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IN VITRO INVESTIGATIONS ON HOST SPECIFICITY OF *RALSTONIA SOLANACEARUM* AMONG SOLANACEOUS CROPS AND ITS BIOLOGICAL CONTROL IN TOMATO

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Abstract

Ralstonia solanacearum, the causal organism of bacterial wilt of solanaceous crops is a major limitation on the production of solanaceous crops worldwide. The present studies were carried out to explore the prevalence, severity, virulence and host specificity range of *R. solanacearum* in chili, tomato, eggplant and potato plants while biocontrol of this pathogen was studied on tomato. The isolation and the initial identification of bacterium were done on TTC and 523 media. Out of 32 isolates, 30 showed positive hypersensitive reaction (HR) with variable response and were confirmed as *R. solanacearum* by performing biochemical tests i.e. Gram staining, KOH, catalase oxidation, Kovacs oxidation, levan production from sucrose, lipase activity on tween 80 agar, production of fluorescent pigment, and oxidation/fermentation of glucose. Race identification studies showed that all the isolates belonged to Race 3 while biovar confirmation tests revealed that 4, 3 and 23 isolates belonged to biovar 1, biovar 2 and biovar 3, respectively. The isolates belonging to distinct biovar class were tested for their wideness of host range by challenging them to chili, tomato, eggplant and potato plants. It was observed that biovar 3 is the most aggressive and has widest host range as compared to counterparts. Biocontrol studies through antagonistic rhizobacteria resulted four antagonistic isolates; PRB10, PAP5, PAT1 and PTR6 having the highest biocontrol activity with 98.75%, 97.5%, 93.75% and 91.25% respectively.

Key words: *R. solanacearum*, Solanaceous crops, Host specificity, Biocontrol, Tomato.

Introduction

R. solanacearum, the causal organism of bacterial wilt, represents the major limitation on the production of solanaceous crops all around the world and is a destructive pathogen due to being soil borne, very widely distributed and has an unusually broad host range (Denny, 2006; Chaudhary & Rashid, 2011). In general, losses depend on local climates, soil types, cropping practices, the choice of crop and plant cultivar, and the virulent characteristics of the *R. solanacearum* local strains (Elphinstone, 2005). Bacterial wilt disease generally occurs in lowlands in tropical or subtropical areas, but one subgroup of *R. solanacearum* known as Race 3 biovar 2 (R3bv2) invades plants at higher altitudes and in temperate zones (Elphinstone, 2005). R3bv2 is an awfully destructive potato pathogen, causing brown rot of potato in the highland tropics of Africa, Asia, and Latin America (Elphinstone, 2005). Five pathogenic races have been described as race 1, which has the highest number of host species, solanaceous crops like chili and sweet pepper, eggplant, potato, tobacco and tomato; non-solanaceous crops like bean, groundnut and sunflower; ornamental plants like *Anthurium* spp., *Dahlia* spp., *Heliconia* spp., etc. (Anon., 2006). For race 2, cooking and dessert bananas, plantain, other *Musa* spp. and wild and ornamental *Heliconia* spp. (Anon., 2006). For race 3, *Capsicum* spp., eggplant, geranium, potato and tomato; weeds like *Solanum dulcamara* and *S. nigrum*. The race is widespread in all the five continents (Anon., 2006). For race 4, ginger and the related plant species mioga and patumma; race 4 occurs in Asia (Elphinstone, 2005). For race 5, *Morus* spp; it is limited to China (Elphinstone, 2005). These races differ in host range, geographical distribution and ability to survive under different environmental conditions (Gillian, 2004).

In Pakistan the pathogen was found in all of the four provinces namely Punjab, Baluchistan, Sindh, and Khyber Pakhtoon Khawa having growing areas for potato, tomato, eggplant and chili (Burney, 1995). Disease incidence was recorded as 1-4% in tomato growing areas of Baluchistan. While in Punjab and Sindh (1993-94), there was 0.5-25% disease attack on chilies. The pathogen was also found in beans, peanut and sunflower plants (Burney, 1995; Burney *et al.*, 1999). Incidence of *R. solanacearum* R3bv2 and R3bv3 was reported in tomato growing areas of Punjab (Begum, 2005) and recently R3bv2 incidence in major potato growing areas of Punjab was observed to be 24.4 % (Tahir *et al*., 2014).

The common control measures used against bacterial wilt include the use of resistant varieties, healthy seed, crop rotation, agronomic practices, biological control and integrated management (Elphinstone & Aley, 1993). Control is difficult due to high variability of the pathogen, limited possibility for chemical control, high capacity of the pathogen to survive in diverse environments and extremely wide host range (Nguyen & Ranamukhaarachchi, 2010). Plant growth promoting rhizobacteria (PGPR) is a group of rhizosphere colonizing bacteria that produces substances to protect the plants against pathogens and increase the growth of plants (Harish *et al*., 2009; Cartieaux *et al.*, 2003). Several studies have reported these PGPR antagonizing several pathogens.

Although lot of work has been done on this disease worldwide but in Punjab (Pakistan), Rawalpindi and Islamabad are major vegetable growing areas without any information about the prevalent race and biovar of the bacterial wilt pathogen. Present study is aimed at the characterization of the pathogen, its host specificity, pathogenicity and its biological control using antagonistic rhizobacteria.

Materials and Methods

The diseased plant samples of potato, tomato, eggplant and chilies infected with bacterial wilt were collected from different field areas of Rawalpindi and Islamabad (33.43°N and 73.04°E) which fall under the category of sub-humid temperate zone and are the areas of vegetables cultivation.

Isolation of *R. solanacearum*: For the isolation of bacterial pathogen, the infected vascular portion of the wilted plant was cut into small pieces, washed with sterilized distilled water and disinfected with sodium hypochlorite. The pieces were placed on 2, 3, 5-triphenyltetrazolium chloride (TTC) media (Kelman *et al*., 1954) and incubated at 28±2°C. This medium was used to differentiate between virulent (white with pink centers) and non-virulent (dark red) colonies.

Hypersensitivity test of pathogen: Hypersensitivity tests were performed by growing tobacco plants (*Nicotiana tabaccum* cv. *Burley*) in sterilized soil. Bacterial suspension (10^8cfu/ml) was prepared and applied on abaxial surface of tobacco leaves with 1 ml disposable syringe. Lighter water spray was applied in order to provide moisture and clear plastic bags were used to cover the plants. The plants were kept at room temperature and 90% relative humidity for 48 hours. Data were recorded on alternate days within a week (Kelman *et al.,* 1954).

Biochemical tests: Following hypersensitive reaction test, bacteria were subjected to other biochemical tests like Gram's staining, catalase oxidase test, Kovacs oxidase test, levan production from sucrose, loop test (Suslow *et al*., 1982) Lipase activity on tween 80 agar, Oxidation and/or fermentation of glucose, production of fluorescent pigment (Schaad *et al*., 2001).

Race identification of *R. solanacearum*: The races of *R. solanacearum* were identified according to Janse (1991) by observing the hypersensitive reaction on tomato and tobacco plants (Table 1). The inoculum was applied to the plants and the reaction results were recorded every day for 10 days.

Biovar identification of *R. solanacearum*: Biovars of *R. solanacearum* were differentiated on the basis of utilization of sugars i.e., lactose (L), maltose (ML), cellubiose (C), mannitol (M), sorbitol (SB) and dulcitol (D). Semi-solid basal medium in bottles was melted in water bath and kept for cooling at 60-70°C. Ten ml of each carbohydrate solution was added in basal media and mixed gently. Cultural plates (90 wells) labeled, dispensed with 200µl of media and kept at room temperature for solidification. 10 ml of distilled water was used as control instead of sugar solution into the basal medium. Individual isolates were taken from 48 hrs old cultures on 523 media plates to make bacterial suspensions (10^8cfu/ml). Media was inoculated by adding 50µl of bacterial suspension to each well containing sugar solution. For each isolate, 2 replicates maintained for individual sugars. Cultural plates were kept for incubation at 30°C and observations were made after 2, 7 and 14 days to confirm the indicator change from olivaceous green to orange color at the surface of the medium.

Pathogenicity test of pathogen: For pathogenicity test, reddish fluidal colonies of bacteria from TTC media were transferred to 523 media and incubated at 30°C overnight. Bacterial cultures were then transferred to test tubes containing 5 ml distilled water in each for each bacterial culture to get turbid bacterial suspensions. These bacterial suspensions were used as inoculums. For the determination of pathogenicity of different isolates, potato, tomato, eggplant and chili were grown in pots containing sterilized soil. Twenty days old seedlings of each plant were used for pathogenicity test to check the host specificity of inoculated strain of the bacteria. Soil drenching (Umesha & Girish, 2005) and leaf detached methods (Winsted & Kelman, 1952) for inoculations were used. For detached leaf method following scale was used: 0 = no symptoms; 1 = partial yellowing of inoculated leaf; 2 = complete chlorosis of inoculated leaf; 3 = total collapse of inoculated leaf. For soil drenching, scale was used: 0 = no symptoms; 1 = partial wilting; 2 = complete wilting; 3 = plant collapsed and dead.

Host specificity test: For testing host specificity with the isolates representing biovar I, biovar II and biovar III of *R. solanacearum*, a total of 132 solanaceous plants (potato, tomato, chilies and eggplant) were inoculated. 5, 4 and 32 plants of each host were grown for testing against isolates of biovar 1, biovar 2 and biovar 3 respectively keeping 1 plant as control in each treatment. 8 weeks old plants having 6-8 expanded leaves, and 10-30 cm tall were selected for treatment application. Inoculum was made by flooding petri plates and collected the suspension in glass beaker, separately. The concentration of inoculums was adjusted spectrophotometrically (1×10^8 cfu/ml) before inoculation. Plants were inoculated by following different methods of inoculation i.e. some plants were inoculated with a hypodermic syringe by injecting 5 ml of bacterial suspension into the roots 5 cm above the soil line and some plants were inoculated by cutting roots with a scalpel along three sides of the pot and pouring 10 ml of bacterial suspension over the freshly wounded roots. Control plants were treated in the same way but with a sterile water suspension. Following treatment application plants were irrigated with 250 ml water per day and were kept in green house. The emergences of disease symptoms on plants were observed weekly for 8 weeks.

Table 1. Race determination in *R. solanacearum* (Janse, 1991).

Race	1	2	3
Tomato	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)

Isolation and *In vitro* evaluation of rhizobacterial isolates against *R. solanacearum*: Isolation of rhizobacteria was made by serial dilution method resulting 105 isolates which were assessed for antagonistic activity against *R. solancearum* using dual culture plate technique. Test rhizobacteria and *R. solanacearum* isolate were co-cultured on the same plate and inhibition zone was observed. RsAR-36 strain of *R. solanacearum* was used for zone inhibition test as it was most virulent.

Identification of rhizobacteria: Twenty isolates were antagonistic to *R. solanacearum* with varying ability to antagonize. Identification of these isolates was done according to procedure described in Bergey's manual (Holt *et al*., 1994).

Evaluation of antagonistic bacteria against bacterial wilt under greenhouse conditions: A potting medium that constitutes a mixture (1/3 soil, 1/3 sand and 1/3 compost) was sterilized at 121°C for 30 min and filled in a sterilized plastic tray. Tomato seeds were surface sterilized with 2% sodium hypochlorite for 2 min (Guo *et al*., 2004), washed thoroughly with sterilized water and planted in plastic tray filled with the sterilized potting medium. The plants were maintained in a greenhouse at temperatures of 24–28°C and 75–90% relative humidity and seedlings were watered with sterile water when required. The pathogen was prepared by culturing in Casamino Acids-Peptone-Glucose (CPG) broth for 48 h at 28°C and 150 rpm on rotary shaker. Cultures were centrifuged at 10,000 rpm for 10 min at 10°C. Bacterial pellets were suspended in distilled water and adjusted to 10^8 cfu/ml. In 400 grams of the sterilized potting medium in pots, 75 ml of *R. solanacearum* having 10^8 cfu/ml was added. One week post incorporation of the pathogen into the soil and one day prior to transplanting the test plants, antagonists were incorporated into the soil at a rate of 50 ml per pot at 10^9 cfu/ml. Following 24 h of antagonist incorporation, four weeks old seedlings raised in plastic tray were root dipped in suspension (10^9 cfu/ml) of antagonistic bacteria for 60 min and transplanted into pathogen-antagonist mixture soil. Plants were kept in the greenhouse at 24-28°C and 75-90% relative humidity in 12 h/12h light/dark conditions. The experiment was conducted twice with a completely randomized design.

Calculation of biological control efficiency: Biological control efficacy (BCE) was calculated using the following formula (Guo *et al*., 2004):

$$\text{Biological control efficacy (\%)} = \frac{\text{[Disease incidence of control – Disease incidence of treatment group]}}{\text{Disease incidence of control}} \times 100$$

Disease assessment: Disease incidence (DI) was calculated with the following formula (Guo *et al*., 2004):

$$\frac{\text{Number of wilted plants per plot}}{\text{Total number of plants per plot}} \times 100$$

Results

Isolation of *R. solanacearum*: A total of 32 isolates of *R. solanacearum* were isolated from the diseased samples. Typical fluidal pinkish red centered colonies were observed on TTC medium when infected plant segments were placed on the medium. Specific colonies were picked and purified for confirmation of bacterial wilt. The bacterial cultures were purified on 523 media and stored in sterile water. Isolates were then confirmed by different tests.

Hypersensitivity test: During the hypersensitivity test, a positive hypersensitive response was observed. All the isolates exhibited chlorosis but with variable intensity and time of expression. Some isolates expressed it within 2 days and some after 8 days. Isolates that exhibited chlorosis within 2 days were considered as strongly virulent, those exhibited in 3-6 days were categorized as moderately virulent and the isolates were rendered slightly virulent showing chlorosis after 6 days. Results revealed that 11 isolates were strongly virulent as chlorosis was observed within 2 days of inoculation. During 3-6 days, 12 more isolates exhibited chlorosis marking them moderately virulent while 7 isolates were noticed exhibiting chlorosis after 6 days thus indicating slightly virulent. Two strains that showed no response were RsAD-17 and RsDS-38 (Table 2).

Biochemical tests: Following the hypersensitivity test, the isolates were subjected to various biochemical tests. In gram staining, 30 isolates out of 32 exhibited negative results thus confirming the bacteria to be gram negative (Table 3). All these gram negative isolates responded positively to loop test as they formed thread like structure when uplifted using a loop (Table 3). In catalase oxidase test, bubble formation was observed on addition of hydrogen peroxide (H_2O_2) indicating the aerobic nature of bacteria. However two isolates i.e. RsAD-17 and RsDS-38 gave negative results (Table 3). Kovacs oxidase test revealed that isolates exhibited variable responses; 14 isolates developed the purple color within 10 seconds and classified as positive for the reaction, 16 isolates produced the color in 60 seconds and considered moderately positive, while 2 isolates; RsAD-17 and RsDS-38 were oxidase negative as they did not show purple color. So, all positive isolates were categorized as *R. solanacearum* (Table 3).

Levan production from sucrose was tested on colonies of *R. solanacearum* over 523 media. Cultures positive to levan production produced raised, convex and mucoid colonies while levan negative cultures produced colonies that were fluidal, opaque and tends to coalesce. Response of the isolates is given (Table 4).

Table 2. HR of tobacco (var. Burley) leaves in response to *R. solanacearum* isolates.

Isolates	24-48 hours	3-6 days	After 6-days
RsSP-1	+	+	+
RsLe-2	+	+	+
RsAD-6	-	+	+
RsLe-8	-	-	+
RsAD-13	-	-	+
RsAD-14	+	+	+
RsAD-16	+	+	+
RsAD-17	-	-	-
RsDN-18	-	+	+
RsCN-19	-	-	+
RsRA-20	-	+	+
RsSH-22	-	+	+
RsAR-23	-	-	+
RsAR-24	+	+	+
RsTR-25	-	+	+
RsTR-28	+	+	+
RsMM-29	+	+	+
RsTT-30	-	+	+
RsRN-31	+	+	+
RsR-32	-	-	+
RsAR-33	-	+	+
RsAR-34	+	+	+
RsAS-35	-	-	+
RsAR-36	+	+	+
RsHS-37	-	+	+
RsDS-38	-	-	-
RsIA-39	-	+	+
RsTA-40	-	-	+
RsAR-42	+	+	+
RsAR-43	-	+	+
RsSR-44	-	+	+
RsSR-45	-	+	+

During Lipase activity on tween 80 agar, dense precipitates around the bacterial growth were formed by the positive isolates, evident mostly after 3-4 days of incubation. All levan negative isolates were positive for lipase production but RsDS-38 and RsCN-19 were positive for both the tests (see table 4). Hydrolysis of Tween 80 is a sign of lipolytic activity (Sierra, 1957) because the substrate is freely soluble in water. After hydrolysis, the precipitated hydrolysed Tween 80 accumulated around colonies representing positive lipase or esterase activity.

Results shown that isolates found positive to levan, were fluorescent while negative were non-fluorescent. Fluorescent strains exhibited green, diffusible fluorescent pigment, whereas non fluorescent isolates produced a brown diffusible pigment which is the characteristic of *R. solanacearum* (King *et al.*, 1954).

For oxidation and/or fermentation of glucose test, pH change was observed at the surface of open tubes resulting in color change from green to yellow. No pH change resulted in sealed tubes indicating that *R. solanacearum* which is aerobic bacterium that is responsible for such type of change in pH.

Biovar Identification of *R. solanacearum*: The race identification of *R. solanacearum* revealed that all the *R. solanacearum* confirmed isolates belonged to race 3 as the isolates were showing chlorosis. The isolates were then tested for biovar confirmation on the basis of utilization of sugars. For this test, olivaceous green to yellow color was documented as positive (+) and no change as negative (-). Among all the tested isolates 4 belonged to Biovar 1, 3 to Biovar 2 or 23 from Biovar 3 (Table 5 & Fig. 1). In terms of number of isolates from each crop, 8 isolates each were obtained from chili and tomato out of which 6 were from biovar 3 and 1 from each biovar 2 and biovar 1. From potato and eggplant 7 isolates each were obtained with 6 and 5 isolates respectively from biovar 3 (Fig. 2)

Pathogenicity test: Among the confirmed *R. solanacearum* isolates, 11 isolates that gave strong hypersensitive response were tested for pathogenicity variability i.e., RsAR-42, RsAR-36, RsAR-34, RsRN-31, RsMM-29, RsTR-28, RsAR-24, RsAD-16, RsAD-14, RsLe-2 and RsSP-1. Disease symptoms became visible after 4 days in soil drenching method. After 8 days, partial wilt symptoms were apparent in most of inoculated plants (Average symptoms scores $\geq$ 1), complete wilting occurred after 12 days (Average symptoms scores $\geq$ 2), death and collapse of seedlings occurred on 14th day (Average symptoms scores $\geq$ 3). In detached leaf method, disease symptoms were observed after 1 day of inoculation. After 4 days of inoculation, most of the leaflets showed partial yellowing (Average symptoms scores $\geq$ 1) and after 10 days, complete chlorosis occurred (Average symptoms scores $\geq$ 2). Total withering and collapse of inoculated leaves was observed on 12th day (Average symptoms scores $\geq$ 3) and some on 14th day of inoculation. 3 leaves showed withering and collapse on 3rd day.

Host specificity test: While investigating the host range of biovars of *R. solanacearum*, out of total isolates that were confirmed to be *R. solanacearum*, 4 belonged to biovar 1, 3 to biovar 2 and 23 isolates belonged to biovar 3. Each biovar was tested on each host plant for its specificity. Results showed that biovar 1 was not so much specific but it was more pathogenic in chili (Table 6). Biovar 2 was more prevalent and pathogenic to tomato and potato (Table 7). Most of the strains belonged to Biovar 3 and they were very much prevalent and highly pathogenic in almost all crops (Table 8).

Isolation and *In vitro* evaluation of rhizobacterial isolates against *R. solanacearum*: One hundred and five (105) rhizobacteria were isolated from the rhizosphere of tomato, potato, eggplant and chili. *In vitro* evaluation of these isolates showed that 20 were antagonistic against *R. solanacearum* with varying zones of inhibition ranging from minimum 0.13 cm to maximum 1.68 cm (Table 9).

Identification of rhizobacteria: Among 20 isolates, 19 belonged to genus *Pseudomonas* and 1 to *Bacillus* (TRB4). Among *Pseudomonas* spp. twelve were identified as *P. fluorescens* (PRH2, PRH3, PRB7, PRB10, ER3, ER5, EB6, EB8, PAC3, CP3, CP4, PTR6), four as *P. aeruginosa* (PAE2, PAP5, PAP7, PAT1) and three as *P. cepacia* (PCT1, PCT4, PCC2).

Table 3. Gram staining, loop test, Catalase oxidase, Kovacs oxidase reaction of *R. solanacearum* isolates.

Isolates	Gram reaction	Loop test	Catalase oxidase test (production of bubbles)	Kovacs oxidase test (production of purple color)
RsSP-1	G -	+	+	+
RsLe-2	G -	+	+	+
RsAD-6	G -	+	+	+ -
RsLe-8	G -	+	+	+
RsAD-13	G -	+	+	+ -
RsAD-14	G -	+	+	+
RsAD-16	G -	+	+	+ -
RsAD-17	G +	-	-	-
RsDN-18	G -	+	+	+ -
RsCN-19	G -	+	+	+ -
RsRA-20	G -	+	+	+ -
RsSH-22	G -	+	+	+
RsAR-23	G -	+	+	+
RsAR-24	G -	+	+	+
RsTR-25	G -	+	+	+ -
RsTR-28	G -	+	+	+
RsMM-29	G -	+	+	+
RsTT-30	G -	+	+	+ -
RsRN-31	G -	+	+	+
RsR-32	G -	+	+	+ -
RsAR-33	G -	+	+	+ -
RsAR-34	G -	+	+	+
RsAS-35	G -	+	+	+ -
RsAR-36	G -	+	+	+
RsHS-37	G -	+	+	+ -
RsDS-38	G +	-	-	-
RsIA-39	G -	+	+	+ -
RsTA-40	G -	+	+	+
RsAR-42	G -	+	+	+
RsAR-43	G -	+	+	+ -
RsSR-44	G -	+	+	+-
RsSR-45	G -	+	+	+ -

Gram staining: + = gram positive, - = gram negative
Loop test: + = made thread loop, - = do not made thread loop
Catalase oxidase test: - = no production of bubbles, **+ =** production of bubbles
Kovacs oxidase: + = production of purple color immediately, - = no production of purple color, +- = production of purple color in 60 sec, (moderately positive)

Evaluation of antagonistic bacteria against bacterial wilt under greenhouse conditions: The isolates that proved antagonistic in laboratory tests were tested for their biocontrol activity under greenhouse conditions on tomato plants. PRB10, PAP5, PAT1 and PTR6 were having the highest biocontrol efficiency as compared to control (Table 9). In some of the isolates, wilt symptoms appeared at initial stage after 15-20 days but later on completely free from the disease.

Discussion

In this study, *R. solanacearum* was isolated from wilt infected solanaceous crops i.e., tomato, eggplant, potato and chilli. Among 32 isolates obtained from the plants, 30 isolates were confirmed to be the *R. solanacearum* by subjecting the bacterium to various biochemical tests. The pinkish red centered colonies obtained on the TTC medium were the key character of the bacterium. It was further characterized for the gram staining, loop test, levan production from sucrose, catalase oxidase test, Kovac's oxidase test, lipase activity on tween 80 agar, production of fluorescent pigment, oxidation and/or fermentation of glucose. Isolates which were positive for hypersensitive reaction, KOH test, catalase test, Kovac's test and oxidation/fermentation of glucose, gave negative response in Gram staining and remained non-fluorescent and were supposed to be *R. solanacearum*. However, some isolates tested, showed differential results for Levan and Lipase production. So, it was found that 30 isolates out of 32 performed uniformly and those were considered as *R. solanacearum*.

Table 4. *R. solanacearum* isolate responses to levan test, lipase activity test and fluorescent production test.

Isolates	Production of Levan	Lipase activity	Production of fluorescence under UV
RsSP-1	-	+	-
RsLe-2	-	+	-
RsAD-6	-	+	-
RsLe-8	+	-	+
RsAD-13	+	-	+
RsAD-14	-	+	-
RsAD-16	-	+	-
RsAD-17	+	-	+
RsDN-18	-	+	-
RsCN-19	+	+	+
RsRA-20	-	+	-
RsSH-22	+	-	-
RsAR-23	+	-	+
RsAR-24	-	+	-
RsTR-25	-	+	-
RsTR-28	-	+	-
RsMM-29	-	+	-
RsTT-30	-	+	-
RsRN-31	-	+	-
RsR-32	+	-	+
RsAR-33	-	+	-
RsAR-34	-	+	-
RsAS-35	+	-	+
RsAR-36	-	+	-
RsHS-37	-	+	-
RsDS-38	+	+	+
RsIA-39	-	+	-
RsTA-40	+	-	+
RsAR-42	-	+	-
RsAR-43	-	+	-
RsSR-44	-	+	+
RsSR-45	-	+	-

Hypersensitive reaction was observed on non-host plant and the strong reaction was exhibited by 11 isolates while the rest 19 isolates were either moderately or slightly virulent. The pathogenicity tests were performed with these isolates in order to evaluate the degree of pathogenicity of the isolates. All of the isolates tested were pathogenic. The bacterium was then further classified for the race and biovar. All of the isolates belonged to race 3. Kado & Heskett (1970) and French *et al*. (1995) reported that *R. solanacearum* isolates used different sugars in varied pattern but Biovar1 did not utilize any sugar from sugar test. In Biovar 2 isolates, utilization of Cellubiose was faster after 2 days followed by Maltose and Lactose which took 3 days. In Biovar 3 isolates, Mannitol was utilized within 2 days and that of Maltose, Sorbitol, Lactose and Cellubiose took 4 days. On the basis of utilization of sugars, Biovar 3 was found more prevalent in Islamabad and Rawalpindi as compared to Biovar 2 and Biovar 3.

The most frequent and aggressive biovar was the R3b2 as 23 (77%) isolates belonged to this group (Fig. 1). The prevalence of R3b2 has been extended to many geographical zones and it is the most widely distributed and considered as the cold tolerant. R3b2 has the ability to persist under harsh cold conditions and thus due to this property, it has acquired the strength to survive under such conditions and cause diseases in wide range of plants. Until now, along with solanaceous plants, plants belonging to other families have been reported to be the host of *R. solanacearum*. Among them, most of the plants have been reported to be the host of the R3b2. According to Kelman *et al*. (1994) *R. solanacearum* is found on every continent and island from warm temperate to tropical regions. In our study, it was observed that *R. solanacearum* is present in every region with different degree of incidence. Current studies have helped in revealing the incidence and prevalence of the bacterial wilt in Rawalpindi and Islamabad which are temperate regions and are humid that makes the conditions favorable for the pathogen.

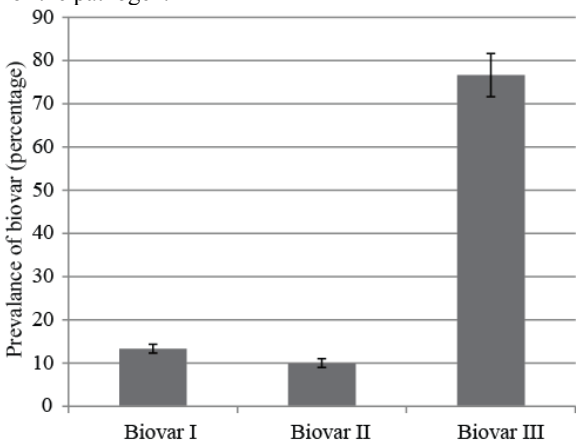

Fig. 1. Prevalence of each Biovar on solanaceous crops (Chilli, tomato, potato and eggplant) of Rawalpindi and Islamabad.

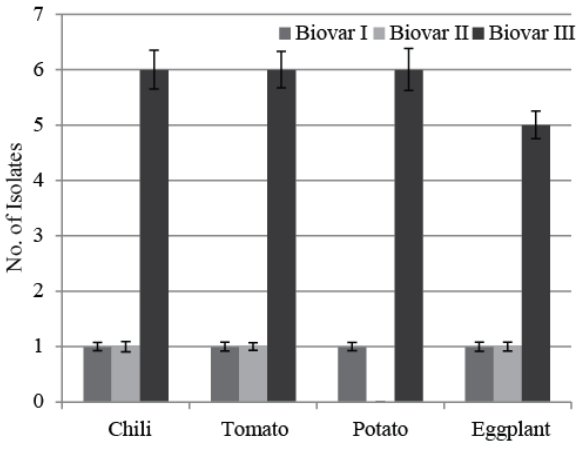

Fig. 2. Number of isolates of each Biovar from Chilli, tomato, potato and eggplant.

Table 5. Grouping of *R. solanacearum* isolates into biovars.

Isolates	Lactose	Maltose	Sorbitol	Mannitol	Cellubiose	Dulcitol	Biovars
RsSP-1	+	+	+	+	+	+	3
RsLe-2	+	+	+	+	+	+	3
RsAD-6	-	-	-	-	-	-	1
RsLe-8	+	+	+	+	+	+	3
RsAD-13	+	+	+	+	+	+	3
RsAD-14	+	+	-	-	+	-	2
RsAD-16	+	+	+	+	+	+	3
RsAD-17	+	+	+	+	+	-	0
RsDN-18	+	+	+	+	+	+	3
RsCN-19	-	-	-	-	-	-	1
RsRA-20	+	+	+	+	+	+	3
RsSH-22	+	+	+	+	+	+	3
RsAR-23	+	+	+	+	+	+	3
RsAR-24	+	+	+	+	+	+	3
RsTR-25	+	+	+	+	+	+	3
RsTR-28	+	+	+	+	+	+	3
RsMM-29	+	+	+	+	+	+	3
RsTT-30	-	-	-	-	-	-	1
RsRN-31	+	+	+	+	+	+	3
RsR-32	+	+	+	+	+	+	3
RsAR-33	+	+	+	+	+	+	3
RsAR-34	+	+	-	-	+	-	2
RsAS-35	+	+	+	+	+	+	3
RsAR-36	+	+	+	+	+	+	3
RsHS-37	+	+	+	+	+	+	3
RsDS-38	+	+	+	+	+	-	0
RsIA-39	+	+	+	+	+	+	3
RsTA-40	+	+	+	+	+	+	3
RsAR-42	-	-	-	-	-	-	1
RsAR-43	+	+	+	+	+	+	3
RsSR-44	+	+	+	+	+	+	3
RsSR-45	+	+	-	-	+	-	2

Table 6. Host range testing for *R. solanacearum* Biovar 1 isolates among solanaceous plants.

Host	Site of inoculation	Biovar 1 isolates	Control
Tomato	Root/Stem	[z]2/4	0/1
Potato	Stem	1/4	0/1
Chilli	Root/Stem	3/4	0/1
Eggplant	Stem	2/4	0/1

[z]The number isolates that were positive/ the number of isolates tested. Controls were treated with sterile water

Table 7. Host range testing for *R. solanacearum* Biovar 2 isolates among solanaceous plants.

Host	Site of inoculation	Biovar 2 isolates	Control
Tomato	Root/Stem	2/3	0/1
Potato	Stem	1/3	0/1
Chilli	Root/stem	2/3	0/1
Eggplant	Stem	1/3	0/1

[z]The number isolates that were positive/ the number of isolates tested. Controls were treated with sterile water

Table 8. Host range testing for *R. solanacearum* Biovar 3 isolates among solanaceous plants.

Host	Site of inoculation	Biovar 3 isolates	Control
Tomato	Root/Stem	20/23	0/1
Potato	Stem	21/23	0/1
Chilli	Root/stem	22/23	0/1
Eggplant	Stem	18/23	0/1

[z]The number isolates that were positive/ the number of isolates tested. Controls were treated with sterile water

Each biovar was then tested on each host plant for its specificity. Biovar 1 was not specific but it was pathogenic on chili. Biovar 2 was more prevalent and pathogenic to tomato and potato. Most of the isolates belonged to Biovar 3 and they were very much prevalent and very pathogenic in almost all crops. The tests elaborated that the symptoms expressed earlier in biovar 3 as compared to other biovars. Moreover, the degree of severity of the biovar 3 was more and number of plant infected by it was more than the other Biovars i.e. it infected all the solanaceous plants i.e. tomato, potato, eggplant and chilli.

R. solanacearum is a major pathogen on tomato, pepper, eggplant, strawberry, bean, ginger, mulberry and other crops (Hayward, 1994). Recently, geographical distribution of the pathogen has been extended to more temperate countries from Europe and North America as the result of the dissemination of strains belonging to race 3, biovar 2. These strains differentiated in Andean plateaus and as such are adapted to cooler environmental conditions.

The biocontrol ability of the rhizobacteria proved that four isolates belonging to *Pseudomonas* spp. i.e., PRB10, PAP5, PAT1 and PTR6 possessed the highest efficiency against the bacterial wilt of tomato under greenhouse conditions. Priou *et al*. (2005) recorded 80% reduction of the same disease on tomato plants under greenhouse conditions using *Pseudomonas putida*. Antagonistic *Pseudomonas* spp. was tested for their ability to suppress bacterial wilt in tobacco and some showed promising results (Liu *et al.,* 1999). Moreover Ran *et al*. (2005) also reported suppression of bacterial wilt in *Eucalyptus urophylla* (Blake) by fluorescent *Pseudomonas* spp.

There are several modes of action known for rhizobacteria applied for the control of plant diseases (Ran *et al*., 2005; Dwivedi & Johri, 2003). *Pseudomonas* exert a protective effect on the roots through antagonism towards phytopathogenic bacteria by producing metabolites that include: lytic enzymes (Berg, 1996); plant hormones and other plant growth promoting substances, e.g., auxins, indole-3-acetic acid, and gibberellins (Ramamoorthy & Samiyappan, 2001); siderophores (Dwivedi & Johri, 2003); and antibiotics (Dwivedi & Johri, 2003; Ran *et al*., 2005). *Bacillus* spp. is also known to produce a wide range of secondary metabolites such as antibiotics, non-volatile and volatile compounds (Parke & Sherman, 2001) and lytic enzymes (Frandberg & Schnurer, 1994).

Present studies helped in assessing the presence, prevalence and characterization of bacterial wilt pathogen in Rawalpindi and Islamabad regions of Pakistan having major cultivation of vegetables. Moreover it was revealed that R3b2 is the most prevalent and aggressive strain causing disease in all solanaceous crops under study. Biological control of this disease in tomato has proven effective in laboratory experiments and under greenhouse conditions, therefore it is suggested to integrate all the possible measures to overcome the devastating pathogen.

Table 9. Efficacy of PGPR *In vitro* and in greenhouse tests.

Isolates	Zone of inhibition (cm)	BCE (%)	Wilt incidence (%)
PRH2	0.37 ± 0.04 ij	68.75 ± 6.61 ghi	25 ± 5.29 efg
PRH3	0.53 ± 0.02 ghij	73.75 ± 7.81 efg	21 ± 6.24 ghi
TRB4	1.15 ± 0.12 b	81.25 ± 3.31 cdef	15 ± 2.65 hijk
PRB7	0.48 ± 0.06 ij	43.75 ± 11.11 kl	45 ± 8.89 bc
PRB10	0.92 ± 0.02 de	98.75 ± 0.43 a	1 ± 0.35 m
ER3	0.41 ± 0.09 ij	73.75 ± 7.60 efg	21 ± 6.08 ghi
ER5	1.03 ± 0.10 bcd	87.50 ± 3.75 abcd	10 ± 3 jklm
EB6	1.11 ± 0.11 bc	77.5 ± 3.31 defg	18 ± 2.65 ghij
EB8	0.53 ± 0.03 ghi	57.5 ± 8.75 ij	34 ± 7 de
PCT1	0.72 ± 0.06 f	71.25 ± 3.31 fgh	23 ± 2.65 fgh
PCT4	0.13 ± 0.05 k	35 ± 13.23 l	52 ± 10.58 b
PCC2	0.36 ± 0.04 j	66.25 ± 5.73 ghi	27 ± 4.58 efg
PAE2	0.49 ± 0.07 hij	61.25 ± 8.75 hij	31 ± 7 def
PAP5	0.78 ± 0.05 ef	97.5 ± 1.25 a	2 ± 1 m
PAP7	0.16 ± 0.01 k	52.5 ± 10.90 jk	38 ± 8.72 cd
PAT1	0.68 ± 0.04 fg	93.75 ± 3.75 ab	5 ± 3 lm
PAC3	0.65 ± 0.13 fgh	73.75 ± 9.92 efg	21 ± 7.94 ghi
CP3	0.39 ± 0.16 ij	76.25 ± 5.73 defg	19 ± 4.58 ghij
CP4	1.68 ± 0.29 a	83.75 ± 6.50 bcde	13 ± 5.20 ijkl
PTR6	0.97 ± 0.09 cd	91.25 ± 1.25 abc	7 ± 1 klm
Control	0 k	0 m	80 ± 9.64 a

Means followed by the same letter within a column are not significantly different as determined by the LSD test (P= 0.05). BCE: Biological control efficiency

Conclusions

This study concluded that race 3 and biovar 3 of *R. solanacearum* is widely prevalent in Rawalpindi and Islamabad and have the ability to infect all vegetables considered in this study. Isolates belonging to Biovar 3 were revealed as the most aggressive ones causing death of host much sooner than others. Biocontrol studies have shown that this method could be employed for the management of this devastating disease but if integrated management is opted this would be the best strategy to control this disease.

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