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Full Length Article

Biochemical and Serological Characterization of *Ralstonia solanacearum* Associated with Chilli Seeds from Pakistan

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Abstract

Ralstonia solanacearum (E.F. Smith) Yabuuchi, the cause of bacterial wilt of chilli, is one of the most severe pathogens of solanaceous crops with a very wide host range. It is mainly soil borne but recently it has also been reported from seed and water. The present studies were carried out to explore the chilli seeds as a potential source of aggravating this problem in the country. The seed samples were collected from major chilli growing areas and research institutes of Pakistan. The isolation and the initial characterization of strains were carried out on 2, 3, 5-triphenyltetrazolium chloride (TTC) and 523 media. Out of all the isolates tested, 72% showed positive hypersensitive reaction (HR) with variable response. Only 40% of these HR positive isolates were confirmed as *R. solanacearum* through biochemical, pathogenicity and Plate Trapped Antigen-Enzyme Linked Immunosorbent Assay (PTA-ELISA) tests. Further characterization was performed by biovar test and concluded that in tested isolates, 9% were biovar 1, 7% were bivar 2 and 84% were biovar 3. ELISA confirmed the presence of *R. solanacearum* with extreme aggressiveness of biovar 3 isolates. © 2015 Friends Science Publishers

Keywords: PTA-ELISA; Bacterial wilt; Biovars; Solanaceous crops; Hypersensitive response

Introduction

Production of vegetables is a profitable business in Pakistan as the environment and other conditions are highly favorable and conducive for the production of a number of vegetables including chilli, which comes after potato and tomato. This crop occupies 20% of the total area under vegetable cultivation and is mainly concentrated in Sindh and Punjab provinces while it is grown on limited area in Khyber Pakhtunkhwa (KPK) and Balochistan. Sindh produces 80% of chilli crop followed by Punjab (about 10%). The crop is concentrated in Layyah district of Punjab and Hyderabad, Kunri, Tharparkar areas of Sindh province. The area and production of chilli crop decreased 65.7% and 78.3%, respectively in Pakistan (GoP, 2012). There are about 150 different types of chilli, characterized on the basis of colour, shape and pungency (Berke, 2002). Chilli is mostly used as flavoring and imparts spiciness to cooked food and vegetables. It provides an excellent income generating opportunity to small farmers and also wealthy source of A and C vitamins (Nano-Womdim, 2001). It contains appreciable amount of minerals such as iron, phosphorus, sulphur, potassium and calcium (Berke, 2002). It gives soothing effects to the digestive system, relief from symptoms of cold, sore throats, fever, pain and arthritis (Milke *et al*., 2006).

Ralstonia solanacearum (Rs) (E.F. Smith) Yabuuchi *et al.* (Syn: *Pseudomonas solanacearum* or *Burkholderia solanacearum*) is one of the most severe and serious pathogen of solanceous hosts in the humid, subtropical and temperate places throughout world (Hayward, 1964, 1991; He *et al.,* 1983). It has also been reported from vegetables and ornamentals (Swanson *et al*. 2005; Ji *et al.* 2006), while Stevens and Van Elsas (2010) observed it from waterways and sediments. In Europe, *R. solanacearum* is a quarantine pest (Council Directive, 1998, 2000; Commission Directive, 2006) and in USA it is an important bioterrorism agent (Madden and Wheelis, 2003).

It is a Gram negative, aerobic, motile and rod shaped bacterium belonging to genus *Ralstonia*, β-subdivision of the class Proteobacteria (Yabuuchi *et al.,* 1995). Distinct geographical distribution of biovars and races of *R. solanacearum* suggest separate evolutionary origins among the strains. Race 1 strains can be found in humid areas throughout the world and attack many solanaceous crops i.e. pepper, tomato, and eggplant. It also attacks tobacco and many plants of other families. It requires an optimum temperature as high as 35°C. Strains of Race 2 attack banana and *Heliconia* and can be found mostly in hot places of South America. Race 3 strains are found at upper altitude in the tropics, subtropical and moderate areas. This race mostly attack potato and, some solanaceous weeds. Race 3 has a

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lesser optimum temperature as 27°C and appears mainly as biovar 2. Strains of Race 4 are mainly destructive on ginger, while strains of Race 5 (biovar 5) are particular on *Morus spp.* (French *et al.,* 1995). *R. solanacearum* is a ubiquitous, highly variable and adaptable plant pathogen on more than 50 plant families in 200 different species (Hayward, 1994; 2000), but causes colossal losses in chilli production (Begum, 2012). Begum (2005) identified Race 3 Biovar 2 and Biovar 3 of *R. solanacearum* in tomato growing areas of Punjab. The pathogen was also found to be seed-borne and Race 3 Biovar 3 was highly virulent on tomato. It has also been isolated and studied genetically and phenotypically from Dutch waterways, sediment and bittersweet plants (Stevens and Van Elsas, 2010).

R. solanacearum is a soil borne plant pathogen (Kelman, 1954), while its seed borne nature has also been reported (Roopali, 1994; Sumithra *et al.,* 2000). The identification of this pathogen was also carried out in Brazil from bell pepper (Garcia *et al.,* 2013) and its presence in hot and sweet pepper was reported by Begum *et al.* (2012). Cultivars showing resistant reactions to bacterial wilt under field conditions may carry the latent infection of the bacterium (Aggarwal and Sood, 2005). Mimura *et al.* (2009) confirmed that accession LS2341 is an appropriate source for the breeding of resistant cultivars of pepper in Japan and is a candidate for a potential source of resistance in other areas of the world. In another experiment seed transmission of the pathogen up to 45% was observed in seeds artificially infected with the pathogen (Umesha *et al*., 2005).

At present, bacterial wilt caused by *R. solanacearum* is extending its host range, aggressiveness and is posing a serious threat to crop production in Pakistan (Begum, 2005, 2011). The disease was first reported in potato growing area of the KPK (Geddes, 1989). Later on survey of Southern KPK, Punjab, Balochistan and some parts of Sindh revealed that the pathogen is present in all the four provinces (Burney and Ahmad, 1997). It has been observed that this pathogen can cause high vegetable production losses in the region (Burney and Ahmad, 1997). The presence of this bacterium in soil and possible spread through seeds is posing a serious threat to the cultivation of solanaceous vegetables in Punjab and Sindh (Burney *et al.,* 1999).

Currently this disease problem is becoming intense and is wreaking havoc to chilli production in Punjab and Sindh. The conditions during monsoon seasons succumb chilies to bacterial wilt, but this problem is often concealed and mystified with other disease problems. Based upon its seed borne nature, wide host range and prevalence of virulent isolates, this study was planned with the following objectives; to study the surveillance of *R. solanaceraum* isolates associated with chilli seeds from different chilli growing areas of Pakistan; to study the pathogenic and virulent behavior of collected isolates of *R. solanaceraum;* to characterize and differentiate the most virulent/aggressive isolates of *R. solanaceraum* based on morphological, pathological, biochemical and ELISA tests.

Materials and Methods

Collection of Samples

A total of 50 chilli seed samples were collected from major chilli growing areas of Pakistan. Seed samples were collected from chilli fields and Research Institutes mainly from Sindh and Punjab, also from seed stores. Mainly the following areas were covered Kunnri, Khair Pur, Mir Pur Khas, Kohat, Qila Saifulla, Faislabad, Kasur and Multan. Seed samples were also obtained from various vegetable seed stores in Rawalpindi, Horticultural Research Institute, National Agricultural Research Center, Islamabad and Federal Seed Certification and Registration Department, Islamabad, Pakistan.

Isolation of Bacterial Pathogens

TTC media (Hugh and Leifson, 1953) and 523 media (Kelman, 1954) were used for isolation and maintenance of bacterial pathogenic isolates. TTC media (one liter) contained Peptone (10 g), Casein Hydrolysate (1.0 g), Glucose (5.0 g) and Agar (15 g), while 523 media (one liter) was made with $MgSO_4.7H_2O$ (0.3 g), K_2HPO_4 (0.2 g), Yeast Extract (4.0 g), Casein Hydrolysate (8.0 g), Sucrose (10 g) and Agar (15 g). In TTC media 5 ml of 1% 2, 3, 5-triphenyltetrazolium chloride was added to the sterilized medium before pouring into the plates. Following methods were used for isolation of *R. solanacearum* from seeds.

1. Seeds were surface sterilized with 4% Clorox for 1 min, directly plated on TTC media. Media plates were incubated for 2-3 days at 30°C. After that colonies of bacteria (reddish pink and fluidal) were picked and streaked on new TTC media plates for purification.
2. Chilli seeds were surface sterilized, placed in test tubes containing nutrient broth medium and kept on shaker at 28°C for 2-3 days. Bacterial suspensions from the medium were streaked on TTC media plates and then purified cultures were made from single colonies.
3. Seeds after surface sterilization were placed in test tubes containing sterilized distilled water and kept on shaker at 28°C for 2-3 days. A loop full of turbid suspension was streaked on TTC plates and then purified cultures were made from single colonies.
4. Seeds after surface sterilization crushed in pestle and mortar containing sterilized distilled water. Then placed in test tubes containing nutrient broth medium and kept in shaker at 28°C for 2-3 days. A loop full of turbid suspension was streaked on TTC plates and then purified cultures were obtained from single colonies. The bacterial cultures were stored in 523 medium.

Characterization of *R. solanacearum*

Preparation of inocula: Strains from preserved cultures were streaked on plates having TTC media. Fluidal colonies

with red or pink center were transferred to 523 media plates and incubated at 30°C for overnight. A very turbid bacterial suspension of bacterial cultures was prepared by transferring bacterial mass from 523 media plates into a tube with 5 mL sterile distilled water for each culture. Cell density was adjusted to 10^8cfu/ml (through Genie spectrophotometer at 620 nm) for each bacterial isolate.

Hypersensitivity test: Hypersensitive reaction was performed by two methods on tobacco plants *(Nicotiana tabacum* cv. *burley)* and detached leaf of *Euphorbia neriifolia* Linn. Plants were raised in the pots *in vitro* and were inoculated at 2-3 leaves stage while the fresh detached leaves of *E. neriifolia* were used for inoculation.

Isolates of *R. solanacearum* for inoculations were multiplied on 523 medium for 48 h prior to inoculation. Bacterial suspensions were prepared in distilled sterilized water and 10^8 cfu/mL, cell density was adjusted through Genie spectrophotometer at 620 nm. Using 1 mL plastic disposable syringe with 25 mm gauge needle, cell suspensions were injected into abaxial side of the mature tobacco leaves and onto the detached leaves of *Euphorbia*. Alternating interveinal regions were infiltrated with bacterial suspension. Each injected area was labeled with appropriate letters. A fine mist of water was lightly sprayed over the whole plants and also on the detached leaves to avoid runoff. Plants were then covered with clear plastic bags (to provide RH of 90%) and placed at room temperature (25-27°C), while detached leaves were placed in Petri dishes containing wet filter papers to maintain RH for almost 24 h. Data were recorded 1, 3 and 7 days after inoculations (Klement *et al.*, 1964).

Biochemical tests for confirmation of *R. solanacearum*: Isolates were studied according to specific biochemical tests for *R. solanacearun* i.e., gram staining (Schaad, 1980), potassium hydroxide test (Suslow *et al.,* 1982), catalase oxidase test (Schaad*,* 1980), kovacs oxidase test, levan production from sucrose (Schaad, 1980), lipase activity on tween 80 agar (Sierra, 1957), production of fluorescent pigment (King *et al.,* 1954) and Oxidation and/or Fermentation of glucose (Hayward, 1964).

Grouping into biovars: Basal medium for the Biovar test was prepared with the same composition and procedure as explained by Hayward (1964). Lactose, Maltose, Cellobiose, Mannitol, Sorbitol and Dulcitol (Each at 10%) were added in sterilized screw-capped test tubes (10 mL each) 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 60 to 70°C. Each 10 mL carbohydrate solution was added and mixed in basal media. 200 µL quantities were dispensed into pre-labeled ELISA plates (90 wells). These medium plates were kept at room temperature for solidification. For control 10 mL of distilled sterilized water instead of sugar solution was used into the basal medium.

Bacterial suspensions from individual isolates (with concentration of 10^8 cfu/mL) were prepared from 48 h old cultures on 523 media plates. Inoculation of the medium was carried out by adding 50 µL of bacterial suspension to each well containing sugar solutions. For each isolate, 2 replicates were maintained for individual sugars. Tissue culture plates were incubated at 30°C and examined after 2, 7 and 14 days for the presence of the indicator change from olivaceous green to orange color at the surface of the medium.

Pathogenicity test: Soil was prepared by the integration of farm yard manure, sand and clay in 1:1:1 ratio. This mixture was sterilized with 37% formalin by 1:9 ratio (1 part formalin and 9 parts soil). Soil mixture was covered with polyethylene sheet and placed for 3-4 days in sun light. Afterwards the sheet was removed and soil was exposed to sun light for about 5 days to release fumes. Then in this soil chilli seeds were grown in pots. 21 day (4-5 leaf stage) old seedlings were used for performing pathogenicity tests.

Inoculation Methods

a). Soil drenching: Plants were not watered a day before inoculation to reduce moisture in the pots. Roots of chilli seedlings were slightly injured by inserting a scalpel in the pots in order to facilitate bacterial infection. About 10 mL of bacterial suspension (10^8 cfu/mL) was poured on the surface of each pot. Inoculated plants were regularly watered and kept at temperature range from 28 to 30°C and 90% relative humidity (Winstead and Kelman, 1952).

b). Detached leaf method: For this method terminal leaves containing 2-3 leaflets were selected and detached from the plants. These leaves were dipped in bacteria suspensions (10^8 cfu/mL) for 1-2 min and put in petriplates (15 cm diameter). These plates were placed at 12 h day and light regime in growth chamber at temperature range from 28-30°C and 90% relative humidity. Inoculated leaves were observed for appearance of symptoms as explained by Winstead and Kelman (1952).

Based on the reaction of various isolates after 14 days of inoculations, pathogenicity was recorded as follows:

Symptoms	Disease severity	Pathogen response
For soil drenching		
No symptom	0	Avirulent
Partial wilting	1	Weakly virulent
Complete wilting	2	Virulent
Plant collapsed and dead	3	Highly virulent
For detached leaf method		
No symptom on inoculated leaf	0	Avirulent
Partial yellowing of inoculated leaf	1	Weakly virulent
Complete chlorosis of inoculated leaf	2	Virulent
Total collapse of inoculated leaf	3	Highly virulent

Plate Trapped Antigen-Enzyme Linked Immunosorbent Assay (PTA-ELISA)

The cultures from 523 medium were grown in nutrient broth medium at 28°C for 3 days on electronic shaker. Culture

tubes were centrifuged at 1000 rpm so that all bacterial culture should be settled down. The following protocol was used for performing the PTA-ELISA for *R. solanacearum,* following Clark and Adams (1977) with some amendments:

1. In the culture tubes, 2mL extraction buffer was added and shaken on vertex mixture vigorously. The material was heated in water bath 70–80^{o}C for 10 min to break the bacterial cells.
2. After extraction, 100 µL of each sample, positive and negative controls was added to the wells of micro-titer plates. All samples and controls were used in duplicate to avoid error.
3. The plates were wrapped tightly in cling film or tissue paper and incubated at 4^{o}C overnight.
4. The plates were washed three times with PBST (Phosphate Buffer Saline+Tween 20). For this purpose, the wells were filled with PBST and plates were inverted to remove buffer. Repeated twice, and then the plates were placed on paper towel for drying.
5. In each well added 200 µL of blocking buffer.
6. Plates were wrapped as explained in (3) and incubated at 37^{o}C for 1 h.
7. Again washed the plates as described in (4) above.
8. Then the probe antibody was diluted and added 100 µL to each test wells.
9. Plates were wrapped as explained in (3) and incubated at 37^{o}C for 2 h.
10. Plates were washed as explained in (4).
11. Conjugate antibody was supplemented in each well @100 µL.
12. Plates were wrapped as explained in (3) and incubated at 37^{o}C for 1 h.
13. The plates were washed 4 times as given above (4). An extra wash stage was included to ensure that the entire unbound conjugate antibody was removed from the wells.
14. Then 100 µL of prepared substrate was added in each well.
15. Plates were wrapped as explained earlier (3) and incubated at room temperature for 1 h.
16. The observations were recorded by reading the absorbance of tested wells at 405 nm by ELISA plate reader.

Statistical Analysis

The disease severity data obtained after pathogenicity tests were analyzed by using statistix and SPSS programs to find out significant difference among isolates, biovars and pathogenicity methods.

Results

Isolation

R. solanacearum causing wilting in chilli plants was isolated from chilli seeds. Fluidal pinkish red centered colonies, typical of *R. solanacearum* were observed on TTC medium when chilli seeds were placed on the medium by different procedures but the best procedure was to crush seeds in distilled sterilized water then put in TTC broth medium for isolation (Fig. 1). Typical colonies were picked and purified for confirmation of *R. solanacearum* (Fig. 2). Total 47 isolates were obtained which are shown in Table 1. Bacterial cultures were purified on 523 media and stored in sterile distilled water for pathogenicity and confirmatory tests.

Hypersensitivity Test

All isolates were tested for hypersensitive response on tobacco plants and detached leaves of *Euphorbia*. In HR positive isolates slight localized chlorosis (+) followed by necrosis (++) and collapse (+++) of whole tissue was evident on Tobacco and detached leaves (Fig. 3-4). Depending upon the appearance of hypersensitive reaction, the isolates producing symptoms within 24 h were considered as strongly pathogenic. Isolates that developed symptoms after 72 h were considered weakly pathogenic, while isolates that showed symptoms in 7 days were considered as slightly pathogenic. Table 1 shows the average reaction of both the procedures. The detached leaves method was fast and clear as compared to the reaction on tobacco plants. The isolates RsLR-8, RsKM-15, RsMT-17, RsHP-21, RsK2-23, RsD-25, RsHA-27, RsHT-28, RsSP-29, RsSP-33, RsLe-40, RsLe-41, RsAS-45, RsAS-48 and RsAS-52, gave strong hypersensitive response (strongly pathogenic), while RsT-4, RsM-7, RsTP-22, RsHT-26, RsKR-30, RsMS-32, RsLe-36, RsHS-39, RsAS-42, RsAS-49 and RsAS-51 showed moderately positive hypersensitive response (weakly pathogenic).

Biochemical Tests

On the basis of all tests performed, isolates, which were positive for hypersensitive reaction, KOH loop test, Catalase test, Kovacs test and oxidation of glucose, while they gave negative response in Gram staining and remained non-fluorescent were assumed as *R. solanacearum.* However, some isolates tested in this study showed differential behavior for Levan and Lipase production. We concluded that 32 isolates out of 47 performed uniformly and those can be considered as *R. solanacearum* (Fig. 5-11; Table 1).

Grouping into Biovars

Biovar test was performed on all isolates that were confirmed to be *R. solanacearum* (on the basis of all biochemical tests carried out). Color change from olivaceous green to yellow was recorded as positive (+) for this test and no change as negative (-). By utilization of specific sugars grouping of these isolates was carried out. All these isolates fell either in Biovar 1, Biovar 2 or Biovar 3 on the basis of utilization of different sugars (Fig. 12) (Table 1). Isolates RsLe-40, RsAS-42, RsAS-49 and RsAS-52 fell in Biovar 1 category, while RsAS-41, RsAS-45 and

Table 1: Results of *R. solanacearum* identification, biochemical and serological characterization

Isolates	Variety	Source	1	2	3	4	5	6	7	8	9	Utilization of sugars						10	11	12
												i	ii	iii	iv	v	vi			
RsLD-1	Longi Dwarf	Sindh	+	-	+	+	+	+	-	+	-	+	+	+	+	+	+	3	-	-
RsLT-2	Longi Tall	Sindh	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	3	-	-
RsT-4	Talhar	Sindh	++	-	+	+	+	-	+	-	+	-	+	+	+	+	-	0	-	-
RsLL-6	Longi Long	Sindh	-	+	-	-	-	+	+	+	-	+	+	+	+	+	+	3	-	-
RsM-7	Maxi	Sindh	++	-	+	+	+	-	+	-	+	+	+	+	+	+	+	3	-	-
RsLR-8	Longi Round	Sindh	+++	-	+	+	+	+	-	-	+	+	+	+	+	+	+	3	+	+
RsK-10	Field sample	Kohat	-	+	-	-	-	+	-	+	-	+	+	+	+	+	+	3	-	-
RsSS-11	Field sample	Faizabad	-	-	+	+	+	+	-	+	-	+	+	+	+	+	+	3	-	-
RsKM-14	Maxi	Kunri	+	-	+	+	+	-	+	-	+	+	+	+	+	+	+	3	-	-
RsKM-15	Field sample	Sindh	+++	-	+	+	+	-	+	-	+	+	+	+	+	+	+	3	+	+
RsKM-16	Field sample	Kunri	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	3	-	-
RsMT-17	Medium Tall	Sindh	+++	-	+	+	+	-	+	-	+	+	+	+	+	+	+	3	+	+
RsSM-18	Shimla March	Sindh	-	-	+	+	+	-	+	-	-	+	+	+	+	+	+	3	-	-
RsK1-19	Kunri 1	Sindh	+	-	+	+	+	-	+	-	+	+	+	+	+	+	+	3	-	-
RsHP-20	Hot Pepper	Sindh	-	-	+	+	+	-	+	-	-	+	+	+	+	+	+	3	-	-
RsHP-21	Hot Pepper	Okara	+++	-	+	+	+	-	+	-	+	+	+	+	+	+	+	3	+	+
RsTP-22	Tall Point	Sindh	++	-	+	+	+	-	+	-	+	+	+	+	+	+	+	3	-	+
RsK2-23	Kunri 2	Sindh	+++	-	+	+	+	-	+	-	+	+	+	+	+	+	+	3	+	+
RsN-24	Nageena	Sindh	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	3	-	-
RsD-25	Desi seed	Punjab	+++	-	+	+	+	-	+	-	+	+	+	+	+	+	+	3	+	+
RsHT-26	Hot Pepper	Bolochistan	++	-	+	+	-	+	-	+	+	+	+	+	+	+	+	3	-	-
RsHA-27	PGRI	Hafizabad	+++	-	+	+	+	-	+	-	+	+	+	+	+	+	+	3	+	+
RsHT-28	Hot Pepper	Punjab	+++	-	+	+	+	-	+	-	+	+	+	+	+	+	+	3	+	+
RsSP-29	Sweet Pepper	Punjab	+++	-	+	+	+	-	+	-	+	+	+	+	+	+	+	3	+	+
RsKR-30	Field	Kunri	++	-	+	+	+	-	+	-	+	+	+	+	+	+	+	3	-	-
RsLT-31	Longi Tall	MirPurKhas	-	+	-	-	-	+	-	+	-	+	+	+	+	+	+	3	-	-
RsMS-32	Maxi Long	Sindh	++	-	+	+	+	+	-	+	+	+	+	+	+	+	+	3	-	+
RsSP-33	Sweet Pepper	Islamabad	+++	-	+	+	+	-	+	-	+	+	+	+	+	+	+	3	+	+
RsLe-34	Le 467	Islamabad	-	-	+	+	+	+	+	+	-	+	+	+	+	+	+	3	-	-
RsLe-35	Le 464	Islamabad	-	+	-	-	-	+	-	+	-	+	+	+	+	+	+	3	-	-
RsLe-36	Le 458	Islamabad	++	-	+	+	+	-	+	-	+	+	+	+	+	+	+	3	-	-
RsC-37	C-4	Islamabad	-	-	+	+	+	+	-	+	-	+	+	+	+	+	+	3	-	-
RsLe-38	Le 231	Islamabad	+	-	+	+	+	-	+	-	+	+	+	+	+	+	+	3	-	-
RsSH-39	Siam Hot	Punjab	++	-	+	+	+	-	+	-	+	+	+	+	+	+	+	3	-	-
RsLe-40	Le 533	Islamabad	+++	-	+	+	+	-	+	-	+	-	-	-	-	-	-	1	+	+
RsAS-41	Field sample	Rawalpindi	+++	-	+	+	+	-	+	-	+	+	+	-	-	+	-	2	+	+
RsAS-42	Field sample	Rawalpindi	++	-	+	+	+	-	+	-	+	-	-	-	-	-	-	1	-	-
RsAS-43	Field sample	Rawalpindi	-	-	+	+	+	-	+	+	+	-	+	+	+	+	-	0	-	-
RsAS-44	Field sample	Rawalpindi	+	-	+	+	+	-	+	-		-	+	+	+	+	-	0	-	-
RsAS-45	Field sample	Rawalpindi	+++	-	+	+	+	-	+	-	+	+	+	-	-	+	-	2	+	+
RsAS-46	Field sample	Rawalpindi	-	+	-	-	-	+	-	+	-	+	+	+	+	+	+	3	-	-
RsAS-47	Field sample	Rawalpindi	-	-	+	+	+	-	+	-	+	+	+	+	+	+	+	3	-	-
RsAS-48	Field sample	Rawalpindi	+++	-	+	+	+	-	+	-	+	+	+	+	+	+	+	3	+	+
RsAS-49	Field sample	Rawalpindi	++	-	+	+	+	+	-	-	+	-	-	-	-	-	-	1	-	+
RsAS-50	Field sample	Rawalpindi	-	-	+	+	+	-	+	+	-	-	+	+	+	+	-	0	-	-
RsAS-51	Field sample	Rawalpindi	++	-	+	+	+	-	+	-	+	+	+	-	-	+	-	2	-	+
RsAS-52	Field sample	Rawalpindi	+++	-	+	+	+	-	+	-	+	-	-	-	-	-	-	1	+	+

Hypersensitive reaction, 2. Gram staining, 3. KOH loop test, 4. Catalase oxidase test, 5. Kovaes oxidase test, 6. Levan production, 7. Lipase production, 8. Fluorescence, 9. Oxidation of glucose, Utilization of sugars (i. Lactose, ii. Maltose, iii. Sorbitol, iv. Mannitol, v. Cellubiose, vi. Dulcitol), 10. Biovars 11. Pathogenicity (only 15 isolates), 12. PTA- ELISA

RsAS-51 under Biovar 2, while all the other isolates were categorized as Biovar 3.

It was concluded that out of all the isolates tested, 72% showed positive HR and were confirmed through different biochemical tests as *R. solanacearum.* Among these, 84% were proved as Biovar 3, while 9% as Biovar 1 and 7% as Biovar 2. Some isolates i.e. RsT-4, RsAS-43, RsAS-44 and RsAS-50 showed variable response against sugars, which didn't match any biovar we assigned as zero (0).

Pathogenicity Test

Among the confirmed *R. solanacearum* isolates, 15 isolates i.e. RsLR-8, RsKM-15, RsMT-17, RsHP-21, RsK2-23, RsD-25, RsHA-27, RsHT-28, RsSP-29, RsSP-33, RsLe-40, RsLe-41, RsAS-45, RsAS-48 and RsAS-52, which gave strong hypersensitive response were studied for pathogenic variability.

In soil drenching method, disease symptoms became visible after 4 days of inoculation. In most of the inoculated plants partial wilt symptoms were apparent after 8 days (average symptom scores ≥1.5), complete wilting occurred after 12 days (average symptom scores ≥2.5), death and collapse of seedlings occurred on 14th day (average symptom scores ≥3). In detached leaf method disease symptoms were obvious after 1 day of inoculation. Most of the leaflets

Fig. 1: Bacterial in TTC broth medium

Fig. 2: Colonies on TTC medium

Fig. 3: HR of Tobacco to an isolate

Fig. 4: HR of Euphorbia

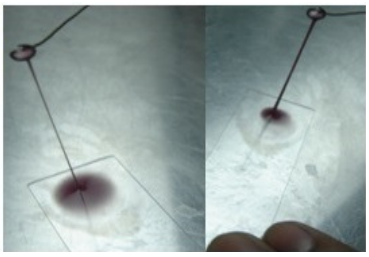

Fig. 5: KOH Test

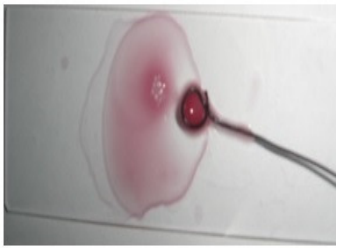

Fig. 6: Catalase oxidase test

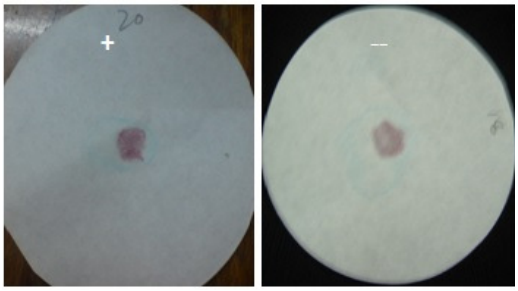

Fig. 7: Kovas oxidase test

Fig. 8: Levan production test

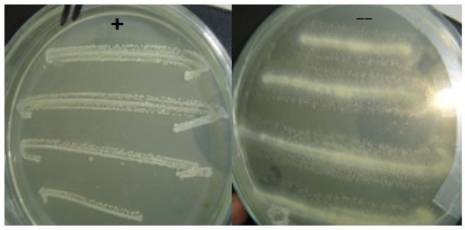

Fig. 9: Lipase Activity

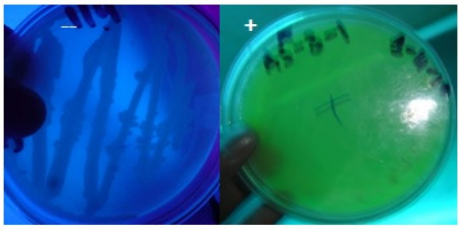

Fig. 10: Fluorescent pigment test

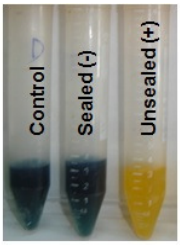

Fig. 11: Oxidation of glucose

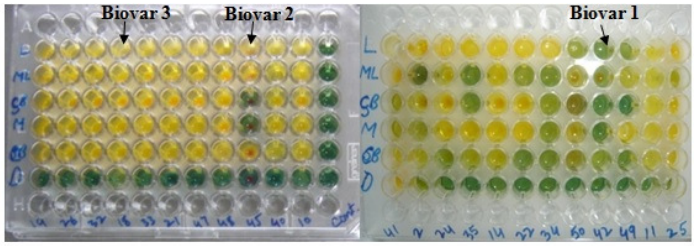

Fig. 12: Biovar test

showed partial yellowing after 4 days of inoculation (average symptom scores ≥1.5). Complete chlorosis occurred after 10 days (Average symptom scores ≥ 2.5), eventually total withering and collapse of inoculated leaves were apparent on 12th day (Average symptom scores ≥3) but some on 14th day of inoculations and, 3 leaves showed wilting and collapse on 3rd day i.e. RsHP-21, Rsk2-23 and RsAS-52.

In *R. solanacearum* pathogenicity test, detached leaf method was more efficient followed by soil drenching method. In both methods of inoculations Biovar 3 isolates i.e. RsKM-15, RsHP-21, RsD-25, RsHA-27, RsHT-28 and RsSP-29 appeared most aggressive in inciting disease

Fig. 13: Soil drenching method

Fig. 14: Detached leaf method

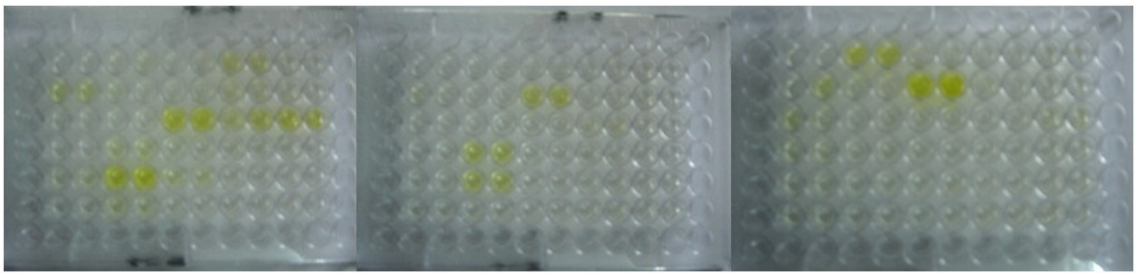

Fig. 15: PTA-ELISA results

symptoms as compared to Biovar 2 isolates, RsAS-41 and RsAS-45 and Biovar 1 isolates, RsLe-40 and RsAS-52 (Table. 1 and Figs. 13-14). Mostly complete death of inoculated plants and leaflets was observed in Biovar 3 but in Biovar 1, inoculated plants were showing wilting and chlorosis of leaflets. Final evaluation after 14 days revealed that Biovar 3 isolates were highly virulent and causing losses in all provinces mostly in Punjab, Sindh and Islamabad while Biovar 1 and 2 were also virulent and causing losses in Punjab and Islamabad, and Punjab respectively. Among all the biovars identified, Biovar 3 was found most prevalent and aggressive (Fig. 16; Table 2).

PTA-Enzyme Linked Immunosorbent Assay

All the 47 *R. solanacearum* isolates were confirmed through PTA-ELISA. The isolates were loaded in ELISA plates along with diseased and healthy controls. The reaction was visually observed in all wells and also measured by ELISA reader at absorbance value of 405nm (Fig. 15). The mean values of positive and negative controls were 0.185 and 0.118, respectively while absorbance in blank well was 0.119. All the isolates showed different level of reaction strength in ELISA test. The isolates RsLR-8, RsKM-15, RsMT-17, RsHP-21, RsTP-22, RsK2-23, RsD-25, RsHA-27, RsHT-28, RsSP-29, RsMS-32, RsSP-33, RsLe-40, RsLe-41, RsAS-45, RsAS-48, RsAS-49, RsAS-51 and RsAS-52 showed positive reaction in PTA-ELISA. The Biovar 3 isolates showed more reaction and their reading ranged between maximum value of 1.147 and minimum value of 0.945. The Biovar 1 and 2 gave less reaction as compared to Biovar 3, their isolates showed reading ranged between maximum value of 0.739 and minimum value of 0.688 and maximum value of 0.855 and minimum value of 0.778, respectively.

Discussion

Cultural traits on different media are important tool for their identification. For *R. solanacearum,* these are best studied by using TTC medium. Virulence of an isolate can be determined on the basis of colony traits on this particular medium. Fluidal colonies with reddish pink center and irregular margins are usually virulent while dark colored non-fluidal colonies with darker colors and smooth margins are normally avirulent. These results are in agreement with the findings of Hugh and Leifson (1953), Kelman (1954), King *et al.* (1954), Kovacs (1956), Sierra (1957), Thornley (1960), Schaad (1980), Suslow *et al.* (1982), Kado and Heskett (1990), French *et al.* (1995), Garcia *et al.* (1999) and Melo *et al.* (1999). Gram reaction by staining is a necessary initial step for the identification and classification of bacteria from any source. The outcome of this test depends upon the fact that Gram negative bacteria have literally fragile cell walls, which are bound by an outer membrane. This membrane is readily disrupted on exposure to 3% KOH releasing the viscous DNA. Gram-positive bacteria by contrast possess a thicker, more rigid wall which resists the disruptive effect of KOH (Suslow *et al.,* 1982). All gram negative bacteria produced gas bubbles when these were mixed with a drop of H_2O_2 on glass slide. H_2O_2 is a byproduct of aerobic respiratory metabolism in aerobic bacteria. Production of gas bubbles gives a clue for presence of aerobic bacteria (Schaad, 1980).

In Kovacs oxidase test positive isolates produced purple color when mass of bacterial growth is rubbed on filter paper impregnated with oxidase reagent. This test is used for differentiation between aerobic and anaerobic bacteria (Kovacs, 1956). The *R. solanacearum* colonies over 523 media were tested for levan production. Colonies of levan negative cultures were fluidal, opaque that tends to

Table 2: Mean disease severity of isolates, biovars and pathogenicity methods

Isolates	Disease Severity	Biovars	Disease Severity	Pathogenicity Methods	Disease Severity
RsHP-21	3.0000±0.0000a	3	2.624±0.0477a	Detached Leaf	2.6711±0.0407a
RsSP-29	2.9167±0.0833ab	1	2.400±0.0444b	Soil Drenching	2.4400±0.0626b
RsHA-27	2.8333±0.1667ab	2	2.333±0.0890b		
RsK2-23	2.7500±0.1708ab				
RsD-25	2.6667±0.1667ab				
RsHT-28	2.6667±0.1667ab				
RsKM-15	2.6333±0.0882ab				
RsAS-52	2.4500±0.0342ab				
RsAS-45	2.3833±0.1302ab				
RsLR-8	2.3833±0.1537ab				
RsSP-33	2.3833±0.1537ab				
RsLe-40	2.3500±0.0806b				
RsMT-17	2.3333±0.1054b				
RsAS-48	2.3000±0.1414b				
RsAS-41	2.2833±0.1302b				
CV	12.30		13.94		13.86

Different letters in the same column show significant difference at 0.05 probability level

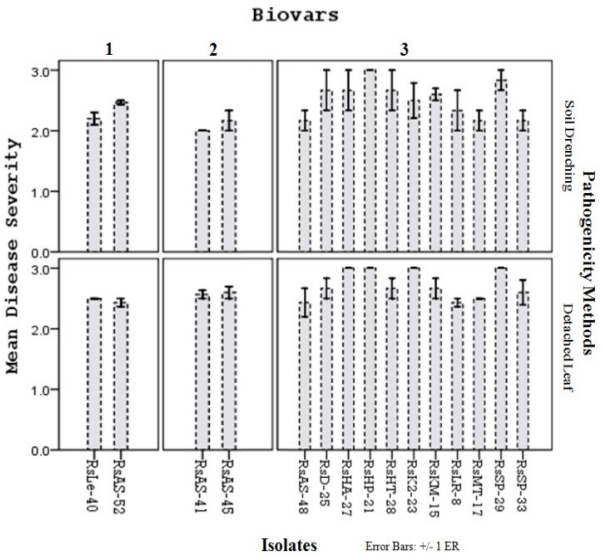

Fig. 16: Mean disease severity of isolates by pathogenicity methods

coalesce. Levan positive cultures produced colonies that were raised convex and mucoid. Levan (poly-fructose) is produced from sucrose by some fluorescent *Pseudomonas spp.* like pathovars of *Pseudomonas syringae*. Production of Levan as an extra cellular capsule or slime layer results in colonies, which are characteristically raised, convex and dome shaped in appearance, mucoid in consistency and opalescent in appearance (Schaad, 1980). This test helps in sorting out fluorescent from non-fluorescent *Pseuodomonas.*

According to Sierra (1957) hydrolysis of Tween 80 is a sign of lipolytic activity or, more correctly, esterase activity, because the substrate is freely soluble in water. After hydrolysis of Tween 80 by *R. solanacearum* isolates, the precipated hydrolysed Tween 80 accumulated around colonies indicating positive lipase/esterase activity. The tested isolates varied in formation of precipitates. The positive isolates of this test formed dense precipitate around the bacterial growth, evident mostly after 3-4 days of incubation. All Levan negative isolates were positive for lipase production but RsKM-16, RsN-24 and RsLe-34 were positive for both the tests. Presence of green, diffusible, fluorescent pigment was evident in fluorescent strains, whereas non fluorescent cultures produced a brown diffusible pigment, which is the characteristic of *R. solanacearum* (King *et al.,* 1954). All the hypersensitive positive (HR +ve) and Levan tested, 48 h old *R. solanacearum* cultures on Kings B Medium were examined under short and long wavelength UV radiation (UV Lamp) and intensity of fluorescence was compared. All Levan positive isolates were found fluorescent while remaining were non fluorescent.

The members of family *Psudomonadaceae* such as genus *Pseudomonas* and *Ralstonia* are oxidative in nature as compared to members of family *Enterobacteriaceae* like *Erwinia,* which are fermentative bacteria (Hugh and Leifson, 1953; Hayward, 1964). There was a gradual pH change at the surface of the open tubes resulting in color change from green to yellow, no pH change was observed in sealed tubes indicating that *R. solancearum,* which is aerobic bacterium, is responsible for such type of change in pH. All the hypersensitive positive isolates were found positive for oxidation of glucose. *R. solanacearum* isolates varied in utilization pattern of different sugars but Biovar 1 didn't utilize any sugar from sugar test (Kado and Heskett, 1990; French *et al.,* 1995). In Biovar 2 isolates, utilization of Cellubiose was faster (after 2 days) than Maltose and Lactose (3 days). In Biovar 3 isolates, Mannitol was utilized within 2 days followed by Maltose, Sorbitol, Lactose and Cellubiose which took 4 days. From sampling sources, Biovar 3 seems more prevalent in all the provinces and in Islamabad, Biovar 2 is restricted to Punjab only while biovar 1 is present in Islamabad and Punjab (Table 1).

Soil drenching is an efficient method for determining pathogenicity of bacteria especially *R. solnacearum*

(Winstead and Kelman, 1952). Detached leaf method has not been used so far for conducting the pathogenicity tests of *R. solnacearum* (Fig. 16; Table 2). Koizumi (1982) used detached leaves to study the relationship between growth of *Xanthomonas citri* and symptom development. An Asiatic strain of *X. citri* multiplied rapidly in detached leaves than in attached leaves of citrus plants. One possible reason for early symptom development with detached leaf method might be that bacteria moved efficiently in the vascular system from injured base of chilli leaflet and caused blockage of vascular system as a result of colonization and tylosis, thereby resulting in yellowish and chlorotic appearance of leaf tissues. While performing the pathogenicity test, isolates showed variable reaction, both for the time taking in appearance of initial symptoms as well as their severity.

In different utilization pattern of various sugars; pathogenic variability and virulence pattern of *R. solanacearum* belonging to Biovar 1, Biovar 2 and Biovar 3 indicates genetic variability among different strains of pathogen. By responding to specific environmental signals, regulatory genes allow bacteria to adapt to changes in their habitat. High virulence of Biovar 3 strains propose their wide host range and compatibility with number of environmental factors favorable for disease appearance such as temperature, rainfall, soil type, inoculum potential, and other soil biological factors such as wilt complexes formed among nematodes (*Meloioigyne spp*), Fungi (*Fusarium spp.*) and *R. solanacearum*.

Occurrence of these Biovars in all chilli seeds collected from field, research stations and seed stores strongly indicate a possible threat of collapse of chilli yield in coming years. *R. solanacearum* is a quarantine pest in potato but not in other solanaceous crops. Moreover current environmental changes of hot humid conditions also favor development of bacterial wilt caused by *R. solanacearum.* This study indicates the possibility of devastating spread of this pathogen from chilli seed infection and also further spread to other solanaceous hosts such as tomato, potato, brinjal and egg plant (Sumithra *et al*., 2000; Aggarwal and Sood, 2005; Abd-Alla and Bashandy, 2008; Wicker *et al.,* 2009). This study could provide basic information for conducting future studies in this regards. Genetic variation represents an alternative adaptive strategy, which by providing some degree of diversity ensures survival in specific niches and/or adaptation to sudden changes in the environment (Perez *et al.,* 2008; Nouri *et al.,* 2009). "There are many bacterial wilts and there are many '*Pseudomonas solanacearums'",* this statement by Buddenhagen (1986) gives us clue for the presence of heterogeneity among the strains of *R. solanacearum.* There has been assumption that survival forms of *R. solanacearum* of any kind, may have developed compromised pathogenicity determinants, the nature of which is poorly understood. It is certainly a challenge for further research to attempt to link the adaptation of stress and pathogenicity determinants (Van Elsas *et al.,* 2005). Although assessments of races and biovars based on phenotypic characterization have been useful for describing strains of *R. solanacearum* world wide, however such classification systems are becoming inconsistent and inadequate as more and more data is being generated from genetic analysis of strains of *R. solanacearum* complex (Priou *et al.,* 2010).

Conclusion

These biovars association with seed indicate that seed may act as a major spreading source of this disease throughout the country. In addition, it may serve as a base for creating genetic variability among the pathogen, leading to presence of three different biovars on the same host. Genetic variability may lead this pathogen to further epidemics on solanaceous hosts. Government should provide healthy and certified seeds. Imported varieties are also susceptible to bacterial wilt, so best management strategy is in use of resistant varieties. Collection of resistant sources from non host and collaborating projects with AVRDC would serve as first step towards bacterial wilt management at national level. It is important report of *R. solanacearum* Biovar 1, Biovar 2 and Biovar 3 association with chilli seeds in Pakistan.

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