

## Determination of Races and Biovars of *Ralstonia solanacearum* Causing Bacterial Wilt Disease of Potato

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### Abstract

A survey was carried out in some selected potato growing districts of Bangladesh during December to February 2011 to know the status of bacterial wilt of potato caused by *Ralstonia solanacearum* in terms of its incidence and severity. The results showed that the highest wilt incidence was recorded in Munshigonj (22.65%), followed by Nilphamari (19.98%) and the lowest incidence was recorded in Jamalpur (9.07%). The highest bacterial wilt severity was recorded in Munshigonj (3.80), while the lowest wilt severity was recorded in Jamalpur (2.90). A total of 44 isolates (*R. solanacearum*) were obtained from the wilted potato plant samples i.e. 20 from Munshigonj, 17 from Nilphamari and 7 from Jamalpur and the isolates were divided into three groups. The production of pink or light red colour with whitish margin on TZC medium by the bacterial isolates indicated all groups of *R. solanacearum* isolates were virulent. The results of pathogenicity test revealed that all groups of *R. solanacearum* isolates were able to cause wilt symptoms in potato plants and brown rot symptoms in potato tuber. On the other hand, all biochemical tests were used for the identification of *R. solanacearum* isolates. The biovar test using the oxidization of disaccharides (sucrose, lactose, maltose) and sugar alcohols (mannitol, sorbitol and dulcitol) by *R. solanacearum* isolates confirmed that all groups of *R. solanacearum* isolates belong to biovar III. The race identification of *R. solanacearum* isolates by pathogenicity test on brinjal, tomato, tobacco and chilli indicating a narrow host range (only in potato) and were categorized in race 3. Therefore, the *R. solanacearum* isolates causing bacterial wilt of potato in Bangladesh were belonging to Biovar III and Race 3.

**Keywords:** biovar, race, *Ralstonia solanacearum*, wilt, potato

### 1. Introduction

Potato (*Solanum tuberosum* L.) is a herbaceous tuber crop belonging to the family Solanaceae. It is one of the three leading staple food crops next to rice and wheat and is of course the most important vegetable grown in Bangladesh and also in the world (Ahmed & Talukder, 1978). It is cultivated and recognized as popular vegetable throughout the entire tropical and subtropical region of the world (Hayward, 1991). Potato is locally known as “Alu”. The crop extends substantial amount of high quality protein and essential vitamins, minerals and trace elements to the human diet (Horton, 1987). It produces more carbohydrates per unit amount than either rice or wheat. In Bangladesh, potato is a crop of great economic significance.

The major potato growing areas of Bangladesh are Munshigonj, Jamalpur, Nilphamari, Jessore, Bogra, Chandpur and Panchagorh. The total acreage of potato is 977 thousand hectares with total annual production of 5268 million tones (BBS, 2009). It contributes alone as much as 54% of the total annual vegetable production of Bangladesh (BBS, 2009). However, the production of potato is quite low as compared to the major potato growing countries of the world. The reasons behind the low yields of potato are different bacterial, fungal, viral and nematodes diseases and poor management practices. Among these, diseases are the most predominant limiting factors the potato bacterial wilt was the most important problem in Bangladesh. A total of fifteen diseases have been recorded in Bangladesh of which early and late blight, wilts (Bacterial, fungal and

nematodes), scab, stem rot, stem canker/scurf, potato leaf roller, potato mosaic virus, dry rot and soft rot are the major diseases of potato in Bangladesh (Christ, 1998).

Bacterial wilt caused by *Ralstonia solanacearum* (Smith, 1986), formerly called *Pseudomonas solanacearum* (Yabuuchi et al., 1976), a soil-borne gram-negative bacterium is a recognized parasite in over 200 families of plants, including potato, brinjal and tomato as well as many native plant species. The bacterium normally invades plant roots from the soil through wounds or natural openings, colonizes the intercellular space of the root cortex and vascular parenchyma, and eventually enters the xylem vessel and spreads up into the stem and leaves. Affected plants suffer chlorosis, stunting, wilting, and usually die rapidly. Losses caused by the disease are known to be enormous but cannot be accurately estimated because of abandonment of wilt-susceptible crops in many parts of the world.

The species *R. solanacearum* is a complex taxonomic unit with broad physiological and genetic diversity. *R. solanacearum* was classified into five races on the basis of different host range (Buddenhagen et al., 1962; He et al., 1983; Pegg & Moffet, 1971), and six biovars according to the ability to oxidize three hexose alcohols and three disaccharides (Hayward, 1964, 1991; Hayward & Hartman, 1994; He et al., 1983). Unlike other phytopathogenic bacteria, race systems of *R. solanacearum* are not based on gene-for-gene interactions i.e., different cultivars carrying different R gene(s). Instead, these are determined based on the pathogenicity of each isolate in different kinds of host plants. Although the biovar and race systems are widely accepted for the classification of *R. solanacearum*, there is no definite correlation between biovar and race. Each race transects the biovars and each biovar contains various races. The only positive correlation between the biovar and race systems exists for biovar 2 and race 3 (Patrice, 2008).

The main control strategy for bacterial wilt has been the use of resistant varieties. However, the stability of bacterial wilt resistance in brinjal, potato and tomato, is highly affected by pathogen density, pathogen strains and several soil factors. The other control methods such as cultural, chemical and biological were not found effective against bacterial wilt disease of potato due to wide host range and genetic diversity of its pathogen, *R. solanacearum*. Information on its pathogen population especially biovars and races are essential to formulate a pathogen-targeted and geographically-targeted integrated management strategy against the disease. Therefore, the present study was undertaken to determine the biovars and races of *R. solanacearum* isolates causing bacterial wilt of potato in Bangladesh at least to step forward for designing an effective management approach.

## 2. Materials and Methods

### 2.1 Surveying and Sampling

A survey was carried out to know the status of bacterial wilt of potato in Bangladesh in terms of its incidence and severity in some selected districts viz. Jamalpur, Munshigonj, Panchagarh, Bogra, Jessore, Chandpur and Nilphamari during December to February, 2011. At least three locations in each district and five farmers' fields from each location were surveyed to record the bacterial wilt incidence and severity. For a quick field diagnosis, the streaming of milky white masses of bacterial cells (ooze) confirmed the disease is bacterial wilt caused by *R. solanacearum* and to distinguish bacterial wilt from vascular wilts caused by fungal pathogen and nematode. At least 10 samples of the diseased plants were collected from each of the surveyed district and were brought to the laboratory for the isolation of different group of isolates of *R. solanacearum*.

### 2.2 Assessment of Disease Incidence and Severity

The status of bacterial wilt of potato was surveyed in terms of its incidence and severity. Data on wilt incidence were recorded in at least three locations from five farmer's fields for each district. Then the per cent wilt incidence was calculated by the following formula:

$$\% \text{ Wilt incidence} = \frac{\text{Number of wilted plants in each field}}{\text{Total number of plants in each field}} \times 100$$

Five plants were randomly selected from each farmer field from each location to calculate the wilt severity in each district. The severity of bacterial wilt was recorded based on the severity scale as described previously by Horita and Tsuchiya (2001). Briefly, 1= No symptom, 2 = Top young leaves wilted, 3 = Two leaves wilted, 4 = Four or more leaves wilted and 5 = Plant dies.

### 2.3 Isolation, Identification, Purification and Preservation of *R. solanacearum*

The wilted plant samples collected from the fields were washed under running tap water to remove sand and soil. The stem of the infected potato plants were surface sterilized with 70% alcohol and cut into two halves and the cut ends were dipped into water in test tubes. After waiting around half an hour, bacterial ooze was seen to come

out into the water from the infected stem. Then a loop full of water was streaked on the Nutrient Agar (NA) plate. The plates were then incubated at 28°C for at least 24 hours to grow the bacterium in the medium. After isolation, *R. solanacearum* isolates were purified by streaking a single colony of each isolate on Triphenyl Tetrazolium Chloride (TTC) plate (Kelman, 1954). The isolates of *R. solanacearum* were then preserved in 10% skim milk and kept at -20°C refrigerator for subsequent studies. The collected isolates from different potato growing districts were classified into three groups based on the obtained districts. To confirm the isolates of *R. solanacearum*, the pathogenicity test was performed on one month old potato seedlings by soil inoculation method with approximately 10<sup>8</sup> CFU/ml. A single colony of *R. solanacearum* showing virulent, fluidal, irregular and creamy white with pink at the centre was selected for each group of isolates and multiplied in a TTC (without adding Triphenyl Tetrazolium Chloride) for pathogenicity test. At 30-40 days age of tobacco plants, bacterial suspension (approximately 10<sup>8</sup> CFU/ml) of each isolate representing a group was injected into the intracellular space of the leaf with a hypodermal syringe. Hypersensitive reaction was observed daily and continued until five days after infiltration.

#### 2.4 Biochemical Characterization of *R. solanacearum*

##### 2.4.1 Identification of Virulent and Avirulent Isolates

The virulent (colonies with pink or light red colour or characteristic red center and whitish margin) and avirulent (smaller, off-white and non-fluidal colonies) strains of *R. solanacearum* were identified in TTC medium containing 0.005% TTC (Kelman, 1954).

##### 2.4.2 Biochemical Tests for the Identification of *R. solanacearum*

Several biochemical tests viz. Gram staining reaction, Potassium hydroxide solubility test, Kovac's oxidase test, Levan test and Sugar fermentation test were performed for confirmation of *R. solanacearum* isolates as described previously by Rahman et al. (2010) and Hossain (2006). Single isolate of *R. solanacearum* from each group was randomly selected for biochemical tests.

##### 2.4.3 Determination of Biovars

The isolates of *R. solanacearum* were differentiated into biovars based on their ability to utilize disaccharides (sucrose, lactose, maltose) and sugar alcohols (manitol, sorbitol and dulcitol) as described previously by Hayward (1964) and He et al. (1983). The biovars were determined in the mineral medium (NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> 1.0 g, KCl 0.2 g, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.2 g, Difco bacto peptone 1.0 g, Agar 3.0 g and Bromothymol blue 80.0 mg per litre) containing 1% sugar. About 200µl of the melted medium was dispensed into the wells of microtitre plate. Inoculums for each group of isolate was prepared by several loop full of bacteria from 24-48 hours old cultures to distilled water to make suspension containing about 10<sup>8</sup> CFU/ml. Then 20 µl of bacterial suspension was added to the wells of microtitre plate incubated at 28°C. The plates were then examined after 3 days of inoculation for changing pH which was indicated by the change of colour (Schaad, 1988).

#### 2.5 Races Identification

The races of *Ralstonia solanacearum* were identified by pathogenicity test on wide host range (Denny & Hayward, 2001). Seedlings of brinjal, tomato, tobacco and chilli were raised in tray. One month (30 days) old seedlings (brinjal, tomato & chilli) were inoculated by soil inoculation method. The incubated plants were then kept in the net house until symptoms development.

### 3. Results and Discussion

#### 3.1 Incidence and Severity of Bacterial Wilt

A total of seven selected potato growing districts viz. Jamalpur, Munshigonj, Panchagarh, Bogra, Jessore, Chandpur and Nilphamari were surveyed to know the status of bacterial wilt of potato in terms of its incidence and severity. However, bacterial wilt infection was noticed in three districts namely Munshigonj, Nilphamari and Jamalpur. A significant variation was observed in terms of bacterial wilt incidence among the selected growing areas surveyed (Table 1). The survey results showed that the highest bacterial wilt incidence was recorded in Munshigonj (22.65%), followed by Nilphamari (19.98%) while the lowest bacterial wilt incidence (9.07%) was recorded in Jamalpur (Table 1). On the contrary, the highest bacterial wilt severity was recorded in Munshigonj (3.80), while the lowest bacterial wilt severity was recorded in Jamalpur (2.90) during the time of survey (Table 1). Moreover, these variations of wilt incidence and severity may be attributed due to the diversity of *R. solanacearum* isolates and also due to the variations in soil factors prevailing in different locations surveyed. Differences of wilt incidence and severity were also reported in eggplant due to the great diversity of host plants

affected by this pathogen, phenotype and genotype of *Ralstonia solanacearum*, its wide geographical distribution, and the range of environmental conditions conducive to bacterial wilt (Rahman et al., 2010).

Table 1. Incidence and severity of bacterial wilt at selected potato growing areas in Bangladesh

Areas Surveyed	Number of isolates	Group	Wilt incidence (%)	Wilt severity (1-5 scale) *
Munshigonj	20	I	22.65a	3.80
Nilphamari	17	II	19.98b	3.00
Jamalpur	7	III	9.07c	2.90
LSD			1.371	
Level of significance			**	

\*\* Significance at 1% level of probability; \*Severity data recorded at the time of survey.

### 3.2 Isolation and Identification of the *R. solanacearum* Isolates

A total of 44 *R. solanacearum* isolates were obtained from the wilted potato plant samples i.e. 20 from Munshigonj (Group I), 17 from Nilphamari (Group II) and 7 from Jamalpur (Group III) (Table 1). Although equal numbers of samples were collected from each of the surveyed area, the number of isolates varied because of failure of isolation of the bacterium from all the infected plants. All of the *R. solanacearum* isolates collected from wilted potato plants produced cream colour or off-white colour colonies on NA media after 24 hours of incubation at 28°C.

### 3.3 Pathogenicity and Hypersensitive Response (HR) Test

The results of pathogenicity test revealed that all the isolate groups of *R. solanacearum* were able to produce wilt symptom in potato plants incubated by soil inoculation method (Table 2). The isolates of *R. solanacearum* were also tested for inducing brown rot symptom on the potato tubers. The results revealed that all isolates groups of *R. solanacearum* were able to produce brown rot symptoms on tubers. On the contrary, the *R. solanacearum* isolate obtained from wilted brinjal plants was not capable to produce any brown rot symptom in the potato tuber. The isolates of *R. solanacearum* collected from the wilted potato plant were tested for hypersensitive reaction in tobacco. The result showed that none of the isolates was able to cause the death of local cell of tissue between veins of tobacco leaves.

Table 2. Pathogenicity, hypersensitive response (HR) and biochemical tests of different isolate groups of *R. solanacearum*

Isolate Name	Number of isolates	Pathogenicity test and HR test on TTC media	Gram staining reaction	KOH solubility test	Kovac's oxidase test	Levan test	Sugar fermentation test				Inference	
							Dextrose	Sucrose	Manitol	Lactose		
Group I	20	+	-	+	+	+	+	+	+	+	+	<i>R. solanacearum</i>
Group II	17	+	-	+	+	+	+	+	+	+	+	<i>R. solanacearum</i>
Group III	7	+	-	+	+	+	+	+	+	+	+	<i>R. solanacearum</i>

+ Positive reaction.

- Negative reaction.

Group I: Munshigonj, Group II: Nilphamari and Group III: Jamalpur.

### 3.4 Biochemical Tests

#### 3.4.1 Gram's Stain

The Gram's staining reaction was performed using crystal violet. The microscopic results showed that all of the isolates of *R. solanacearum* did not retain violet colour i.e. the isolates retained counter stain (pink colour). Therefore, all isolates of *R. solanacearum* representing each group are gram negative and straight or curved rod shaped which is the characteristic feature of any plant pathogenic bacteria (Table 2).

#### 3.4.2 Potassium Hydroxide Solubility Test

The gram negative test of *R. solanacearum* was also confirmed by Potassium hydroxide solubility test. The result revealed that a elastic thread or viscous thread was observed when loop raised from the bacterial solution by toothpick, a few centimeters from glass slides in case of all group indicating that all groups of *R. solanacearum* isolates are gram negative (Table 2).

#### 3.4.3 Kovac's Oxidase Test

Kovac's oxidase test was also carried out to know the oxidation ability of *R. solanacearum* isolates. the result showed that all groups of *R. solanacearum* isolates were able to develop deep blue colour with oxidase reagent within few seconds which indicated that the tested group of *R. solanacearum* isolates were Gram negative (Table 2).

#### 3.4.4 Levan Test

Levan is an intracellular bacterial polysaccharide (beta-2, 6-1 linked D-fructan), whose potential and actual uses are similar to those of dextrans (Avigad, 1968). In this study, induction of the Levan was performed in NA medium containing 5% sucrose, Levan sucrose (E.C.2.4.1.10), which catalyzes the synthesis of Levan from sucrose, is produced by a number of bacteria including *R. solanacearum*. The result showed that all group of *R. solanacearum* isolates were able to produce distinctive domed shaped or round colonies due to production of levan in sucrose containing NA medium (Table 2).

#### 3.4.5 Sugar Fermentation Test

The *R. solanacearum* is able to oxidize the sugars which are indicated by colour change (reddish to yellow). The results of sugar fermentation test clearly showed that all groups of *R. solanacearum* isolates obtained from the wilted potato plants samples were able to oxidize the four (4) basic sugars (Dextrose, sucrose, manitol and lactose) by producing acid and gas. The acid production in sugar fermentation test by bacterial isolates were indicated by the colour change from reddish to yellow, gas production was noted by the appearance of gas bubbles in the inverted Durham's tubes and the oxidation of sugar manitol by the bacterial isolates indicated by the production of yellow to red colour (Table 2). The isolates were also characterized by the hypersensitive response (HR) test in tobacco and also by different biochemical tests. These results were supported by the findings of Dhital et al. (2001) who observed that *R. solanacearum* was able to produce wilt symptoms in potato, HR in tobacco leaves and was found positive by a series of biochemical tests. Also, the Gram staining and KOH solubility test indicated that the isolates of *R. solanacearum* are gram negative. Suslow et al. (1982) reported that the KOH technique is far easier and faster to distinguish gram negative and gram positive bacteria than the traditional Gram-staining test in which dyes are employed. Like other gram negative bacteria, the isolates of *R. solanacearum* were able to develop blue colour in Kovac's test by oxidizing the sugars and were able to ferment four basic sugars (Dextrose, sucrose, manitol and lactose) to produce acid and gas. The isolates of *R. solanacearum* were also able to produce round or circular domed shaped colonies in sucrose medium. When the bacteria were grown on a medium containing sucrose, the production of an extracellular enzyme (levan sucrose) was induced and sucrose was converted to levan and glucose. During this fermentation process, the bacteria utilize sucrose for maintenance and their growth.

#### 3.5 Identification of Virulent/Avirulent Strains of *R. solanacearum*

The virulent and avirulent isolates of *R. solanacearum* were differentiated by Kelman Tetrazolium Chloride (TZC) agar test. In this test, virulent isolates produce pink or light red colour colonies or colonies with characteristic red centre and whitish margin and avirulent isolates produce smaller, off-white and non-fluidal or dry on TZC medium after 24 hours of incubation. Result of this test showed that all groups of *R. solanacearum* isolates collected from different growing areas produced pink or light red colour colonies or colonies with characteristic red centre and whitish margin on TZC medium (Table 2). This indicates that all *R. solanacearum* isolates were virulent. Kelman (1954) reported that avirulent colony types of *R. solanacearum* could be easily differentiated by the pigmentation from the wild virulent types. *R. solanacearum* developed two types of colonies on tetrazolium chloride (TZC) medium on which virulent colonies appear as white with pink centres and non-virulent colonies appear as small off-white colonies. On this medium, typical bacterial colonies appear fluidal, irregular in shape, and white with pink centres after 2 to 5 days incubation at 28°C as reported by Champoiseau (2008). *R. solanacearum* produced fluidal colonies with pink or light red colour on TZC media after 24 hours of inoculation as reported previously by Rahman et al. (2010).

### 3.6 Biovar Differentiation

The result of the biovar test showed that all *R. solanacearum* isolates oxidized disaccharides (sucrose, lactose, and maltose) and sugar alcohols (manitol, sorbitol and dulcitol) within 3-5 days. The oxidation reaction was indicating by the change of colour. The results revealed the change of colour blue to yellow indicating the oxidization of sugars by bacterial isolates. Therefore, all groups of *R. solanacearum* isolates belong to biovar III as shown in Table 3. On the other hand, all the control plates of different sugar and sugar alcohol remain unchanged. The differentiation of biovars of *R. solanacearum* based on the utilization of carbohydrates was reported previously by Hayward (1964), He et al. (1983), Kumar et al. (1993). Also, they observed that biovar III oxidizes both disaccharides and hexose alcohols, biovar II oxidizes only disaccharides whereas Biovar I oxidizes hexose alcohols only, and biovar IV oxidizes only alcohols.

Table 3. Differentiation of *Ralstonia solanacearum* into biovars and races

Isolate group	Utilization of carbohydrates						Biovars	Races
	Dextrose	Maltose	Lactose	Sorbitol	Manitol	Dulsitol		
1	+	+	+	+	+	+	III	3
2	+	+	+	+	+	+	III	3
3	+	+	+	+	+	+	III	3

+ Positive reaction, - Negative reaction.

Isolate Group 1: Munshigonj, Isolate Group 2: Nilphamari and Isolate Group 3: Jamalpur.

### 3.7 Identification of Races

There is no biochemical test for race identification of bacterial wilt pathogen *R. solanacearum*. The races of *R. solanacearum* were identifying by pathogenicity tests in wide host range such as brinjal, tomato and chilli. The result of the pathogenicity test showed that none of the group of *R. solanacearum* isolates tested in the study was not able to cause wilt symptom in inoculated brinjal, tomato and chilli plants indicating a narrow host range but the isolates produced wilt symptom in potato seedlings (Table 2). Therefore, all groups of *R. solanacearum* isolates causing bacterial wilt of potato collected from three selected growing areas belong to race 3. On the other hand, isolate obtained from wilted brinjal plant inducing wilt symptom in tomato, chilli and brinjal which is belonging to race 1. Denny and Hayward (2001) identified race of *R. solanacearum* by host range. The findings of the present study are also supported by Buddenhagen et al. (1962) who classified *R. solanacearum* into three races who found only one race. Race 1 infects many solanaceous plants such as brinjal, tomato, tobacco, pepper and other plants including some weeds. In addition to race 2 that causes wilt of triploid banana (*Musa* spp.) and *Heliconia* spp., while race 3 affects potato and tomato but it is weakly virulent on other solanaceous crops. Aragaki and Quinon (1965) reported that race 4 infected ginger in the Philippines. He et al. (1983) reported race 5 from mulberry in China. Five races have been described so far, but they differ in host range, geographical distribution and ability to survive under different environmental conditions (French, 1986). Patrice (2008) reported that *R. solanacearum* was initially subdivided into races and biovars based on variability in host range. He added that five races have been identified within the species. Strains of *R. solanacearum* have also been divided into five host-specific races by Pradhanang et al. (2000). However, the results of this study primarily indicated that bacterial wilt pathogen of potato; *R. solanacearum* is belonging to race 3 although no wilt symptom was observed in tomato.

## 4. Conclusion

The incidence of bacterial wilt varied in the major potato growing areas may be due to the species complex of the pathogen, *R. solanacearum* and also for various soil factors. Biovar III and Race 3 of *R. solanacearum* was only prevalent in all growing areas of potato in Bangladesh. The findings of the present study will be useful for designing the study of the population structures of *R. solanacearum* using the molecular approaches with special emphasis on its integrated management.

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