of bacterial blight. Isolation was done by employing the streak plate method using Yeast glucose chalk agar (YGCA) medium. Repeated isolation from the infected plant parts yielded well separated, typical, yellow, mucoid, colonies of bacterium on Yeast glucose chalk agar (YGCA) medium after 72 hours of incubation at 27 ± 2^0C. Colonies were purified and maintained on Master plates containing Nutrient sucrose agar (NSA) medium.The pathogen isolated from each location was designated according to name of region as presented in table 1.

Lipopolysaccharide (LPS) profiling of *Xanthomonas axonopodis*pv. *punicae*Lipopolysaccharide profiling of *X. axonopodis* pv. *punicae* was done according to method suggested by Neeraj *et al*[7]. The data presented in the Table 2 revealed that theamount of lipopolysaccharides in the wall of different *X. axonopodis*pv. *punicae* isolates varied from 46.86 to 139.9 µg/mg. Higher lipopolysaccharide content was found in isolate KD-11 whereas lowest lipopolysaccharide containing isolate was RH-22. The higher lipopolysaccharide containing isolate KD-11 was more virulent (produce water soaking reaction in 5 days) whereas less lipopolysaccharide containing isolate RH-22 was less virulent (produce watersoaking in 11 days). Lipopolysaccharides (LPSs) constitute one of the main component of the outer membrane of almost all Gram negative bacteria where they play an essential role for bacterial growth and survival. In particular, their external location enables them to communicate with the environment which, in the case of plant bacteria, is mainly constituted by a plant host. The main roles played by LPSs in the bacteria–plant interaction are to provide a structural barrier to plant-derived antimicrobial compounds, plant recognition, plant adhesion, induction of defence-related responses and infection in plant. Drigues *et al*.[5], showed that, composition of the *Pseudomonas solanacearum* lipolysaccharide (LPS) is similar to that described for the LPS of enterobacteria. Antonio Molinaro isolated the Lipopolysaccharide from *Xanthomonas hortorum*pv. *vitians*, prepared the lipid A and the polysaccharide moieties thereof, and characterized all preparations by compositional analysis. Main sugar components were rhamnose and 3-acetamido-3, 6-dideoxy-galactose which presumably furnish the O-specific poly-saccharide. Other sugars were mannose, glucose, 6-deoxygalactose (fructose), and galacturonic

acid and were core region constituents, and glucosamine, which builds up the carbohydrate backbone of lipid A. The LPS contains several phosphate groups, most of which were present in the core region. The main fatty acids in the lipid A were C10:0, 3-OH-C10:0 and 3-OH-C12:0. The latter is the only amide-linked fatty acid.

Exopolysaccharide (EPS) profiling of *Xanthomonas axonopodis*pv. *punicae*

The amount of EPS among different *X. axonopodis*pv. *punicae*was determined according to the method followed by Damery and Alexander[4]. Isolates were differed in their capability of the production of exopolysaccharides. The data presented in the Table 2 revealed that the amount of exopolysaccharides in the wall of different *X. axonopodis*pv. *punicae* isolates varied from 98.94 to 183.9 µg/mg. Higher exopolysaccharides was found in isolate KD-11 whereas lowest exopolysaccharides containing isolate was GBV-21.Rudolf *et al*., investigated bacterial exopolysaccharides (EPS) for their role as virulence factors of leaf spot diseases caused by pseudomonads and xanthomonads. The capacity of these bacteria to induce persistent water-soaking in leaves plays a crucial role during pathogenesis that seems to be accomplished by a synergistic interaction between bacterial EPS and plant polymers. The main EPS components were alginate and levan (*Pseudomonas*), xanthan (*Xanthomonas*), as well as lipopolysaccharides (LPS) and a small amount of proteins. LPS may be involved in specific interactions with plant polymers leading to agglutination and precipitation (incompatibility) or gel-formation (compatibility). Borkar and Verma[1] showed that exopoly-saccharide (EPS) of virulent *X. campestris*pv. *malvacearum* produced persistent water soaking on leaves of sensitive Acala-44 and 1- 10B cotton cultivars but not in leaves of the resistant cultivar 101-102B at minimum concentration of 10 mg EPS/ml (3 mg/inoculation point). Water soaking produced by EPS of the more virulent race 32 persisted for a longer period compared with that of less virulent race 8. The experiment showed that EPS of virulent races did not induce a necrotic reaction inducing factor. The water soaking inducing factors of the virulent race was nonspecific and produced persistent water soaking in tobacco, castor and cowpea, suggesting that, the production of EPS containing water soaking inducing factor was controlled in incompatible host bacterium interaction. Borkar and Verma[2] proved that the exopoly-saccharide (EPS) of avirulent *X. campestris*pv. *malvacearum* race-32 did not contain the watersoaking (WS)-inducing factor but contained necrotic reaction (NR)-inducing factor and induced NR on resistant cotton (cv. 101-102B) on which the viable cells of the same avirulent race-32 produced hypersensitive reaction (HR). NR and HR were differentiated on the basis of the induction period required, visible reaction on infiltrated areas, bacterial constituents or metabolite responsible, involvement of host constituent during these reactions and their chemical inhibition.

Table 1: *Xanthomonas axonopodis*pv. *punicae* various regions of India

#	Indian State	District	Taluka	Region/ village	Designated isolate
1	**Maharashtra**	Nashik	Satana	Chaugaon	MNSC-01
			Satana	Lakhmapur	MNSL-02
			Satana	Nampur	MNSN-03
			Deola	Deola	MND-04
			Malegaon	Umrana	MNMU-05
		Ahmednagar	Sangamner	Nimbgaon	MASN-06
		Solapur	Mangalvedhe	Bavachi	MSMB-07
			Mangalvedhe	Maroli	MSMM-08
			Sangola	--------	MSS-09
2	**Karnataka**	Tumkur	Tumkur	--------	KT-10

		Davangere	Davangere	--------	KD-11
		Hospet	Hospet	--------	KH-12
		Chitradurga	Holalkere	--------	KCH-13
		Bellary	Bellary	--------	KB-14
		Bijapur	Bijapur	--------	KB-15
3	**Tamil Nadu**	Coimbatore	Thondamuthur	Nursipuram	TCTN-16
		Erode	Gobi	Ganpatipalayam	TEGG-17
4	**Andhra Pradesh**	Mehbubnagar	Mehbubnagar	--------	AM-18
		Anantpur	Anantpur	--------	AA-19
5	**Gujrat**	Vadodara	Vadodara	Waghodi	GVW-20
		Bharuch	Bharuch	Valia	GBV-21
6	**Rajasthan**	Hanumangarh	Hanumangarh	--------	RH-22
7	**Madhya Pradesh**	Burhanpur	Burhanpur	Icchapur	MBI-23

Table 2: Lipopolysaccharide and exopolysaccharide profiling of *Xanthomonas axonopodis*pv. *punicae* isolates

#	Isolates	LPS (µg/mg)	EPS (µg/mg)
1	MNSC-01	73.50	124.03
2	MNSL-02	94.36	154.85
3	MNSN-03	111.43	111.79
4	MND-04	103.36	139.81
5	MNMU-05	116.57	128.65
6	MASN-06	87.36	139.67
7	MSMB-07	81.79	158.84
8	MSMM-08	111.57	124.84
9	MSS-09	70.29	131.24
10	KT-10	124.03	172.58
11	KD-11	139.89	183.87
12	KH-12	104.83	179.24
13	KCH-13	98.14	180.47
14	KB-14	133.89	158.30
15	KB-15	84.34	168.36
16	TCTN-16	122.86	159.86
17	TEGG-17	68.90	107.86
18	AM-18	77.71	114.92
19	AA-19	58.24	112.03
20	GVW-20	54.52	112.79
21	GBV-21	74.38	98.94
22	RH-22	46.86	105.23
23	MBI-23	68.25	99.917

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REFERENCES

1. Borkar, S. G. and Verma, J. P., Exopolysaccharide, a watersoaking inducing factor produced by *Xanthomonas*

*campestris*pv. *malvacerum* the bacterial blight pathogen of cotton. *Cotton Fibre Trop Rev*. **44:** 149–153 (1989).

2. Borkar, S. G. and Verma, J. P., Inhibition of susceptible water soaking and or Hypersensitive reaction in cotton by Exopolysaccharide of avirulent *Xanthomonas campestris*pv. *malvacearum*. Folia Microbiol, **36(2):** 173-176 (1991b).
3. Castro, C. D. A. Molinaro, R. Lanzetta, A. Silipo and Parrilli, M., Lipopolysaccharide structures from Agrobacterium and Rhizobiaceae species, Carbohydrate Research., **(343):** 1924–1933 (2008).
4. Damery, J. T., Alexander, M., Physiological differences between effective and ineffective strains of Rhizobium.*Soil. Sci*, **108:** 209- 215 (1969).
5. Drigues,P.,Lafforgue,D.D.,Trigalet,A.,Du pin,P.,Samain, D., and Asselineau, J., Comparative Studies of Lipopolysacch aride and Exopolysaccharide from a Virulent Strain of *Pseudomonas solanacearum* and from Three Avirulent Mutants. *Journal of Bacteriology,* **(5):** 504-509 (1985).
6. Gross, M. and Rudolph, K., Studies on the Extracellular polysaccharides (EPS) produced in *vitro* by *Pseudomonas* phaseolicola. I. Indications for a Polysaccharide Resembling Alginic Acid in Seven *P. syringae* Pathovars J. Phytopathology, **118:** 276-287 (1987).
7. Neeraj, S. S. Gaurav, S. Chatterjee and Chandra, M., Exopolysaccharides and lipopolysaccharide production by *Sinorhizobiobium fredii* tn5 mutants infecting *Vigna radiate*. *ARPN Journal of Agricultural and Biological Science*., **5(4):** 32-38 (2009).
8. Osborne, M. J., Studied on Gram- negative cell wall. I. Evidence for the role of 2 Keto 3- deoxy- octonate in the lipopolysaccharide of *Salmonella typhimurium*. *Proceedings of the National Academy of sciences of the United States of America* **50:** 499 (1963).
9. Schaad, N. W., *Xanthomonas*. *In:* Laboratory Guide for Identification of Plant Pathogenic Bacteria, II Ed. *International Book Distributing Co. Lucknow*: 165 (1992).