Race and biovar determination of *Ralstonia solanacearum* in the north west of Pakistan

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**Abstract**

Multiple comprehensive surveys were conducted during 2012, in order to know the current status of bacterial wilt (BW) of tomato caused by *Ralstonia solanacearum* (*R. solanacearum*), in the commercial tomato growing districts of Khyber Pakhtunkhwa (KP), Pakistan. A total of 74 locations covering all the 26 districts of the 7 divisions of KP were visited for the presence of tomato plants showing BW symptoms. According to Polymerase chain reaction (PCR), the expected 281bp band was amplified from 25 candidates of *R. solanacearum* isolates, thus genetically confirming them to be *R. solanacearum*. These *R. solanacearum* isolates were subjected to race identification and biovar determination tests. Race differentiation was done using hypersensitive response (HR) test on tobacco plants; whereas 10% solutions of six different sugars including disaccharides (i.e. sucrose, lactose and maltose), and sugar alcohols (i.e. manitol, sorbitol and dulcitol) in Triphenyl tetrazolium chloride (TTC) medium, were used for biovars determination. Results indicated that all PCR-confirmed *R. solanacearum* isolates belonged to race-1 and biovar-3. However, two isolates i.e. (*R. solanacearum*11-DIK1 and *R. solanacearum*10-MDN2) were found to belong to race-3, biovar-2.

**Keywords:** Tomato, *Ralstonia solanacearum*, PCR, race, biovar determination

1. **Introduction**

Tomato (*Solanum lycopersicum* L.), is one of the most important vegetable crops of the world, and is a member of Solanaceae family. It is perennial in nature, but sometimes it grows as an annual plant in temperate climates. Tomato can be considered as sack of nutrients with 2.9-3.9% sugars and carbohydrates contents; respectively. It contains 0.9% proteins, 1.2 % dietary fiber, and 0.2 % fats. In addition, different vitamins (vitamin A, C and E), elements (e.g. Potassium), zeaxanthin, carotene, lycopene and lutein are also present in a considerable amount in this crop (USDA. 2011).

According to Zhang et al., (2009); Schidfar et al., (2011), tomato is believed to beneficial to the heart thus reducing cardiovascular diseases, as well
as playing a positive role in the control of cancer and neurodegenerative diseases. The powerful natural anti-oxidant lycopene; is also present in tomatoes. Lycopene helps protects humans against UV-skin damage and prostate cancer (Allen, 2008). The lead producers of tomatoes are China, India, United States, and Turkey; producing 50.55, 18.23, 12.57, and 11.82 million tons annually; respectively. With annual production of 0.57 million tons, Pakistan ranked 34th in the world in terms of tomato production, and 142th in terms of per hectare yields of tomatoes (FAOSTAT. 2015). In addition to other reasons; various tomato diseases caused by viruses, fungi, nematodes and bacteria play major roles in reducing the per hectare yield of tomatoes.

Tomato bacterial wilt (BW) caused by *R. solanacearum* (Smith) is considered to be one of the most important diseases due to its economic impact. *R. solanacearum* is a Gram-negative rod; and is a major threat to the production of tomatoes, potatoes, tobacco and other solanaceous plants in both tropical and temperate zones. Up to 30% annual losses in tomatoes and potatoes were common in Pakistan. More than 200 different plant species were attacked by this pathogen including; ground nuts, chillies, cotton, rubber, cassava, caster beans, brinjals, ginger and many weeds.

*R. solanacearum* is a species complex consisting of groups, races, biovars, bio-types, sub-races and strains (Hossain, 2013; She et al., 2015, Yuan et al., 2015). It is highly variable and consists of at least six biovars based on biochemical properties; and five races based on host range (Fegan and Prior, 2005). *R. solanacearum* race 3 biovar 2 is classified as a select agent, and strict quarantine regulations were in place to exclude it from disease-free zones of the world including the continental U.S. It is critical to develop a clear understanding of the pathogen strains and their variability’s in order to allow accurate detection and diagnosis; as well as application of effective management strategies such as deployment of disease-resistant varieties.

Since no scientific work has been carried out on BW in tomato crop in KP; there was no information regarding the races and biovars of this pathogen (A. Bibi, personal communication). To fill the vacuum of this valuable information about pathogenic *R. solanacearum*, the present research was initiated with the objective to identify all races and biovars of this pathogen consistently present in north west of Pakistan.

2. Materials and methods

2.1. Collection of diseased tomato plant samples

Tomato growing districts of the KP (Table 1) were surveyed multiple times throughout the growing period (i.e. April-August, 2012) for collection of samples of diseased plants showing typical symptoms of BW. KP was divided into seven administrative divisions; thus a total of 74 sites were surveyed for sampling. Three to ten fields were surveyed within each location. In each field, two-three spots (each having about 8-10 plants) were randomly selected.

2.2. PCR-based confirmation of *R. solanacearum* isolates

*R. solanacearum* isolates were confirmed by PCR using species-specific primers i.e. 5'GTCGCCGTCAACTCACTTTCC3' and 5'GTCGCCGTAGCAATGCGGAATCG3'; with amplified 281bp fragment (Umesha and Avinash, 2014).

2.2.1. Isolation of nucleic acids (DNA) from *R. solanacearum* isolates

DNA was extracted as described by Pastrik and Maiss, (2000). For the isolation of bacterial genomic DNA, a loopful of a bacterial culture was suspended in 1ml PBS buffer (0.14 M NaCl, 2.7 mM KCl, 10 mM NaHPO4, 1.8 mM KH2PO4; pH 7.4), and then centrifuged for 2 min. at 13,000 Xg and 4°C.
Table 1: List of administrative divisions of Khyber Pakhtunkhwa (KP) surveyed for bacterial wilt (BW) of tomato plant

| S. no. | Administrative Division          | Districts                                                                 |
|--------|----------------------------------|---------------------------------------------------------------------------|
| 1      | Bannu Division                   | Bannu, Lakki Marwat                                                       |
| 2      | D.I. Khan Division               | Dera Ismail Khan, Tank                                                    |
| 3      | Hazara Division                  | Abbottabad, Battagram, Haripur, Mansehra, Tor Ghar, Lower Kohistan, Upper Kohistan |
| 4      | Kohat Division                   | Hangu, Karak, Kohat                                                       |
| 5      | Malakand Division                | Buner, Chitral, Lower Dir, Upper Dir, Malakand, Shangla, Swat              |
| 6      | Mardan Division                  | Mardan, Swabi                                                             |
| 7      | Peshawar Division                |Charsadda, Nowshera, Peshawar                                              |

The pellet was re-suspended in 320 μl lysis-buffer (100 mM NaCl; 10 mM Tris-HCl, 1mM EDTA, pH 8.0), placed on a heating block at 95°C for 10 min. and then cooled on ice for 5 min. 80 μl of lysozyme stock solution (50mg/ mllysozyme in 10mM Tris-HCl, pH 8.0) was added, and then the sample was incubated for 30 min. at 37°C. The DNA was purified using the Easy-DNA-Extraction-kit (Invitrogen, DeSchelp, Netherlands), containing two solutions (i.e. A and B). 220μl of the solution A was added; and then the mixture was incubated for 30 min. at 65°C. After addition of 100 μl of solution B and mixing; 500 μl of chloroform was added and the mixture was centrifuged for 20 min. at 20,000 Xg. The aqueous phase was separated and DNA was precipitated by addition of 70 %ethanol; the resulting pellet was washed with 80 % ethanol. After the final centrifugation, the DNA was re-suspended in 100 μl of sterile ultra-pure water.

2.2.2. PCR Amplification with species-specific primers

The extracted genomic DNA from each isolate of *R. solanacearum* was PCR-amplified using specific primers according to (Bibi *et al.*, 2015). The volume of the PCR mixture used was 25 μl each. This mixture per tube consisted of 1 μl of each primer (0.2 μM); 1 μl of Taq-DNA polymerase, 3 μl template DNA (1ng), 2.25 μl buffer (KCL-50mM-Tris-HCl-2mM, pH 8.4), 2 μl MgCl₂ (1 mM); and 2.5 μl (100 μM) of each dNTP. A 30 PCR cycle was performed for all candidate isolates. The conditions used for this PCR were: 3 min. at 95°C (one time at the start); 20 sec. at 94°C; 50 sec. at 50°C; 30 sec. at 72°C; 5 min. at 72°C (one time at the end); and then stored at 4°C. A template-less negative control was also included.

2.2.3. Gel electrophoresis

To separate the PCR-amplified DNA bands, 2 % agarose gel (0.67 g agarose dissolved in 30ml TBE buffer) was prepared, cooled, tray-solidified and sub-merged in TBE buffer. Blue tracking (3 μl/sample) was added to each PCR-product sample (25 μl), and each sample was carefully loaded to the wells of the gel. A 1 kb size ladder was also used. Electrophoresis was performed as per procedure of Bibi *et al.*, (2015) at 200 volts for 30 min.

2.2.4. Gel staining and imaging

To visualize the different bands; 0.5 μg methidiumbromide solution was used for gel staining for 15 min., and then washed in sterile water for another 15 min. UV tech machine was used to observe the bands under UV light; while images were saved for future use.
2.3. Race determination and tobacco hypersensitive reaction (HR)

The race differentiation was carried out based on tobacco hypersensitive response (HR). One month old tobacco seedlings were transplanted into plastic pots containing sterilized soil. Four weeks after transplantation; tobacco leaves were infiltrated at their lower surfaces with 1 ml of *R. solanacearum* suspension (10^8 cells/ml) using a disposable plastic syringe. Control leaves were infiltrated with autoclaved suspension of the bacterium. Leaf reactions were examined as early as possible on the day following infiltration and again 24 h later (Gebreel *et al.*, 2000).

2.4. Biovar determination of *R. solanacearum* isolates through carbohydrate utilization tests

Biovars determination was done using carbohydrate utilization test according to Chaudhry and Rashid, (2011). Solutions of 10% of six different sugars including: disaccharides such as: sucrose, lactose, maltose, and sugar alcohols such as: manitol, sorbitol and dulcitol; were prepared and then sterilized by heating for 30 min. at 100°C. (TTC) medium was prepared and then 10 ml of this medium were aseptically added in each test tube already containing 10 ml of the sterilized 10% sugar solution. About 50 μl of 48 h old bacterial suspension containing about 10^8 cells/ml, were inoculated into each tube of TTC-sugar mixture. The tubes were incubated at 28°C and examined after 72 h for change in pH indicated by changing color of medium from green to orange.

3. Results

3.1. Molecular identification of *R. solanacearum* isolates

Using species-specific primers, PCR was used to finally confirm the identity of *R. solanacearum* isolates. The 281bp specific band was amplified from 25 isolates, confirming that they all belong to the genus *R. solanacearum* (Fig. 1.).

3.2. Race determination using tobacco hypersensitive reaction (HR)

The PCR-confirmed *R. solanacearum* isolates were subjected to race identification test. HR test was negative for 23 isolates. All isolates except (RS11-DIK1 and RS10-MDN2); produced dark brown lesions 24 h after leaves infiltration (Table 2). After 48 h the dark brown lesions on leaves became necrotic and were surrounded by yellow halos. The two isolates (RS11-DIK1 and RS10-MDN2) produced no visible symptoms on the inoculated tissue; however, they caused yellowing of this tissue, indicating that they belong to Race-3. After 72 h; yellowing and reversible wilting symptoms were observed in tissues inoculated by both isolates. After one week, (RS11-DIK1 and RS10-MDN2) inoculated leaves showed wilting symptoms and thus confirming that they belong to Race-3, whereas, all the remaining 23 isolates exhibited complete wilting of plants thus designed as Race-1. After one month, the 23 isolates killed the inoculated plants; however, those plants inoculated with (RS11-DIK1 and RS10-MDN2) showed severe wilting symptoms. The same pathogenic *R. solanacearum* isolates were re-isolated and then re-identified from the infested plants, confirming their pathogenicity.

3.3. Biovar determination of *R. solanacearum* isolates
Results of biovar determination tests shown in Table (3) revealed that all the 25 isolates of *R. solanacearum* oxidized disaccharides (i.e. sucrose, lactose, and maltose) and sugar alcohols (i.e. mannitol, sorbitol and dulcitol). The oxidation reaction was indicated by the change of color of TTC medium from green to yellow. All isolates were designed as biovar III except isolates (RS10-MDN2 and RS11-DIK1) which failed to oxidize sugar alcohols; and were thus classified as biovar II (Table 4). All the biovar III isolates belong to Race-1, while the 2 biovar II isolates belong to Race-3.

**Table 2: Race determination of 25 *R. solanacearum* isolates recovered from different locations of Khyber Pakhtunkhwa (KP)**

| S. no | Isolates | Location | Symptoms produced on tobacco plants |
|-------|----------|----------|-------------------------------------|
|       |          |          | 24 h | 48 h | 72 h | One week | One month | Race |
| 1     | RS1-SGL1 | Shangla  | DBL  | NYH  | Y+W  | CPW      | WD       | 1     |
| 2     | RS2-BAN1 | Bannu    | DBL  | NYH  | Y+W  | CPW      | WD       | 1     |
| 3     | RS3-HAR1 | Haripur  | DBL  | NYH  | Y+W  | CPW      | WD       | 1     |
| 4     | RS4-KAR1 | Karak    | DBL  | NYH  | Y+W  | CPW      | WD       | 1     |
| 5     | RS5-SWB1 | Swabi    | DBL  | NYH  | Y+W  | CPW      | WD       | 1     |
| 6     | RS6-SWT1 | Swat     | DBL  | NYH  | Y+W  | CPW      | WD       | 1     |
| 7     | RS6-SWT2 | Swat     | DBL  | NYH  | Y+W  | CPW      | WD       | 1     |
| 8     | RS7-NSH1 | Noshera  | DBL  | NYH  | Y+W  | CPW      | WD       | 1     |
| 9     | RS7-NSH2 | Noshera  | DBL  | NYH  | Y+W  | CPW      | WD       | 1     |
| 10    | RS8-BNR1 | Buner    | DBL  | NYH  | Y+W  | CPW      | WD       | 1     |
| 11    | RS8-BNR2 | Buner    | DBL  | NYH  | Y+W  | CPW      | WD       | 1     |
| 12    | RS9-KOH1 | Kohat    | DBL  | NYH  | Y+W  | CPW      | WD       | 1     |
| 13    | RS10-MDN1| Mardan   | DBL  | NVS  | YIT   | W        | SW       | 3     |
| 14    | RS10-MDN2| Mardan   | DBL  | NVS  | YIT   | Y+RW    | W        | SW     | 3     |
| 15    | RS10-MDN3| Mardan   | DBL  | NVS  | YIT   | Y+RW    | W        | SW     | 3     |
| 16    | RS11-DIK1| D.I. Khan| NVS  | YIT   | Y+RW  | W        | SW       | 3     |
| 17    | RS12-CHD1|Charsadda| DBL  | NYH  | Y+W  | CPW      | WD       | 1     |
| 18    | RS12-CHD2|Charsadda| DBL  | NYH  | Y+W  | CPW      | WD       | 1     |
| 19    | RS12-CHD3|Charsadda| DBL  | NYH  | Y+W  | CPW      | WD       | 1     |
| 20    | RS13-DLO1|Dir Lower| DBL  | NYH  | Y+W  | CPW      | WD       | 1     |
| 21    | RS13-DLO2|Dir Lower| DBL  | NYH  | Y+W  | CPW      | WD       | 1     |
| 22    | RS14-PES1|Peshawar  | DBL  | NYH  | Y+W  | CPW      | WD       | 1     |
| 23    | RS15-MKD1|Malakand  | DBL  | NYH  | Y+W  | CPW      | WD       | 1     |
| 24    | RS15-MKD2|Malakand  | DBL  | NYH  | Y+W  | CPW      | WD       | 1     |
| 25    | RS16-HAN1|Hangu     | DBL  | NYH  | Y+W  | CPW      | WD       | 1     |

Where; NVS = No visible symptoms, DBL = Dark brown lesion, YIT = Yellowing of inoculated tissue, NYH = Necrosis surrounded by yellow halo, W= wilting, Y = Yellowing, RW = reversible wilting, CPW = Complete plant wilting, SW = severe wilting WD= wilted and dead.
Table 3: Biovar determination of 25 *R. solanacearum* isolates recovered from different locations of Khyber Pakhtunkhwa (KP)

| S. no | Isolates | Location | Disaccharides | Sugar Alcohols | Biovars |
|-------|----------|----------|---------------|----------------|---------|
|       |          |          | Sucrose | Lactose | Maltose | Mannitol | Sorbitol | Dulcitol |       |
| 1     | RS1-SGL1 | Shangla   | +       | +       | +       | +        | +        | +        | III    |
| 2     | RS2-BAN1 | Bannu     | +       | +       | +       | +        | +        | +        | III    |
| 3     | RS3-HAR1 | Haripur   | +       | +       | +       | +        | +        | +        | III    |
| 4     | RS4-KAR1 | Karak     | +       | +       | +       | +        | +        | +        | III    |
| 5     | RS5-SWB1 | Swabi     | +       | +       | +       | +        | +        | +        | III    |
| 6     | RS6-SWT1 | Swat      | +       | +       | +       | +        | +        | +        | III    |
| 7     | RS6-SWT2 | Swat      | +       | +       | +       | +        | +        | +        | III    |
| 8     | RS7-NSH1 | Noshera   | +       | +       | +       | +        | +        | +        | III    |
| 9     | RS7-NSH2 | Noshera   | +       | +       | +       | +        | +        | +        | III    |
| 10    | RS8-BNR1 | Buneer    | +       | +       | +       | +        | +        | +        | III    |
| 11    | RS8-BNR2 | Buneer    | +       | +       | +       | +        | +        | +        | III    |
| 12    | RS9-KOH1 | Kohat     | +       | +       | +       | +        | +        | +        | III    |
| 13    | RS10-MDN1| Mardan    | +       | +       | +       | +        | +        | +        | III    |
| 14    | RS10-MDN2| Mardan    | +       | +       | +       | +        | +        | +        | III    |
| 15    | RS10-MDN3| Mardan    | +       | +       | +       | +        | +        | +        | III    |
| 16    | RS11-DIK1| D.I. Khan | +       | +       | +       | -        | -        | -        | II     |
| 17    | RS12-CHD1| Charsadda | +       | +       | +       | +        | +        | +        | III    |
| 18    | RS12-CHD2| Charsadda | +       | +       | +       | +        | +        | +        | III    |
| 19    | RS12-CHD3| Charsadda | +       | +       | +       | +        | +        | +        | III    |
| 20    | RS13-DLO1| Dir Lower | +       | +       | +       | +        | +        | +        | III    |
| 21    | RS13-DLO2| Dir Lower | +       | +       | +       | +        | +        | +        | III    |
| 22    | RS14-PES1| Peshawar  | +       | +       | +       | +        | +        | +        | III    |
| 23    | RS15-MKD1| Malakand  | +       | +       | +       | +        | +        | +        | III    |
| 24    | RS15-MKD2| Malakand  | +       | +       | +       | +        | +        | +        | III    |
| 25    | RS16-HAN1| Hangu     | +       | +       | +       | +        | +        | +        | III    |

Where; (+): Color of TTC medium changed from green to yellow, (-): Color of the medium did not change

Table 4. Over all PCR identification, race and biovar determination of the 25 *R. solanacearum* isolates

| S. no | Location | PCR confirmed *R. solanacearum* isolates | Race | Biovar |
|-------|----------|------------------------------------------|------|--------|
| 1     | Shangla  | RS1-SGL1                                 | 1    | III    |
| 2     | Bannu    | RS2-BAN1                                 | 1    | III    |
| 3     | Haripur  | RS3-HAR1                                 | 1    | III    |
| 4     | Karak    | RS4-KAR1                                 | 1    | III    |
| 5     | Swabi    | RS5-SWB1                                 | 1    | III    |
| 6     | Swat     | RS6-SWT1                                 | 1    | III    |
| 7     | Swat     | RS6-SWT2                                 | 1    | III    |
| 8     | Noshera  | RS7-NSH1                                 | 1    | III    |
| 9     | Noshera  | RS7-NSH2                                 | 1    | III    |
| 10    | Buneer   | RS8-BNR1                                 | 1    | III    |
| 11    | Buneer   | RS8-BNR2                                 | 1    | III    |
| 12    | Kohat    | RS9-KOH1                                 | 1    | III    |
| 13    | Mardan   | RS10-MDN1                                | 1    | III    |
| 14    | Mardan   | RS10-MDN2                                | 1    | III    |
| 15    | Mardan   | RS10-MDN3                                | 1    | III    |
| 16    | D.I. Khan| RS11-DIK1                                | 3    | II     |
| 17    | Charsadda| RS12-CHD1                                | 1    | III    |
| 18    | Charsadda| RS12-CHD2                                | 1    | III    |
| 19    | Charsadda| RS12-CHD3                                | 1    | III    |
| 20    | Dir Lower| RS13-DLO1                                | 1    | III    |
| 21    | Dir Lower| RS13-DLO2                                | 1    | III    |
| 22    | Peshawar | RS14-PES1                                | 1    | III    |
| 23    | Malakand | RS15-MKD1                                | 1    | III    |
| 24    | Malakand | RS15-MKD2                                | 1    | III    |
| 25    | Hangu    | RS16-HAN1                                | 1    | III    |
4. Discussion

To confirm the identity of microbial isolates which give fallacy results, it is necessary to use molecular identity-confirmation methods such as PCR (Champoiseau and Jones, 2009). Thus to confirm the identity of current isolates as *R. solanacearum*; we used species-specific primers that amplified the specific 281bp fragment confirmed from previous reports of Kyaw *et al.* (2014); Umesha and Avinash, (2014); Ying *et al.*, (2017).

As a highly diversified pathogen; *R. solanacearum* is considered as a species complex in a heterogeneous group consisting of hundreds of genetically distinct strains (Fegan and Prior, 2005). The pathogen has developed several races, biovars (Tahir *et al.*, 2014). According to Denny and Hayward, (2001); this species complex was subdivided into races based on host range, and into biovars based on its ability to produce acid from a panel of sugars. Umesha and Avinash, (2014) added that; a more phylogenetically meaningful classification scheme based on sequences of several genes, divided this species complex into four phylotypes. This scheme previously grouped strains according to geographical origin such that; strains from Asia were in phylotype I, those from the Americas were in phylotype II, those from Africa were in phylotype III, and those from Indonesia which is the apparent center of diversity, were in phylotype IV (Fegan and Prior, 2005). Phylotypes themselves can be sub-grouped into sequevars which were clusters of isolates with highly conserved DNA sequences.

Race 1 biovar 1 (*R*1*B*1) isolates of *R. solanacearum* were considered to be the most virulent. Members of *R*1*B*2 typically infect potatoes; but they can also infect tomatoes and geranium (Ornamental) plants (Swanson *et al.*, 2005). In the present study, we obtained two *R*1*B*2 strains (RS11-DIK1 and RS10-MDN2) isolated from tomato plants samples collected from D.I. Khan and Mardan districts; respectively, which were pathogenic to tomatoes. These *R*3*B*2 strains were widely distributed in Asia Africa, South and Central America; and were found in some soils and waterways in Europe (Elphinstone, 2005). According to Alvarez *et al.*, (2005), strains of *R*4*B*4 have been reported from North America, while *R*3*B*1 were reportedly found in some coastal parts of West Africa.

Results of the current study revealed that; 23 isolates of *R. solanacearum* belonged to Race 1, while two only belonged to Race 3. Race 1 strains attack tobacco, tomato, many other solanaceous crops; and certain diploid bananas. However, Race 3 causes wilting in potato, tomato and rarely other solanaceous plants. Race 2 and 4, fortunately were not common in Asia. Race 2 (found in Africa) was known to infect triploid banana (*Musa acuminata*) and *Heliconia* spp., while Race 4 attacks mulberry (OEPP/EPPO. 2004). Five races have been described so far; but they differ in their host range, geographical distribution and in ability to survive under different environmental conditions (Popoola *et al.*, 2015).

Conclusions

Race and biovar determination of 25 *R. solanacearum* isolates recovered from different BW infected tomato plants growing areas of KP; Pakistan, indicated that 92% of these isolates belonged to race 1, biovar 3; while 8 were race 3, biovar 2.

Conflict of interests

The authors declare no conflict of interests.
5. References

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