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SURVEILLANCE OF *RALSTONIA SOLANACEARUM* INFECTING POTATO CROP IN PUNJAB

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A B S T R A C T

Potato is the most consumed vegetable in Pakistan and is regarded as source of food and energy for millions of people around the world. Bacterial wilt of potato caused by *Ralstonia solanacearum* is an important disease causing huge losses worldwide. Punjab is the major potato producing province in Pakistan contributing 80% production. This study was conducted to record the incidence and severity of bacterial wilt disease in Pakistan's major potato growing areas of Punjab and also to characterize the pathogen. Survey of several fields from each area viz; Lahore, Okara, Faisalabad, Sahiwal, Sialkot and Taxila was done for the collection of samples of plants and soil. *R. solanacearum* was isolated on Triphenyl-tetrazolium chloride (TTC) agar media from infected samples followed by pathogenicity tests on potato plants. Race of the isolates of *R. solanacearum* was determined and the isolates were characterized biochemically and identified on molecular basis using the *R. solanacearum*-specific primer pair 759/760 which amplified 280 bp fragment of DNA. Data obtained from the survey of potato growing areas revealed bacterial wilt as a common problem. The highest incidence of bacterial wilt (24.4%) was observed in Okara. Areas including Sahiwal, Lahore, Faisalabad, Sialkot and Taxila were having 21.7, 18.0, 23.2, 19.8 and 8.5 percent incidence respectively while disease prevalence was 100% in all areas except Taxila (60%). The isolation from the infected samples was done and the isolates were determined to be belonging to race 3 biovar 2. According to the molecular identification tools, 21 isolates were confirmed to be *R. solanacearum*.

Keywords: Potato, bacterial wilt, *Ralstonia solanacearum*, incidence.

INTRODUCTION

Potato (*Solanum tuberosum* L.) is the most important vegetable crop of the world, including Pakistan. The climate of Pakistan, especially of Punjab, is very suitable for the production of potatoes. Punjab is the chief potato producer of Pakistan and more than 80% of the potato comes from it. *Ralstonia solanacearum* causes bacterial wilt, a soilborne vascular disease that is arguably one of the most economically important bacterial diseases in the world. It attacks over 450 plant species including crops as tomatoes, tobacco, potatoes, eggplant and bananas (Allen *et al.,* 2005). *R. solanacearum* is a species complex with considerable diversity. It contains many strains that differ in host range, geographical distribution, pathogenicity and biochemical properties (Hayward, 1994). It represents the major limitation in the production of solanaceous crops all around the world and is a destructive pathogen which causes significant damage under disease-favorable weather conditions (Doan and Nguyen, 2005). In Pakistan, this disease may jeopardize the future cultivation of many solanaceous vegetables in Punjab and Sindh provinces where its frequency extended from 5-25 % (Burney *et al.,* 1999).

R. solanacearum has been considered as the main constraint to growing potatoes (*Solanum tuberosum* L.) in tropical and subtropical regions and countries with warm climates (Hayward, 1995). The worldwide dissemination of *R. solanacearum* race 3 is associated with seed potato tubers, because latent *R. solanacearum* populations can colonize the vascular tissues of the tuber without inducing symptoms of disease (Hayward, 1991). As Pakistan relies mainly on import for potato

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seed, this has introduced this disease in Pakistan as latent infections in tubers. Crop in the northern areas of Pakistan have already been reported to be infected with bacterial wilt and losses greater than 30% are estimated. So the present study was planned to assess the incidence of bacterial wilt pathogen in major potato growing areas of Punjab where no exclusive study has been done with reference to this disease. The objectives of the study were to survey the major potato growing areas for determination of incidence, prevalence and characterization and molecular identification of the bacterial wilt pathogen.

MATERIALS AND METHODS

Survey: Survey was conducted in major potato growing areas of Punjab (Table 1). From each location 3 fields were selected and 3 spots were randomly selected from each field. On the basis of symptoms, two rows of selected spots were observed for bacterial diseases. The wilted plants were uprooted and the disease was confirmed by cutting the wilted plants from the lower end of stem and dipped into water to observe the bacterial streaming. This test is characteristic test for bacterial pathogens and helped to identify the bacteria initially (Priou *et al.,* 1999). The samples that showed the oozing of bacteria were collected. The tubers and soil of these infected plants were also collected. The samples were preserved in polyethylene bags, taken to the laboratory and stored at 4 °C for further processing.

Area	Location Surveyed	Sr.	Area	Location Surveyed
Faisalabad	Sadhar Pansara Chaba Tandianwala Bangla Koraywala Buraywala	4	Okara	Baman Shah Burj Jeeway Khan Moza Ameer Aman Salwal Qadirabad
Sahiwal	Chak 86/6r Chak 95/6r Chak 30/14L	5	Lahore	Mujaki Mangaal Lakhodair Ganja Sindhuwa Awan Dihiwala Gurki
Taxila	Taxila fields Hazro	6	Sialkot	Claswala Dhilam Gunah Chicharwali Ghoinkey Sehjokala

Disease incidence and prevalence were determined by the following formula:

$$Disease\ incidence\ \% = \frac{No.of\ plants\ wilted}{Total\ no.of\ plants\ observed} \times 100$$

$$Disease\ prevalence\ \% = \frac{No.of\ fields\ infected}{Total\ no.of\ field\ surveyed} \times 100$$

Isolation of *Ralstonia solanacearum*: *R. solanacearum* was isolated on 2,3,5-triphenyltetrazolium chloride (TTC) agar media (Hugh and Leifson, 1953) that is specific for isolation and also differentiates between virulent and avirulent strains. Vascular portion of infected stem sections were cut into small pieces, washed and sterilized with 1% NaOCl followed by washing thrice with distilled water and directly placed on TTC plates. From soil, serial dilution technique was used for isolation of the pathogen. An aliquot (0.1 ml) was placed on media and the plates were incubated at 28-30°C for 2-3 days. After incubation the virulent *R. solanacearum* colonies (white with pink center) were selected and purified.

Pathogenicity of *R. solanacearum:* Pure bacterial cultures were transferred to test tubes containing 5 ml distilled water to obtain turbid bacterial suspensions and used as bacterial inoculums. Twenty days old seedlings raised in pots were used for pathogenicity test to check the host specificity of inoculated strain of *R. solanacearum*. The experiment was performed in sterilize conditions and soil was also sterilized prior to the experiment. The potato plants were kept and observed regularly for the appearance of symptoms for 4

weeks. The experiment was performed with three replications. The rapid appearance of the wilt symptoms within a week was indication of high virulence of isolates and plants were killed within 10 days and isolates were categorized as highly virulent (+++). Moderately virulent isolates were shown by '++' and characterized by mild appearance of wilt symptoms initially which intensified and killed the plants between 10 and 15 days of inoculation and in weakly virulent isolates, wilt symptoms were clear after 15 days and isolates were marked as '+' and plants may or may not be killed even after 4 weeks.

Identification of race in *R. solanacearum:* Race differentiation was done based on host range and hypersensitivity reaction (HR) on tomato and tobacco (Janse, 1991). Bacterial inoculum (10^8 cfu/ml) was applied to the plants of tomato and tobacco grown under sterilized conditions. The plants were kept under controlled conditions for 30 days and the isolates were categorized as highly virulent, moderately virulent and weakly virulent based on the appearance of the symptoms after inoculation.

Table 2. Race determination in Ralstonia solanacearum (Janse, 1991).

Reaction in	Race		
	1	2	3
Tomato/aubergine	Wilting	No reaction	Wilting
Tobacco cv. White Burley plants (stem inoculation)	Wilting	No reaction	No reaction
Tobacco cv. White Burley leaves (hypersensitivity test)	Necrosis (48 h) and wilting (7–8 days)	HR (12–24 h)	Chlorosis (2–8 days)

* adapted from Bulletin OEPP/EPPO Bulletin 34, 155-157, available at;
http://archives.eppo.int/EPPOStandards/PM7_DIAGNOS/pm7-21(1).pdf

Identification of Biovars in *R. solanacearum*: The isolates were differentiated into biovars, based on their ability to utilize disaccharides (lactose, maltose and cellobiose) and hexose alcohols (mannitol, sorbitol and dulcitol), according to Hayward (1964), and Kumar *et al*. (1993). Ten ml of each 10% lactose, maltose, cellobiose, mannitol, sorbitol and dulcitol was added in sterilized screw-capped test tubes and then heated at 100°C for 30 min to sterilize these solutions. Bottles of semi-solid basal medium were melted in water bath and cooled to 70 °C. Carbohydrate solution (10 ml) was added and mixed in basal media. Five ml solution was poured into labeled tubes. Media was then allowed to solidify at room temperature. Control was kept by adding 10ml sterilized distilled water instead of sugar solution into the basal medium. Bacterial suspensions from individual isolates (with concentration of 10^8 cfu/ml) were prepared from 48 hrs old cultures. Inoculation of the medium was carried out by adding 1-2 drops of bacterial suspension to each tube containing sugar solutions, three replicates of sugar solution and control were maintained for each isolate. Test tubes were incubated at 30°C and examined after 2, 7 and 14 days for the presence of indicator change from olivaceous green to orange colour on the surface of medium.

Biochemical tests of *R. solanacearum*: Biochemical tests were conducted for the characterization of the isolates that included gram reaction, Potassium hydroxide test, Catalase oxidase test, Levan production from sucrose, Kovac's oxidase test, Lipase activity on Tween 80 agar, Oxidation / fermentation of glucose and Gelatin liquefaction. All these tests were performed as described by Schaad *et al.* (2001).

Molecular Identification of *R. solanacearum*: For the molecular identification of *R. solanacearum*, genomic DNA was extracted using cetyltrimethyl ammonium bromide (CTAB).

Thermal cycler was used for PCR amplification and specie specific primers (i.e. 759/760) of *R. solanacearum* were used which amplifies the specific 280 bp fragment (Fegan and Prior, 2005). Reaction mixture contained 1xPCR buffer (10 mM Tris HCl with pH 8.3, 50mM KHCl), 1.5 mM $MgCl_2$, 0.05mM of each dNTP, 25 pmol of primers 759 (5'GTCGCCGTCAACTCACTTTCC3') and 760 (5'GTCGCCGTCAGCAATGCGGAATCG3'), 1µl of genomic DNA as template and 0.5 U of Taq DNA polymerase. Samples were denatured at 94°C for 3 min, annealed at 53°C for 1 min and extended at 72°C for 1.5 min, followed by 30 cycles of 94°C for 15 s, 60°C for 15 s, 72°C for 15 s, and a final extension of 72°C for 5 min (Opina *et al.,* 1997). PCR products were separated in 1.5% agarose gel, stained with ethidium bromide at 0.5µg/ml and were visualized and photographed under ultraviolet light.

RESULTS

Survey: The survey of potato growing areas in Punjab revealed that bacterial wilt was present in all fields. Nearly 90 fields (total 30 locations from six areas and 3 fields from each location) were visited from several areas and each field was observed to be exhibiting the disease. Disease prevalence (DP) of bacterial wilt was found 100 % in central Punjab except Taxila where disease prevalence was recorded to be 60% (3 fields out of 5 were observed attacked). Average highest disease incidence was recorded at Okara (24.4%) followed by Sahiwal (21.7%) (Table 3).

Table 3. Disease incidence and disease prevalence (%) in potato fields of Punjab.

Area	Location	DI (%)	DP (%)
Okara	Baman Shah	28	100
	Burj Jeeway Khan	22	100
	Moza Ameer Aman	23	100
	Salwal	25	100
	Qadirabad	24	100
	Average	24.4	
Sahiwal	Chak 86/6r	18	100
	Chak 95/6r	24	100
	Chak 30/14L	23	100
	Average	21.7	
Lahore	Mujaki	15	100
	Mangaal	18	100
	Lakhodair	21	100
	Ganja Sindhuwa	17	100
	Awan	16	100
	Dihiwala	19	100
	Gurki	20	100
	Average	18.0	
Faisalabad	Sadhar	19	100
	Pansara	25	100
	Chaba	23	100
	Tandianwala	23	100
	Bangla	25	100
	Koraywala	27	100
	Buraywala	21	100
	Average	23.2	
Taxila	Taxila fields	10	60
	Hazro	7	60
	Average	8.5	
Sialkot	Claswala	20	100
	Dhilam	15	100
	Gunah	29	100
	Chicharwali	20	100
	Ghoinkey	18	100
	Sehjokala	17	100
Average		19.8	

Isolation of *R. solanacearum*: The bacterium produces pink/reddish centered colonies which indicate the virulent isolates. A total of 21 isolates were obtained. The isolation was done from the samples which were severely infected with the pathogen.

Pathogenicity of *R. solanacearum*: Among the isolates collected, 5 were found highly virulent, 10 as moderately virulent and 6 as weakly virulent (Table 4).

Identification of Race in *R. solanacearum*: Race identification test revealed that all the isolates were belonging to race 3. Wilt symptoms were observed on tomato plants while on tobacco leaves, hypersensitivity test was performed which demonstrated the isolate are exhibiting chlorosis of leaves. The results were in

accordance with Janse (1991).

Identification of Biovars in *R. solanacearum* isolates: The biovar confirmation test was performed and kept under observation up to 14 days for color change in lactose, maltose and cellobiose which explained that all the isolates were from biovar 2 (Table 5).

Table 4. Virulence of isolates

Isolate	Virulence*	Isolate	Virulence*
FSR3-1	++	OBJK7-4	+++
FSR3-5	++	OQ3-1	++
FBN4-2	++	OA3-2	++
FC1-3	++	LMu1-3	++
FTW2-7	+	LG2-4	+++
FTW6-5	+	LA3-1	++
TF8-2	+	LA3-3	+
TF3-1	+	SK2-1	+++
SC863-1	+++	SK2-2	+++
SC14-2	++	SK2-3	+
OBM3-4	++		

*Highly virulent (+++), moderately virulent (++) and weakly virulent (+).

Table 5. Biovar determination.

Sr. No.	Isolates	Control	Lactose	Maltose	Sorbitol	Mannitol	Cellobiose	Dulcitol	**Biovars**
1	FSR3-1	-	+	+	-	-	+	-	2
2	FSR3-5	-	+	+	-	-	+	-	2
3	FBN4-2	-	+	+	-	-	+	-	2
4	FC1-3	-	+	+	-	-	+	-	2
5	FTW2-7	-	+	+	-	-	+	-	2
6	FTW6-5	-	+	+	-	-	+	-	2
7	TF8-2	-	+	+	-	-	+	-	2
8	TF3-1	-	+	+	-	-	+	-	2
9	SC863-1	-	+	+	-	-	+	-	2
10	SC14-2	-	+	+	-	-	+	-	2
11	OBM3-4	-	+	+	-	-	+	-	2
12	OBJK7-4	-	+	+	-	-	+	-	2
13	OQ3-1	-	+	+	-	-	+	-	2
14	OA3-2	-	+	+	-	-	+	-	2
15	LMu1-3	-	+	+	-	-	+	-	2
16	LG2-4	-	+	+	-	-	+	-	2
17	LA3-1	-	+	+	-	-	+	-	2
18	LA3-3	-	+	+	-	-	+	-	2
19	SK2-1	-	+	+	-	-	+	-	2
20	SK2-2	-	+	+	-	-	+	-	2
21	SK2-3	-	+	+	-	-	+	-	2

Biochemical Tests of *R. solanacearum*: Biochemical tests performed for the characterization of each isolate included Gram reaction, Potassium hydroxide test, Catalase oxidase test, Levan production from sucrose, Kovac's oxidase test, Lipase activity on Tween 80 agar, Oxidation / fermentation of glucose and Gelatin liquefaction (Table 6).

Molecular identification of *R. solanacearum*: Molecular identification of *R. solanacearum* was carried out using primer pair 759/760 and the characteristic amplicon size of 280 bp was observed on 1.5% agarose gel (Fig. 1). All 21 the isolates were confirmed to be *R. solanacearum*.

Table 6. Some of biochemical tests of *R. solanacearum* isolates.

Isolates	Staining	KOH	Catalase	Levan	Kovacs	Lipase	*O/F glucose	Gelatin
FSR3-1	-	+	+	-	+	+	+	-
FSR3-5	-	+	+	-	+	+	+	-
FBN4-2	-	+	+	-	+	+	+	-
FC1-3	-	+	+	-	+	+	+	-
FTW2-7	-	+	+	-	+	+	+	-
FTW6-5	-	+	+	-	+	+	+	-
TF8-2	-	+	+	-	+	+	+	-
TF3-1	-	+	+	-	+	+	+	-
SC863-1	-	+	+	-	+	+	+	-
SC14-2	-	+	+	-	+	+	+	-
OBM3-4	-	+	+	-	+	+	+	-
OBJK7-4	-	+	+	-	+	+	+	-
OQ3-1	-	+	+	-	+	+	+	-
OA3-2	-	+	+	-	+	+	+	-
LMu1-3	-	+	+	-	+	+	+	-
LG2-4	-	+	+	-	+	+	+	-
LA3-1	-	+	+	-	+	+	+	-
LA3-3	-	+	+	-	+	+	+	-
SK2-1	-	+	+	-	+	+	+	-
SK2-2	-	+	+	-	+	+	+	-
SK2-3	-	+	+	-	+	+	+	-

*O/F: Oxidation/Fermentation.

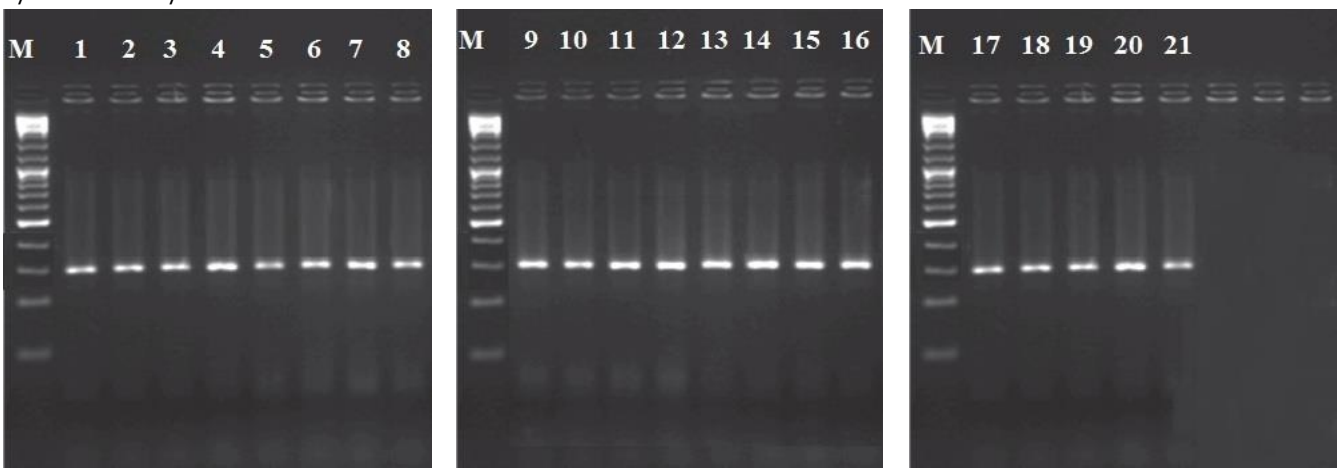

Figure 1. The characteristic amplicon of 280 bp size of *R. solanacearum*; M is molecular weight marker of 100 bp and the isolates are numbered from 1 to 21.

DISCUSSION

It is evident from the worldwide reports that bacterial wilt caused by *Ralstonia solanacearum* is the most important bacterial pathogen that is still going on increasing its host range and infecting more and more crops posing serious threats to crop production specially vegetables. The presence of pathogen as hidden infections has let it to distribute more widely. This is the reason that the pathogen has developed several races, biovars and possesses huge diversity. The occurrence of bacterial wilt disease in Pakistan was first reported by Kamal and Muhgal (1968). Later on, Khan *et al*. (1985)

during the survey of potato diseases in district Swat also reported the disease with minor incidence. Surveys of potato crop from several researchers (Hafiz, 1986; Turkensteen, 1986, 1987; Geddes, 1989; Burney, 1994; Ahmed *et al*., 1995) were done and they reported bacterial wilt disease in several areas. They also noticed that incidence of disease was increasing and until 1995, the disease had dispersed to 4 potato zones in Pakistan. In zone 2 (Irrigated plains of Central Punjab and South East KPK) which is major potato production zone, bacterial wilt was classified as minor disease (Ahmed *et al*., 1995). But our study has elaborated the current status of bacterial wilt disease in major potato growing zone of Pakistan where this disease has now 100% prevalence and has developed as a common disease of potato with maximum incidence in Okara (24.4%).

Regarding the characterization of bacterial wilt pathogen in Pakistan, very few studies have been conducted so far. Initial data on survey has been collected by several scientists but no one characterized this pathogen. Chaudhary and Rashid (2011) collected samples from tomato plants from various parts of Soan Skesar valley of Punjab, Pakistan and isolated *R. solanacearum* on TTC media. They characterized the pathogen for several tests and concluded pathogen positive for Gram staining, KOH test, Kovacs oxidase test, Catalase test, Oxidation/fermentation of glucose, Hydrolysis of tween 80 and negative for Arginine dihydrolase, Levan production, Salt tolerance, Lecithinase detection, Gelatin hydrolysis and production of Fluorescent pigment. In this study, *R. solanacearum* isolates were characterized for several biochemical tests and the results were in accordance with Chaudhary and Rashid (2011). Isolates were positive to KOH, Catalase, Kovacs oxidase, Lipase and oxidation/fermentation tests and negative for Levan production and Gelatin liquefaction.

Race 3 biovar 2 (R3bv2) strains causing potato brown rot, which ranks among the most destructive diseases of potato in Africa, Asia, and Central and South America (CABI, 2003). Although the host range of R3bv2 was once thought to be limited to potatoes, eggplant, and tomato, it is now known that these strains can potentially infect many ornamental and weed species as well (Janse *et al.*, 2004). Our findings are also coherent to the previous findings and the isolates infecting potato crop in major potato growing areas of Punjab were also revealed to be from R3bv2.

Several primers have been developed for the molecular identification of *R. solanacearum*. Fegan and Prior (2005) described the molecular identification procedures of *R. solanacearum* upto the sequevar level based on endoglucanase (*egl*) gene. They explained that *R. solanacearum* can be identified up to specie level by using several primers. All *R. solanacearum* and *R. syzygii* generate 280 bp fragment with primer set of 759 and 760. In our study, all the isolates suspected to be *R. solanacearum* were confirmed following this approach.

CONCLUSION

The study has elucidated the important out coming regarding bacterial wilt status in Pakistan's major potato growing areas. The study concludes that disease has developed as a common disease and causing huge losses especially in zone 2 (Irrigated plains of Central Punjab and South East KPK) which is major potato producing zone in the country. It was revealed that strains were belonging to R3bv2 which is most destructive in potato. Strict measures should be taken to avoid its entry and further spread in the country and attention must be given for its management in the field.

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