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BIOCHEMICAL CHARACTERIZATION OF *Ralstonia solanacerum* CAUSING BACTERIAL WILT OF BRINJAL IN BANGLADESH

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ABSTRACT

Ralstonia solanacearum causes bacterial wilt of solanaceous crop plants including brinjal, a most devastating disease in humid tropic. A survey was conducted on the status of bacterial wilt incidence and severity in major brinjal growing areas and to characterize the isolates of *R. solanacearum* causing bacterial wilt of brinjal in Bangladesh. The wilt incidence was recorded maximum (22.52) in Rangpur followed by Jessore (20.56) and Panchagarh (20.0) while the lowest wilt incidence was recorded in Jamalpur (6.12). On the contrary, the wilt severity was recorded highest (4.00) in Jhinaidah and the lowest (2.80) was recorded in Jamalpur followed by Jessore (2.93) at the time of survey. The isolates of *R. solanacearum* were obtained from different locations surveyed were arranged in seven groups based on the location. Gram's staining and Potassium hydroxide solubility test revealed that all groups of *R. solanacearum* isolates are gram negative. The isolates of *R. solanacearum* fermented four basic sugars (Dextrose, sucrose, manitol and lactose). These results of all biochemical tests in combination with the pathogenicity test confirmed the isolates were *R. solanacearum* causing bacterial wilt of brinjal. All groups of *R. solanacearum* isolates were found virulent producing pink or light red color or characteristic red center and whitish margin on TZC medium after 24 hours of incubation. On the biovar test clearly revealed that all groups of *R. solanacearum* isolates oxidized disaccharides (Sucrose, lactose, and maltose) and sugar alcohols (manitol, sorbitol and dulcitol) within 3-5days and confirmed biovar as III. Pathogencity test on tomato and chilli indicating wide host range of *R. solanacearum* isolates and categorized them in race 1. Therefore, it may be confirmed that *R. solanacearum* causing bacterial wilt of brinjal in Bangladesh belong to Biovar III and Race 1.

Key Words: Characterization, *Ralstonia solanacerum,* Wilt, Brinjal

INTRODUCTION

Ralstonia solanacearum, the causal agent of bacterial wilt disease, is a severe obstacle to the production of solanaceous plants in both tropical and temperate regions. As a diverse species complex, *R. solanacearum* has developed an extremely broad host range throughout the world, including >450 host species representing 54 plant families (Wicker *et al.* 2007). Traditionally, *R. solanacearum* was classified into five races (r) (Buddenhagen

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et al. 1962; He *et al.* 1983 and Pegg and Moffet 1971), on the basis of differences in host range, and six biovars (bvs), according to the ability to oxidise three hexose alcohols and three disaccharides (Hayward 1964, 1991, 1994 and He *et al.* 1983). 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.

Brinjal (*Solanum melongena* L) belongs to the family solanaceae and is cultivated and recognized as popular vegetable throughout the entire tropical and subtropical region of the world (Hayward, 1991). Brinjal is locally known as "Begun" and its early European name is "Egg plant". However, the production of brinjal is far below as compared to the major brinjal growing countries of the World. Brinjal suffers a total of twelve diseases in Bangladesh of which phomopsis blight/fruit rot, wilts (Bacterial, fungal and nemic) and collar rot are the major. Although comprehensive research progresses have been made concerning the management of phomopsis blight/fruit rot, collar rot, fungal and nemic wilt in Bangladesh. The comprehensive effort has not been made on the bacterial wilt of brinjal and characterization of its pathogen in Bangladesh. 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. However, a devastating annual loss exceeding $ 950 million was estimated for about three million farm families in 80 countries incurred by bacterial wilt (Walker and Collion, 1998).

The use of bacterial wilt-susceptible commercial cultivars grafted onto bacterial wilt-resistant rootstocks has been demonstrated to be an effective management tool in both the Philippines and Bangladesh. The stability of bacterial wilt resistance in brinjal, potato and tomato, is highly affected by pathogen density, pathogen strains and several soil factors. Other control methods such as soil amendments, crop rotation, biological control, and field sanitation are often not effective mostly due to the complex diversity of the pathogen. Hence it is clear that a better understanding of the population structure of this highly diverse pathogen is needed in order to develop pathogen-targeted and, possibly, geographically targeted management practices. The present study was undertaken to know the status of bacterial wilt of brinjal in terms of its incidence and severity in major brinjal growing areas and to characterize different *R. solanacearum* strains causing bacterial wilt in brinjal upto biovars and races exist in Bangladesh.

MATERIALS AND METHODS

Surveying and sampling

A survey was carried out to know the status of bacterial wilt of brinjal in Bangladesh in terms of its incidence and severity in seven major brinjal growing areas viz. Panchagarh, Rangpur, Pabna, Jessore, Jhinaidah, Jamalpur and Mymensingh during November to December, 2009. At least three locations in each area and five farmer's field for each growing areas were surveyed to record the bacterial wilt incidence and severity. For a

quick field diagnosis of brinjal wilt caused by bacterium, *R. solanacearum* and to distinguish bacterial wilt from vascular wilts caused by fungal pathogen and nematode, bacterial streaming from infected plant material was performed and confirmed by streaming of milky white masses of bacterial cells (ooze) (Shew and Lucas, 1991). At least 10 samples of the diseased plants were collected from each of the surveyed area and were brought into the laboratory for the isolation of different groups of isolate of *R. solanacearum*.

Assessment of disease incidence and severity

The status of bacterial wilt of brinjal was assayed based on wilt incidence and severity. Data on wilt incidence were recorded in at least three locations from five farmer's field for each growing area. 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 in each location to calculate the wilt severity in each growing area. 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.

Isolation, idendification, purification and preservation of Ralstonia solanacearum

The isolates of *R. solanacearum* were isolated in Nutrient Agar (NA) plate from the wilted brinjal plants collected from the different locations of each of the surveyed areas by streaking the bacterial ooze streamed out into the water from the infected stem. The plates were then incubated at 28^0C for at least 24 h. After isolation, *R. solanacearum* isolates were purified by streaking a single colony of each isolate on Triphenyl Tetrazolium Chloride (TTC or TZC) medium as described by Kelman (1954). The isolates collected from different brinjal growing areas were classified into seven groups based on the growing areas from where the isolates were obtained. To confirm the isolates of *R. solanacearum*, the pathogenicity test was performed in one month old brinjal seedlings by soil inoculation method. A single colony of *R. solanacearum* showing virulent, fluidal, irregular and creamy white with pink at the center was selected for each group of isolates for pathogenicity test. At 30-40 days age of tobacco plants, bacterial suspension (approximately 10^8 CFU/ml) of each isolate representing a group was injected in the leaves. Bacterial suspensions were injected into the intracellular space of the leaf with a hypodermal syringe. Hypersensitive reaction was observed daily and continued up to five days of infiltration. The isolates of *R. solanacearum* were preserved in 10% skim milk kept at -20^0C refrigerator for subsequent biochemical studies.

Biochemical characterization of Raslstonia solanacearum

Identification of virulent and avirulent isolates

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

Biochemical tests for the identification of Raslstonia 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 Hossain *et al.* (2007). Single isolate of *R. solanacearum* from each group was randomly selected for biochemical tests.

Determination of biovars

The isolates of *R. solanacearum* were differentiated into biovars based on their ability to utilize disaccharides (Sucrose, lactose, and maltose) and sugar alcohols (manitol, sorbitol and dulcitol) as described previously by Hayward (1954) and He *et al.* (1983). The biovars were determined in the mineral medium ($NH_4H_2PO_4$ 1.0g, KCl 0.2g, $MgSO_4.7H_2O$ 0.2g, Difco bacto peptone 1.0g, Agar 3.0g and Bromothymol blue 80.0 mg per litre) containing 1% sugar. About 200 µl of the melted medium is dispensed into the wells of microtitre plate. Inocula for each group of isolates was prepared by adding several loopful of bacteria from 24-48h old cultures to distilled water to make suspension containing about 10^8 CFU/ml. Then 20 µl of bacterial suspension was added to the wells of microtitre plate incubated at 28-32^{0}C. The tubes were then examined at 3 days after inoculation for change in p^H by a color change (Schaad *et al.*, 2001).

Races identification

The races of *R. solanacearum* were identified by pathogenicity test on wide host range (Schaad *et al.*, 2001).Seedlings of tomato and chilli were raised in tray and one month old seedlings (tomato & chili) were inoculated by soil inoculation method (Kersten *et al.*, 1998). The incubated plants were then kept in the net house until symptoms development.

RESULTS AND DISCUSSION

Incidence and severity of bacterial wilt

A total of seven major brinjal growing areas viz. Panchagarh, Rangpur, Pabna, Jessore, Jhinaidah, Jamalpur and Mymensingh were surveyed to know the status of bacterial wilt of brinjal in terms of its incidence and severity. A significant variation was observed in terms of bacterial wilt incidence among the major growing areas surveyed (Table 1). The survey results showed that the highest (22.52%) bacterial wilt incidence was recorded in Rangpur followed by Jessore and Pachagarh with 20.56 and 20.0 % wilt incidence while

the lowest (6.12%) bacterial wilt incidence was recorded in Jamalpur. The incidence of bacterial wilt of brinjal was recorded 9.98, 10.67 and 14.36%, in Jhinaidah, Pabna and Mymensingh, respectively. On the contrary, the highest (4.0) bacterial wilt severity was recorded in Jhinaidah while the lowest (2.80) bacterial wilt severity was recorded in Jamalpur followed by Jessore with values 2.93. The severity of bacterial wilt of brinjal was recorded 3.13, 3.16 and 3.26 in Mymensingh, Pabna, and Rangpur, respectively (Table 1). The survey results indicated a regional variation in bacterial wilt incidence and severity. Differences of wilt incidence and severity were also reported due to the great diversity of host plants affected by this pathogen, phenotype and genotype of *R. solanacearum,* its wide geographical distribution, and the range of environmental conditions conducive to bacterial wilt (Chatterjee *et al.,* 1997).

Table 1. Incidence and severity of bacterial wilt of brinjal at major growing areas in Bangladesh

Areas surveyed	Wilt incidence (%)	Wilt severity (1-5 scale)*
Panchagarh	20.00a	3.00
Rangpur	22.52a	3.26 ≈ 3
Pabna	10.67c	3.16 ≈ 3
Jessore	20.56a	2.93 ≈ 3
Jhinaidah,	9.98c	4.00
Jamalpur	6.12d	2.80 ≈ 3
Mymensingh	14.36b	3.13 ≈ 3
LSD	2.927	
Level of significance	**	

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

Isolation and identification of the R. solanacearum isolates

A total of 51 *R. solanacearum* isolates were obtained from the wilted brinjal plant samples collected from different locations surveyed. 24 isolates were obtained from Panchagarh, 5 from Rangpur, 4 from Pabna, 5 from Jessore, 4 from Jhinaidah, 4 from Jamalpur and 5 from Mymensingh (Table 2). Although equal numbers of infected plants were collected from each of the surveyed area, the number of isolates varied because of the failure of isolation of the bacterium from all the infected plants or might be due to other wilt symptoms. All of the *R. solanacearum* isolates collected from wilted brinjal plants produced cream color or off-white color colonies on NA media after 24 hours of inoculation.

Pathogenicity and hypersensitivite reaction (HR) test

The results of pathogenicity test revealed that all the isolate groups of *R. solanacearum* were able to cause wilt symptoms in brinjal seedlings (Table 3). The isolates of *R. solanacearum* collected from the wilted brinjal plants were tested for hypersensitive

reaction in tobacco. Result showed that all the isolates were able to cause rapid death of local cell of tissue between veins of tobacco leaves. These results are in accordance with the findings reported by Dhital *et al.* (2001) who observed that *R. solanacearum* was able to produce HR in tobacco leaves. These results clearly indicated that like other plant pathogenic bacteria, *R. solanacearum* isolates possess Hrp type III secretion system which is responsible for inducing HR on tobacco leaves.

Table 2. Isolates of *Ralstonia solanacearum* collected from major brinjal growing areas in Bangladesh

Areas surveyed	Group of Isolates	Number of isolates obtained in each area	Culture in NA medium
Panchagarh	Group 1	24	Cream or off-white color colonies
Rangpur	Group 2	5	Cream or off-white color colonies
Pabna	Group 3	4	Cream or off-white color colonies
Jessore	Group 4	5	Cream or off-white color colonies
Jhinaidah	Group 5	4	Cream or off-white color colonies
Jamalpur	Group 6	4	Cream or off-white color colonies
Mymensingh	Group 7	5	Cream or off-white color colonies

Biochemical tests

Gram's stain test

The Gram's staining reaction were performed using crystal violate. The microscopic results showed that all of the isolates of *R. solanacearum* did not retain violet color i.e. the isolates retained counter stain (pink color). 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).

Potassium hydroxide solubility test

All of the plant pathogenic bacteria are usually gram negative except C*lavibacter* and *Streptomyces*. The gram negative test of *R. solanacearum* was also confimed by Potassium hydroxide solubility test. The positive test indicated by a elastic thread or viscious thread observed when loop raised from the bacterial solution by toothpick a few centimeters from glass slides in case of all group of isolates of *R. solanacearum* indicating that all groups of *R. solanacearum* isolates are gram negative (Table 3). 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-strain in which dyes are employed.

Table 3. Biochemical tests of *Ralstonia solanacearum* of different isolated groups

Isolate Name	Pathogenecity test and color test on TTC media	HR Test	Gram staining reaction	KOH solubility test	Kovac's oxidase test	Levan test	Sugar fermentation test				Inference
							Dextrose	Sucrose	Manitol	Lactose	
Group 1	+	+	+	+	+	+	+	+	+	+	*Ralstonia solanacearum*
Group 2	+	+	+	+	+	+	+	+	+	+	*Ralstonia solanacearum*
Group 3	+	+	+	+	+	+	+	+	+	+	*Ralstonia solanacearum*
Group 4	+	+	+	+	+	+	+	+	+	+	*Ralstonia solanacearum*
Group 5	+	+	+	+	+	+	+	+	+	+	*Ralstonia solanacearum*
Group 6	+	+	+	+	+	+	+	+	+	+	*Ralstonia solanacearum*
Group 7	+	+	+	+	+	+	+	+	+	+	*Ralstonia solanacearum*

Group 1 = Panchagarh, Group 2 = Rangpur, Group 3 = Pabna, Group 4 = Jessore, Group 5 = Jhinaidah, Group 6 = Jamalpur, Group 7 = Mymensingh

Kovac's oxidse test

Kovac's oxidse test was also performed 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 color with oxidase reagent within few seconds which indicated that the tested group of *R. solanacearum* isolates were gram negative (Table 3).

Levan test

Levan is an extracellulr bacterial polysaccharide (bête-2, 6-1 linked D-fructan), whose potential and actual uses are similar to those of dextran (Avigad, 1968). Induction of the Levan was performed in NA medium containing 5% sucrose, Levan sucrase (E.C. 2.4.1.10), which catalyzes the synthesis of Levan form 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 3). When the bacteria were grown on a medium containing sucrose, the production of an extracellular enzyme (levan sucrase) was induced and sucrose was converted to levan and glucose. During the fermentation process, the bacteria also utilize sucrose for maintenance and growth. Levan production was also reported by soil-borne bacterium, *Bacillus licheniformis* by Ghaly *et al.* (2007).

Sugar fermentation test

The isolates of *R. solanacearum* is able to oxidize the sugars which are indicated by color change (reddish to yellow). The results of Sugar fermentation test clearly showed that all groups of *R. solanacearum* isolates obtained from the wilted brinjal plants were able to oxidize the four (4) basic sugars (Dextrose, sucrose, manitol and lactose) by producing acid and gas (Table 3). The acid production in sugar fermentation test by bacterial isolates was indicated by the color change from raddish to yellow, gas production was noted by the apperence of gas bubbles in the inverted Dhuram's tubes and the oxidation of sugar manitol by the bacterial isolates indicated by the production of yellow to red color.

Identification of virulent/avirulent strains of Ralstonia solanacearum

The virulent and avirulent isolates of *R. solanacearum* were differentiated by Kelman Tetrazolium choloride (TZC) agar test. The results showed that virulent isolates produce pink or light red color colonies or colonies with characteristic red center and whitish margin and avirulent isolates produce smaller, off-white and non-fluidal or dry on TZC medium after 24 hours of incubation. The result showed that all groups of *Ralstonia solanacearum* isolates collected from different growing areas produced pink or light red color colonies or colonies with characteristic red center and whitish margin on TZC medium. This indicates that all groups of *Ralstonia solanacearum* isolates were virulent (Table 3). Kelman (1954) reported that avirulent colony types of *R. solanacearum* could

easily be differentiated by the pigmentation from the wild virulent types of *R. solanacearum*. *R. solanacearum* developed two types of colonies on tetrazolium chloride (TZC) medium on which virulent colonies appeared white with pink centers and non-virulent colonies appeared dark red. On this medium, typical bacterial colonies appear fluidal, irregular in shape, and white with pink centers after 2 to 5 days incubation at 28^{o}C reported by Champoiseau (2008).

Biovar differentiation

The biovar of *R. solanacearum* isolates was identified by utiliztion of disaccharides and hexose alcohols. The result of the biovar test showed that all seven groups of *R. solanacearum* isolates oxidized disaccharides (Sucrose, lactose, maltose) and sugar alcohols (manitol, sorbitol and dulcitol) within 3-5days (Table 2 & 3). The oxidation reaction was indicated by the change of color. The results revealed a change of color blue to yellow color indicating the oxidization of sugars by bacterial isolates. Therefore, all groups of *R. solanacearum* isolates belong to biovar III as shown in (Table 4 and Fig. 1). On the other hand all the control plates of different sugars and sugar alcohols 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) and Kumar *et al*. (1993). They observed that biovar III oxidizes both disaccharides and hexose alcohols whereas Biovar I oxidize hexose alcohols but not disaccharides, biovar II oxidizes only disaccharides and biovar IV oxidizes only alcohols.

Table 4. Differentiation of *Ralstonia solanacearum* into biovars and races

Isolate group	Utilization of carbohydrates						Biovars	Races
	Dextrose	Maltose	Lactose	Sorbitol	Manitol	Dulsitol		
1	+	+	+	+	+	+	III	I
2	+	+	+	+	+	+	III	I
3	+	+	+	+	+	+	III	I
4	+	+	+	+	+	+	III	I
5	+	+	+	+	+	+	III	I
6	+	+	+	+	+	+	III	I
7	+	+	+	+	+	+	III	I

Summary and conclusion

Bacterial wilt incidence varied in the major brinjal growing areas may be due to the species complex of the pathogen at molecular level and various soil factors. Pathotypes and biotypes of bacterial wilt pathogens of brinjal were remained same in major brinjal growing areas of Bangladesh. Biovar III and Race I of *R. solanacearum* was only prevalent in all growing areas of the country. The findings of the present study will step forward in

determination of the population structures of *R. solanacearum* to design an effective molecular based analysis of *R. solanacearum* causing bacterial wilt disease with special emphasis on its integrated management.

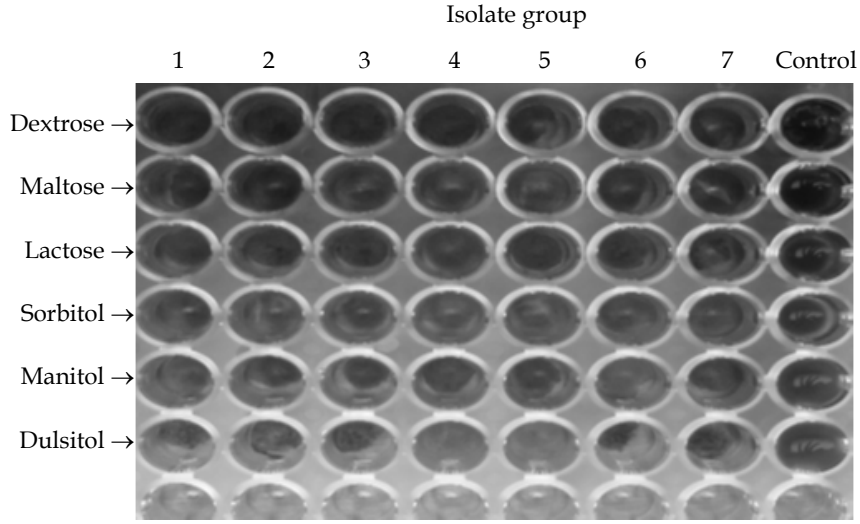

Fig. 1. Biovar test showing positive (+ve) yellow color and negative (-ve) green color reaction indicating the utilization of sugar and alcohol by *Ralstonia solanacearum* isolates in microtitre plate

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