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Article

Bacterial wilt of tobacco in Bangladesh: a pilot study for assessment of the status, detection of seed-borne nature and genetic variation of its pathogen, *Ralstonia solanacearum*

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Abstract: Bacterial wilt of tobacco caused by *Ralstonia solanacearum* poses a significant threat to tobacco cultivation in Bangladesh. In this study, fourteen tobacco growing areas of Bangladesh were surveyed to assess the status of bacterial wilt disease in tobacco. The result revealed that the higher level of bacterial wilt incidence and severity was recorded in Bandarban followed by Naikhonchari, Lama, Rangpur, and Lalmonirhat and the lower level was in Chakaria, Kushtia, Meherpur, Chuadanga, and Jhenaidah. However, a moderate level of incidence and severity was recorded in Manikgonj, Khagrachori, and Tangail. After estimating the bacterial population in the soil of respective regions it was observed that the highest bacterial population was counted at 22.4 x 10^7 CFU/g soil in Chakaria and the lowest was counted at 4.3 x 10^7 CFU/g soil in Tangail. Among the seed samples, sample no BWT Seed 21 had the highest level of infection (93%), sample no BWT Seed 37 had a medium level (39.46%), and sample no BWT Seed 57 had the lowest level of infection (5.14%) with *R. solanacearum* as detected by plating tobacco seed samples by TTC agar medium. Biovar and races of *R. solanacearum* were identified through the sugar oxidation test and pathogenicity test and it was found that *R. solanacearum* isolates from all the fourteen growing areas were grouped into race 1 and biovar 1, biovar 2, biovar 3. Genetic diversity of *R. solanacearum* analysis was done by using REP and ERIC primers where both primers showed three clusters (I, II, and III) at 60% similarity. The results are very crucial for the development of sustainable management of bacterial wilt of tobacco and for future population analyses of *R. solanacearum*.

Keywords: bacterial wilt; tobacco; seed-borne nature; genetic variation; *Ralstonia solanacearum*

1. Introduction

Tobacco (*Nicotiana tabacum* L.) is one of the important cash crops in the world including Bangladesh. However, this crop is badly affected by bacterial wilt caused by *Ralstonia solanacearum* species complex, including other solanaceous and musaceae family crops (Ahmed *et al.*, 2022). The bacterial wilt of tomato, tobacco, eggplant, and some ornamental plants, as well as brown rot of the potato, are among the numerous crop plants whose output is severely constrained by this disease in tropical, subtropical, and warm temperate parts of

the world (Patil *et al.*, 2012; Jiang *et al.*, 2017). This bacterium survives in soil, water, or reservoir plants after destroying the host and then is released into the soil or other environments as a saprophyte until it contacts a new host (Hossain *et al.*, 2022). The symptoms of bacterial wilt include quick drooping of the plant's foliage and the sign occurs as discoloration of the vascular system from pale yellow to dark, stunting of the plant, and may die rapidly (Manda *et al.*, 2020).

R. solanacearum is considered a "species complex" because of the strong genetic diversity that occurs inside the organisms (Ahmed *et al.*, 2013). The species *R. solanacearum* has been split into five biotypes based on its capacity to oxidize sugars (sucrose, maltose, and lactose) and sugar alcohols (sorbitol, mannitol, dulcitol) and is a complex taxonomic entity with wide physiological and genetic variation and five races of *R. solanacearum* were determined based on variations in host range (Razia *et al.*, 2021).

Unlike other phytopathogenic bacteria, race systems of *R. solanacearum* are not dependent on gene-for-gene interactions *i.e.*, different cultivars carrying different R genes. Instead, these are established based on each isolate's pathogenicity in several host plant types. Every race crosses the biovars, and every biovar has a variety of races. Only biovar 2 and race 3 exhibit a favorable association between the biovar and race systems (Patrice, 2008). The phylogenetic relationships between *R. solanacearum* isolates were investigated using a variety of DNA analysis methods, including restriction fragment length polymorphisms (RFLP), polymerase chain reaction (PCR)-RFLP, 16S rRNA gene sequence analysis, repetitive extragenic palindromic (REP)-PCR, and amplified fragment length polymorphism (AFLP) (Horita and Tsuchiya, 2001; Liu *et al.*, 2017). Hossain *et al.*, 2022 discovered that the REP and ERIC markers are widely used in conjunction with the rep-PCR (repetitive sequence PCR) technique, which comprises evaluating repetitive sequence DNA polymorphism present in the bacterial genome. Each strain has a varied distribution of these sequences, which makes it possible to distinguish between the strains. Information related to the status of bacterial wilt such as the soil-borne nature of this pathogen in different solanaceous vegetables (chili, brinjal, tomato) and genetic diversity using a diverse range of molecular markers (REP, ERIC) is very crucial for the development of sustainable management of bacterial wilt of tobacco (Horita and Tsuchiya, 2001; Genin, 2010). The investigation by a number of researchers in Bangladesh has revealed that bacterial wilt caused by *Ralstonia solanacearum* incidence and severity may vary in different crops like tomato, brinjal, chili, potato, and other solanaceous crops due to different geographical locations (Rahman *et al.*, 2010; Ahmed *et al.*, 2013). As a result, there is no information available about the prevalence of tobacco bacterial wilt, the biovars, and races that cause it, or the diversity of those organisms in Bangladesh. In this study, the status of bacterial wilt of tobacco, determination of the seed-borne nature, biovars and races of *R. solanacearum* isolates and the phylogenetic relationship among *R. solanacearum* isolates obtained from the tobacco growing areas using repetitive element (REP and ERIC) were investigated.

2. Materials and Methods

2.1. Ethical statement

Ethical approval was not required for this study.

2.2. Materials

2.2.1. Survey and samples collection and assessment wilt incidence and severity

A survey to know the status of bacterial wilt disease in terms of the incidence and severity was done in the selected tobacco fields of fourteen districts of Bangladesh viz. Naikhongchari, Lama, Chakaria, Bandarban, Khagrachari, Manikganj, Tangail, Rajbai, Meherpur, Kushtia, Jhenaidah, Chuadanga, Lalmonirhat and Rangpur (Figure 1). At least three locations in each growing area and five farmer’s fields in each location were surveyed. After collecting the samples of the diseased tobacco plants from each of the surveyed areas they were brought for the detection of *R. solanacearum* in the laboratory. The bacterial wilt incidence was recorded in at least five fields in each growing area surveyed and calculated using the following formula:

% Wilt incidence = (Number of infected plants/ Total number of plants) × 100

Based on the severity scale, the degree of bacterial wilt that was recorded was previously described by Horita and Tsuchiya, 2001. Briefly, 1 denotes no symptom, 2 denotes wilted top young leaves, 3 denotes two wilted leaves, 4 denotes four or more wilted leaves, and 5 denotes plant death.

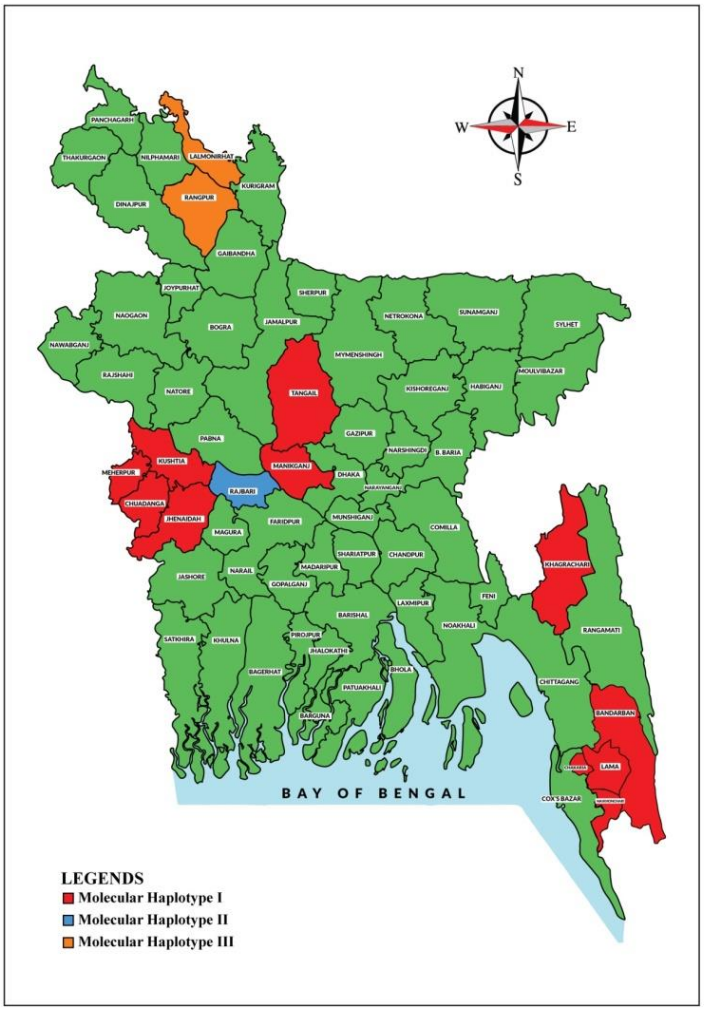

Figure 1. Geographical distribution of different molecular haplotypes of *R. solanacearum* in major tobacco-growing areas of Bangladesh.

2.3. Detection of *R. solanacearum*

2.3.1. Detection of *R. solanacearum* in soil samples and tobacco seeds from infected fields and plating the seed on TTC media

The dilution plate technique was followed for the detection of *R. solanacearum* in the soil samples to assess the status of *R. solanacearum* population in the soil. The Zig-zag method was followed to collect the soil samples from each surveyed area. Around 300-400 g rhizosphere soil (a composite preparation) was collected for the determination of *R. solanacearum* population per gram soil following the dilution plate technique. Briefly, a stock soil suspension was prepared by taking a 1 g soil sample in 9 ml sterile water. Then the solution was diluted serially until the countable number of colonies was grown on the TTC medium (2, 3, 5-triphenyltetrazolium chloride) and the results were expressed in CFU per gram soil (Lin *et al.*, 2009). The seed samples were surface sterilized for one minute with a solution of one percent sodium hypochlorite, washed three times with distilled water between each wash, blotted to dry, and then immediately plated on TTC agar medium. In each plate, 25 tobacco seeds were plated by hand and were incubated for 36-48 hours at 28°C. The plates were then observed for the presence of *R. solanacearum* which grew around seeds as a colony with a red center and white margin. Sixteen plates containing 400 seeds for each sample were maintained.

Virulent: The characteristic red center with whitish margin colony on TTC medium or pink or light red color was used to identify the virulent isolates of the bacteria (Ahmad *et al.*, 2013).

Avirulent: The avirulent isolates have smaller, off-white, and non-fluidal or dry colonies on the TTC medium (Ahmad *et al.*, 2013).

The seed infection was then calculated as follows:

% Seed infection = (Number of infected seeds/ Total number of seeds) × 100

2.4. Isolation, purification, and preservation of *R. solanacearum* by PCR

The isolation of the bacterial colony was done and again plated in a nutrient agar (NA) medium. For purification well separated, having an irregular shape, dull white in color, fluidal appearance with pink centered single colony were isolated and sub-cultured on NA medium. *R. solanacearum* isolates preserved by 3-4 loopful of the virulent colonies were suspended in CPG broth containing 25% glycerol in the Cryovial. For further study, the tubes were stored in a refrigerator at -80°C. According to Chandrashekara *et al.,* 2012 CPG (casamino acid, peptone, and glucose) broth was prepared for the liquid culture of *R. solanacearum*. The required chemicals for 100 ml CPG preparation are 100 ml distilled water, 0.1g casamino acid, 1g bacteriological peptone, and 0.5g glucose. After mixing all ingredients, 5ml of the mixture was taken in 15ml falcon tubes and sterilized at 121°C under 15 PSI pressure for 15 minutes. A characteristic single colony of *R. solanacearum* from TTC media was streaked onto NA medium and incubated for about 24 hours at 28°C. A loopful bacterial culture from NA media was transferred in CPG broth and shaken by an electrical shaker for 18 hours at 28°C. Wizard® Genomic DNA Purification Kit: pH 7.0 (Promega, Madison, WI, USA) was used for the extraction of the Genomic DNA of *R. solanacearum* according to the manufacturer's instructions. Finally, the genomic DNA samples of all isolates were preserved in deep freeze at -20°C for further use.

2.5. Primers, PCR condition, and electrophoresis

According to Hossain *et al.,* 2022, Specific primers Y-2 /OLI-1 were used to confirm the *R. solanacearum* through PCR (Hossain *et al.,* 2010). The forward primer {Y2-(FP) CCCACTGCTGCCTCCCGTAGGAGT (length 24, 66.4 Tm°C, Product size 288 bp)} is designed along one strand in the direction toward the reverse primer {OLI-1 (RP) GGGGGTAGCTTGCTACCTGCC (length 21, 63.4 Tm° C, Product size 288 bp)}. Likewise, the reverse primer is designed from the complementary strand. PCR reactions were carried out in a 25 µl volume containing 50 pmol each of two opposing primers, 625 mM deoxynucleoside triphosphates (dNTPs), and 2U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus). The PCR reaction mixture consists of 12.5 µl of GoTaq® G2 Green Master Mix (Promega, Madison, WI, USA) (1X PCR reaction buffer (pH 8.5), 400 µM of each of the four deoxyribonucleotide triphosphates (dNTPs), 4 mM $MgCl_2$, 0.5 units Taq DNA polymerase, 1 µl of each primer, 9.5 µl of Nuclease free water, and 1 µl of DNA template. Initial denaturation, denaturation, annealing, extension, ultimate extension, and visualization were performed.

2.6. Determination of biotypes and races

2.6.1. Culture of *R. solanacearum* isolates

To inoculate tobacco, brinjal, and chili seedlings under artificial conditions, *R. solanacearum* was multiplied in casein peptone glucose (Casamino acid 1g L^{-1}, Peptone 10g L^{-1}, Glucose 10g L^{-1} pH 7.2) for 24 hrs.

2.6.2. Preparation of inoculum and raising of seedlings and inoculation

According to Chandrashekara *et al.,* 2012, the concentration of the bacterial cells was adjusted to 5 x 10^8 cfu/ml using spectrophotometer readings at a wavelength equal to A600nm =0.8 to 1.0. The bacterial cells were suspended in sterile water. Viable tobacco, brinjal, and chili seedlings were cultivated in trays under net house conditions. Tobacco, eggplant, and chili seedlings that were about a month old were carefully removed, the soil cleaned out, and a few tertiary roots were trimmed using sterile scissors and dipped in bacterial culture for ten minutes. The seedlings that had been inoculated were moved into earthen pots with soil that had been sterilized. As a control, plants that had received similar sterile water spraying were used. The number of days it took for symptoms to manifest was observed. The plants were given two days to completely wilt once the first signs of wilt showed. With a sterile scalpel, 5 cm of the stem above the soil level was cut to confirm the presence of *R. solanacearum* infection. One milliliter of sterile, distilled water was used to rehydrate 100 mg of contaminated tissue for 10 minutes. The presence of *R. solanacearum* was next verified by plating the sample on a TTC medium.

2.6.3. Determination of biovars

The isolates of *R. solanacearum* were classified into different biovars through the generation of acids by the utilization of sugar sources (Tahat and Sijam, 2010). The various steps include the preparation of mineral medium for the Biovars test, the preparation of sugar solution, and the test described (Rahman *et al.,* 2010).

2.6.4. Identification of races

R. solanacearum was once loosely categorized into "races" depending on the host range (Chandrashekara *et al.,* 2012). Race 1 strains infect and spread disease to mulberry trees, are only found in China, and are restricted to

musaceous species like *Heliconia spp*. and triploid bananas. Race 3 strains target potatoes the most, while Race 4 strains are particularly virulent on ginger. Race 5 strains exclusively infect and spread disease to mulberry trees. As a result, the aforementioned classification was used for *R. solanacearum* isolates.

2.7. Genetic diversity of *Ralstonia solanacearum,* primers, and PCR conditions

R. solanacearum is a heterogeneous organism that shows both phenotypic and genotypic diversity. Five races and five biotypes make up the species. This work used rep-PCR to assess the diversity of *R. solanacearum* isolates obtained from significant tobacco-growing regions. Collectively, the markers REP and ERIC were used to find out the ancestor of these *R. solanacearum* isolates. The primers for rep-PCR are as follows: ERIC (ERIC 1R (RP) [5'-ATGTAAGCTCCTGGGGATTCAC-3'] and ERIC 2 (FP) [5'-AAGTAAGTGACTGGGGTGAGCG3']) and REP (REP IR (RP) [5'-IIIICGICGICATCIGGC-3'] and REP2I (FP) [5' - ICG I CTTATC I GGCCTAC – 3'] (Hossain *et al.,* 2022).

2.8. Data analysis

The percentage of infected seeds and the bacterial population in the soil were examined using the statistical program R 4.2.0. Each band represents the phenotype at a single allelic locus because these markers are dominant (William *et al.,* 1990). The size of the amplification products was estimated using a single molecular marker, a 100 bp DNA ladder, by comparing the distance traveled by each fragment with that of the known-sized pieces of the molecular weight marker. As a result, each isolate and primer was given a unique number according to the position on the gel, and all different bands or fragments were scored visually on the basis of their existence (1) or absence (0). A single data matrix was produced using all the scores. A computer-based program called NT SYS PC 2.02i was used to calculate polymorphic loci, Nei's gene diversity (Nei, 1972), population differentiation (Gst), gene flow (Nm), gene distance (D), and to create a UPGMA (Unweighted Pair Group Method of Arithmetic Means) dendrogram among populations (Hossain *et al.,* 1999).

3. Results

3.1. Assessment of the status of bacterial wilt of tobacco with its incidence and severity in Bangladesh

The infection levels were classified as low, moderate, or high based on the percent wilt occurrence. According to Figure 2, the lowest (0.33%) incidence of bacterial wilt was found in Meherpur, Kustia, Jhenaidah, Chuadanga, and Chakaria districts, while the highest (8.33%) incidence was found in Bandarban, followed by the second highest (7.66%) incidence in Naikhonchari and Lama, followed by Rangpur (6.00%) and Lalmonirhat (5.33%). Manikgonj, Khagrachari, and Tangail, on the other hand, had medium wilt incidences of 2.33%, 1.66%, and 0.66%, respectively. There was no evidence of bacterial wilt in Rajbari. The highest (4.67) bacterial wilt severity was observed in Bandarban, where infected plants almost died, followed by the second highest (4) disease severity in Lalmonirhat and Rangpur, and the lowest (0.67) severity was observed in Chakaria, Tangail, Khagrachari, Kushtia, Chuadanga, Meherpur, and Jhenaidah, all of which indicated the presence of *R. solanacearum* without displaying symptoms. Lama, Naikhonchari, and Manikganj had medium levels of bacterial wilt severity, with severity scores of 3.67, 3.33, and 2.33, respectively.

3.2. Bacterial population in soil (CFU/g×10^7soil)

The survey results of fourteen tobacco-growing areas revealed that the maximum bacterial population was counted in the Rangpur and Chakaria districts. Similar level bacterial populations were recorded in soils collected from Meherpur, Kushtia, Chuadanga, and Lalmonirhat. Bacterial populations were the same in the Brahmaputra Alluvium soil tract (Tangail, Manikganj, Rajbari) and hill tract except in Khagrachari (Table 1).

Table 1. The bacterial population (CFU/g soil) in the tobacco-growing areas of Bangladesh.

Locations	Bacterial population (CFU/g× 10^7 soil)
Tangail	4.3 ef
Rajbari	4.7 ef
Manikganj	5.7 de
Bandarban	5.7 de
Chakaria	22.4 a
Naikhongchari	5.1 def
Khagrachari	8.6 c
Lama	4.6 ef

Table 1. Contd.

Locations	Bacterial population (CFU/g× 10^7 soil)
Meherpur	7.5 cd
Kushtia	8.7 c
Jhenaidah	13.3 b
Chuadanga	6.0 c
Rangpur	20.0 a
Lalmonirhat	8.4 c
P-Value	0.001
Level of significance	***
CV (%)	16.93

Same letter shows similar level of significance
*** indicates the value is significant at a 0.01% level

3.3. Detection of *R. solanacearum* in tobacco seed samples

A total of sixty-three tobacco seed samples showing significant levels of infection were tested to assess the status of tobacco seeds in terms of the presence of *R. solanacearum*. The maximum (93%) *R. solanacearum* infection was recorded in only one seed sample (BWT Seed sample number 21) in 2016 while the minimum (5.14%) *R. solanacearum* infection was recorded in only one seed sample (BWT Seed sample number 57) of the same year (Figure 2). However, fourteen seed samples were found with 0-10% *R. solanacearum* infection, 11-20% *R. solanacearum* infection was recorded in 18 seed samples, 20-30% *R. solanacearum* infection was noticed in 10 seed samples, 30-40% *R. solanacearum* infection was found in 7 seed samples, 6 seed samples were found with *R. solanacearum* infection of 40-50%, 7 seed samples were found with *R. solanacearum* infection 50-70% and only one seed sample was found with more than 93% *R. solanacearum* infection (Table 2).

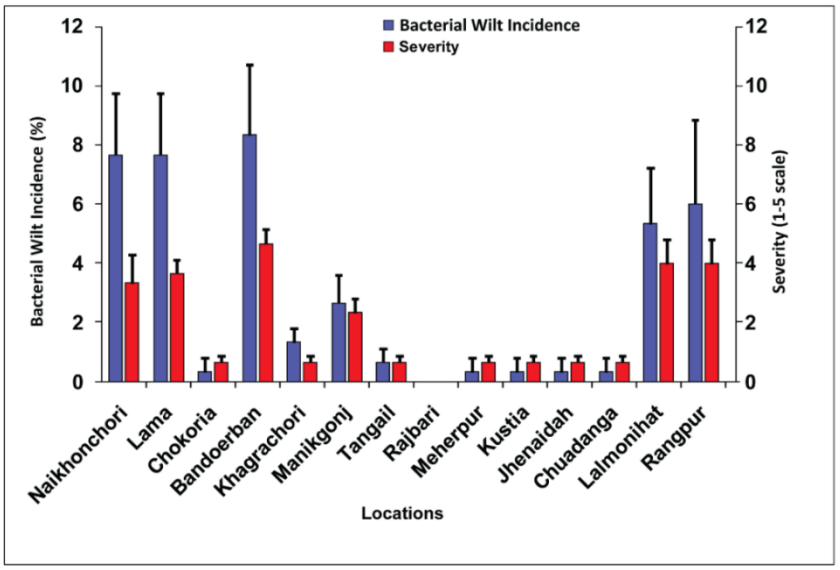

Figure 2. Disease incidence (percent plant infection) in different tobacco growing areas and Severity (1-5 scale) of bacterial wilt in different tobacco growing areas. 1= No symptom, 2 = Top young leaves wilted, 3 = Two leaves wilted, 4 = Four or more leaves wilted and 5 = Plant dies. Data are the averages of the five farmer's fields of each location.

Table 2. Tobacco seed samples with different levels of *R. solanacearum* infection.

Seed sample	% Infection	Seed sample	% Infection	Seed sample	% Infection
1	14.847 a-z	22	10.780 a-z	43	8.870 a-z
2	9.683 a-z	23	18.253 q-y	44	8.620 a-z
3	16.747 a-z	24	18.016 q-y	45	16.180 a-z
4	22.840 n-t	25	7.28 b-d	46	23.510 m-t

Table 2. Contd.

Seed sample	% Infection	Seed sample	% Infection	Seed sample	% Infection
5	10.653 a-z	26	6.880 cd	47	43.180 f-j
6	13.63 a-z	27	15.767 a-z	48	51.837 d-f
7	28.380 l-p	28	14.863 a-z	49	42.747 g-j
8	17.080 a-z	29	21.147 o-v	50	34.533 j-l
9	24.377 m-s	30	16.880 a-z	51	56.743 c-e
10	17.970 q-y	31	43.430 f-j	52	51.397 d-g
11	19.206 q-x	32	10.580 a-z	53	59.870 cd
12	9.650 a-z	33	21.990 o-u	54	60.933 c
13	26.737 l-q	34	72.503 b	55	22.837 n-t
14	19.627 p-w	35	47.817 f-h	56	18.053 q-y
15	25.00 m-r	36	20.527 o-v	57	5.143 d
16	12.920 a-z	37	39.467 h-k	58	17.307 a-z
17	18.340 q-y	38	38.763 i-k	59	45.077 f-i
18	9.793 a-z	39	31.223 k-n	60	50.110 e-g
19	8.350 a-d	40	5.120 d	61	31.366 k-n
20	9.773 a-z	41	10.340 a-z	62	28.573 l-o
21	93 a	42	58.833 c-e	63	31.873 k-m
P-Value	0.001	P-Value	0.001	P-Value	0.001
LSD	8.91	LSD	8.91	LSD	8.91
Level of significance	***	Level of significance	***	Level of significance	***
CV (%)	20.847	CV (%)	20.847	CV (%)	20.847

Same letter shows a similar level of significance
*** indicates the value is significant at a 0.01% level

3.4. PCR-based detection of *R. solanacearum* obtained from soil of the infected area and tobacco seeds

R. solanacearum isolates in soils and seeds of different varieties of tobacco in different tobacco growing areas were detected by PCR using Y2/OL-1 primers as developed by Hossain *et al.*, 2022. The PCR results showed an amplicon size of 288 bp DNA from all isolates, which was specific to *R. solanacearum*. The control sample was filled with nucleus-free water and the figure had no band in the control sample (Figure 3).

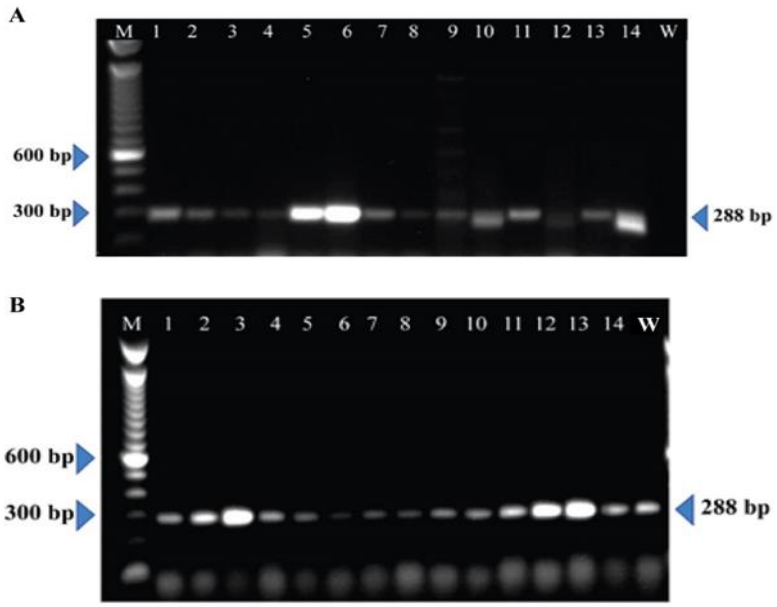

Figure 3. PCR-based detection of *R. solanacearum* using primers Y2/OL-1 from A) different tobacco growing area soils and B) tobacco seeds in Bangladesh. 1: Tangail, 2: Rajbari, 3: Manikganj, 4: Bandarban, 5: Chakaria, 6: Naikhongchari, 7: Khagrachari, 8: Lama, 9: Meherpur, 10: Kustia, 11: Jhenaidah, 12: Chuadanga, 13: Rangpur, 14: Lalmonirhat.

3.5. Determination of biovar and races of *Ralstonia solanacearum* isolates obtained from tobacco

Bacterial wilt is a widespread bacterial disease that can affect people in tropical, subtropical, and certain temperate regions of the world (Fegan and Prior, 2005). *R. solanacearum* strains were divided into five races according to their host range, and into five biovars according to how differently they produced acid from a panel of carbohydrates (Denny, 2006). Races and biovars are not generally correlated. The host ranges and geographic distributions of *R. solanacearum*'s five races vary.

3.5.1. Determination of biovar

The fourteen isolates of *R. solanacearum* were assessed for biovar determination based on their ability to metabolize disaccharides (sucrose, lactose, and maltose) and sugar alcohols (mannitol, sorbitol, and dulcitol), (Razia *et al.*, 2021). The findings demonstrated that within 3-5 days, all *R. solanacearum* isolates contain sugar alcohols and oxidized disaccharides. The color change of the solution occurs from blue to yellow due to p^H change indicating that oxidation of sugar and sugar alcohols were taken place. The result of the test showed that ten isolates (Lama, Kushtia, Rangpur, Manikganj, Bandarban, Lalmonirhat, Chakaria, Naikhongchari, Khagrachari, Chuadanga) of tobacco belong to the biovar 3, six isolates (Tangail, Rajbari, Manikganj, Bandarban, Chakaria and Meherpur) belong to biovar 1 and four isolates (Lama, Kushtia, Jhenaidah, and Meherpur) belong to biovar 2 which is shown in Table 3.

Table 3. Utilization of sugar and alcohol by isolates, determination of biovars and races from different tobacco growing regions of Bangladesh.

Isolate ID	Sugar utilization						Biovar	Race determination			Race
	Glucose	Maltose	Lactose	Sorbitol	Manitol	Dulcitol		Pathogenicity on tobacco	Pathogenicity on brinjal	Pathogenicity on chili	
RS-01	+	−	−	−	−	−	1	+	+	+	1
RS-02	+	−	−	−	−	−	1	+	+	+	1
RS-03	+	+	+	+	+	+	1,3	+	+	+	1
RS-04	+	+	+	+	+	+	1,3	+	+	+	1
RS-05	+	+	+	+	+	+	1,3	+	+	+	1
RS-06	+	+	+	+	+	+	3	+	+	+	1
RS-07	+	+	+	+	+	+	3	+	+	+	1
RS-08	+	+	+	+	+	+	2,3	+	+	+	1
RS-09	+	+	+	−	−	−	1,2	+	+	+	1
RS-10	+	+	+	+	+	+	2,3	+	+	+	1
RS-11	+	+	+	−	−	−	2	+	+	+	1
RS-12	+	+	+	+	+	+	3	+	+	+	1
RS-13	+	+	+	+	+	+	3	+	+	+	1
RS-14	+	+	+	+	+	+	3	+	+	+	1

3.5.2. Determination of races

In order to determine the race of *R. solanacearum*, fourteen isolates were collected. Pathogenicity tests on a variety of hosts allowed for the differentiation of *R. solanacearum* races (Denny and Hayward, 2001). Tobacco, eggplant, and chili seedlings were infected with bacterial suspensions of each tobacco isolate for this test using the soil inoculation method. After three days of inoculation, the incubated plants were inspected for pH changes, identified by a color change, and retained in the net house until symptoms developed (Schaad *et al*., 2001). The pathogenicity test result revealed that all *R. solanacearum* isolates were capable of producing the wilt symptom in tobacco, eggplant, and chili plants, indicating a wide host range as shown in Figure 4.

Therefore, with the exception of the *R. solanacearum* isolates from Rajbari and Tangail, the majority of the examined *R. solanacearum* isolates collected from various growing regions (Bandarban, Chakaria, Naikhongchari, Khagrachari, Lama, Meherpur, Kushtia, Jhenaidah) belonged to race 1. They are belonging to either Race 2 Race 3 or Race 4. Representative isolates of *R. solanacearum* from Bandarban, Chakaria, Naikhongchari, Khagrachari, Lama, Meherpur, Kushtia, and Rangpur were found more virulent as compared to isolates from Tangail, Rajbari, Chuadanga, Jhenaidah when inoculated artificially on tobacco plants. Representative isolates of *R. solanacearum* from Tangail, Meherpur, Jhenaidah, and Rangpur were observed more virulent as compared to the isolates from other growing regions when inoculated artificially on eggplants. Representative isolates of *R. solanacearum* from Bandarban, Naikhongchari, Meherpur, Jhenaidah, and Chuadanga were observed more virulent as compared to the isolates obtained from other growing areas when inoculated artificially on chili plants.

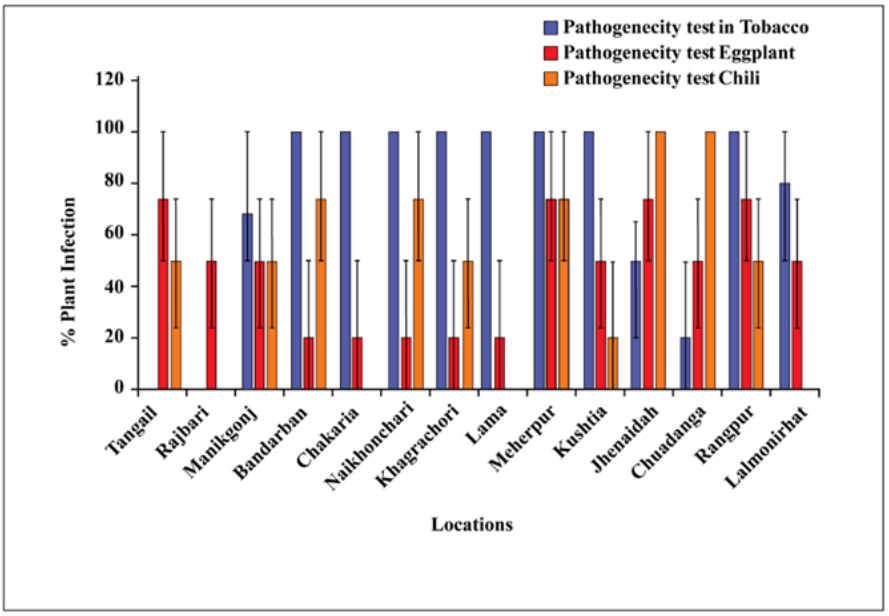

Figure 4. Percent plant infection by *R. solanacearum* isolates when inoculated onto tobacco plants, eggplants, and chili plants.

3.6. Genetic diversity analysis and dendrogram of *R. solanacearum* obtained from different tobacco growing areas using REP marker, ERIC primer

In spite of morphological and cultural similarities of *R. solanacearum* isolates of tobacco by using REP primer and ERIC primer, the genetic variation in the banding pattern of fourteen isolates of *R. solanacearum* was estimated. REP primer produced a total of 11 bands of which all bands (100%) were considered polymorphic. The banding pattern of *R. solanacearum* of tobacco samples using the primers were REP [(FP-ICGICTTATCIGGCCTAC); (RP-IIICGICGICATCIGGC)]. On the other hand, ERIC primer produced 19 bands of which all bands (100%) were considered polymorphic. The banding pattern of *R. solanacearum* of tobacco samples using the primer were ERIC [(FP-AAGTAAGTGACTGGGGTGAGCG); (RP-ATGTAAGCTCCTGGGGATTCAC)].

Dendrogram constructed with UPGMA based on the polymerase chain reaction DNA fingerprint data analyzed with NTSYS software for a collection of fourteen representative isolates of *R. solanacearum* of different tobacco growing areas shown in Figure 1 using REP primer, ERIC primer, and combined both primers are shown three clusters (I, II, and III) at 60% similarity. When using REP primer, cluster I is composed of eleven isolates (Rs-01, Rs-05, Rs-12, Rs-06, Rs-11, Rs-09, Rs-03, Rs-04, Rs-08, Rs-07, Rs-10), and cluster II composed of one isolate (Rs-02) and cluster III composed of two isolates (Rs-12, Rs-14). On the other hand, while using ERIC primer twelve isolates were included in cluster I, and one isolate was included in clusters II and III respectively. When analyzed using both the primers together similar results came as described above for using REP primer (Figure 5).

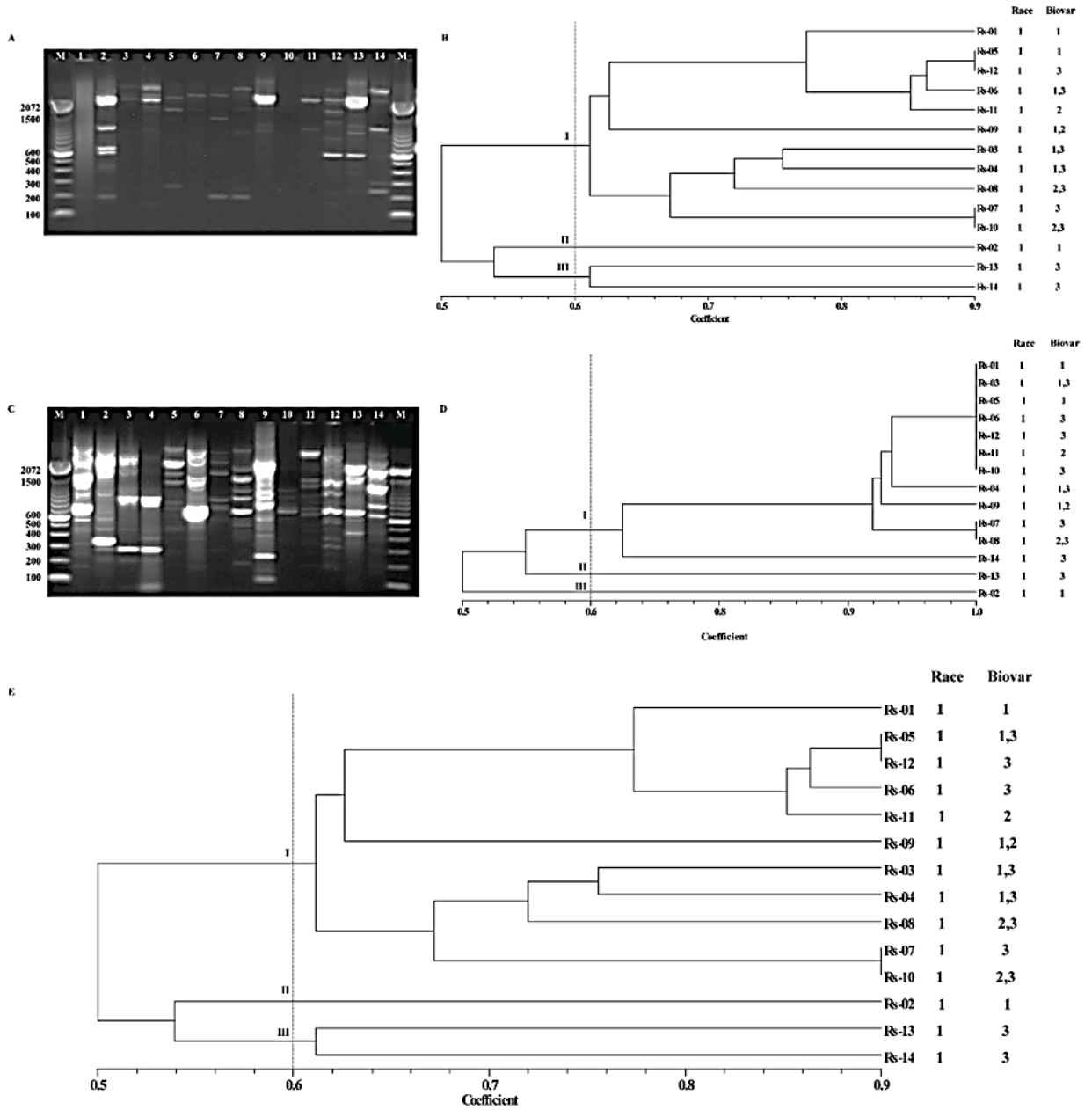

Figure 5. DNA fingerprint patterns of *R. solanacearum* isolates obtained from different tobacco growing areas of Bangladesh by PCR generated with A) REP primers and C) ERIC primers. Dendrogram constructed with UPGMA based on the polymerase chain reaction DNA fingerprint data analyzed with NTSYS software for a collection of 14 representative isolates of *R. solanacearum* of different tobacco growing areas using B) REP primer D) ERIC primers E) Combinedly with REP primer and ERIC primer. Similarity percent was marked with co-efficient. The dotted line indicated the similarity index level for clustering among the *R. solnacearum* isolates. Fourteen isolates are designated as Rs-01-Tagail, Rs-02-Rajbari, Rs-03-Manikganj, Rs-04-Bandarban, Rs-05-Chakaria, Rs-06-Naikhonchari, Rs-07-Khagrachari, Rs-08-Lama, Rs-09-Meherpur, Rs-10-Kushtia, Rs-11-Jhenaidah, Rs-12-Chuadanga, Rs-13-Rangpur and Rs-14-Lalmonirhat.

4. Discussion

Bacterial wilt has been identified as one of the main tobacco diseases that affect tobacco production in many tobacco-growing countries (Zhou *et al.*, 2012). From the results of the survey, it is observed that the prevalence of bacterial wilt in tobacco ranged from 8.33% to 0.33% and the mean incidence of bacterial wilt was 4.73% and it is prevalent in all sampled tobacco growing areas except Rajbari. Similar to this experiment's technique, the prevalence of bacterial wilt diseases of tobacco was calculated by counting the proportion of plants in a plot

that showed disease-related foliar symptoms (Ma *et al.,* 2018). According to Liu *et al.,* 2017 the incidence of tobacco bacterial wilt is about 15–75% causing 50–100% yield losses. In case of tomatoes, the disease incidence ranged from 9.41% to 15.28% in Egypt (Elnaggar *et al.,* 2018). From the result of this study, it is revealed that the bacterial wilt severity ranged from 4.67-0.67 (on the basis of a 1-5 scale) in the sampled locations. The severity of the disease depends upon the prevailing temperature and soil moisture at the time of transplanting and sowing (Mondal *et al*., 2014). In the case of brinjal, the severity may vary from 2.80 to 4.00 (on the basis of a 1-5 scale) which was revealed by Rahman *et al.,* 2010. Ahmed *et al*., 2013 reported that the incidence and severity of the disease were influenced by the diversity of the *R. solanacearum* strains, variation in soil types, survivability of the pathogen in the soil for a longer period of time due to continuous growth of the same crops and intercropping with susceptible host and disease development were favored by environmental conditions such as warm and humid soil conditions *Ralstonia solanacearumin* was detected in all asymptomatic and symptomatic tomato plants, with population counts ranging from 10.5 to 86.7 × 10^5 cfu/g reported in China (Zheng *et al*., 2014). The soil population shown in the experiment ranged from 4.3 x 10^7 CFU/g to 22.4 x 10^7 CFU/g which varied from region to region among the sampled areas. According to the Yi *et al*., 2007 scale, the prevalence and severity of tobacco bacterial wilt were shown on each treatment where: 0= plants with no symptoms, 1= occasionally having chlorotic spots on stems or having fewer than half the leaves wilt on unilateral stems, 3=(30-50%) having a black streak that is less than half the height of the stem or between half and two-thirds of the leaves wilt on unilateral stems, 5= (50-70%) having a black streak that is more than half the height of the stem but does not reach the top of the stem; more than two-thirds of the leaves are wilted but do not extend to the top of the stem; more than two-thirds of the leaves are wilted on unilateral stalks; 7 = (70–90%) black streak reaching the top of the stem; all leaves wilted; and 9 = death of plants. The variations in wilt incidence and severity since *R. solanacearum* affects a wide variety of host plants have a global geographic spread and thrive in a variety of environmental circumstances (Chatterjee *et al*., 1997).

A total of sixty-three isolates from seed and fourteen isolates from soil were collected from fourteen tobacco-growing regions of Bangladesh. All isolates of the study produced a pink color colony with a whitish margin on the TTC medium. Fluidal colonies with pink or light red color were produced upon inoculation of virulent *R. solanacearum* on TTC media after 24 hours of inoculation (Dhital *et al*., 1996; Rahman *et al*., 2010; Ahmed *et al*., 2013; Sharma and Sharma, 2014). By the pigmentation from the wild virulent types of *R. solanacearum,* avirulent colonies could be easily differentiated where small off-white colonies of bacteria are formed which is discussed by Hossain *et al.,* 2022. For better confirmation of the bacteria molecular-based detection method was performed through PCR using species-specific primer Y-2/OLI-1. DNA of all isolates showed banding at 288 bp. This pattern was in agreement with the result of Pastrik and Maiss who reported that when the DNA of *R. solanacearum* amplified with the specific primer Y-2/ OLI-1 it produced a 288 bp band size (Pastrik and Maiss, 2000). *R. solanacearum* produced a 288 bp band size when the DNA of that pathogen was amplified with the specific primer Y-2/ OLI-1 (Hossain *et al.,* 2022) The 288 bp amplicon was detected in bacterial wilt-infected samples of tomato in Egypt (Elnaggar *et al*., 2018).

Based on their capacity to use disaccharides and sugar alcohols oxidation by isolates from various tobacco growing regions in Bangladesh within 3-5 days, biovars were determined, and it was discovered that every isolate used in the study belonged to three biovars (Biovar 1, Biovar 2 and Biovar 3). The ability to make acid from a panel of carbohydrates allowed the *R. solanacearum* complex to be further subdivided into biotypes (Kumar *et al*., 2004). Biotype 3 oxidized both hexose alcohols and disaccharides while biotypes 1, 2, and 4 exclusively oxidized alcohols (Paudel *et al.,* 2020). Biotype 1 oxidized hexose alcohols but not disaccharides. All groups were identified as belonging to biotype 3 by Rahman *et al.,* 2010 after characterizing the *R. solanacearum* isolates of eggplants gathered from seven districts in Bangladesh. *R. solanacearum* was confirmed to be positive by a number of biochemical assays, and Ahmed *et al.,* 2013 showed that it might cause wilt symptoms in potato and tobacco leaves. Due to *R. solanacearum* high sensitivity to serological testing, a pathogenicity test was used to identify it in a variety of hosts, including tobacco, eggplant, and chili. Aslam *et al*., 2015 found that from 42 *R. solanacearum* strains almost all the isolates from chili were included in biovar 3 (82%), and the rest of the isolates were included in biovar 4 (18%). The number and pathogenicity of *R. solanacearum* were diverse, as reported by Zheng *et al.,* 2014 within various hosts, in various areas of plants, and at various phases of disease development in the field. Denny and Hayward identified the race of *R. solanacearum* by host specificity (Denny and Hayward, 2001). All the isolates obtained from wilted tobacco produced wilt symptoms in tobacco, chili, and eggplant in the experiment. Therefore, all tobacco isolates gathered from significant growing regions belonged to race 1. In China, indicating race 1 *R. solanacearum* isolated from tobacco also supports the study's findings (Liu *et al*., 2017). The host range, geographic

distribution, and capacity for survival of five different races of *R. solanacearum* varied (Ahmed *et al.*, 2013), *R. solanacearum* was initially categorized into races and biotypes based on host range variability (Patrice, 2008). Genetic diversity refers to the variation in genetic constituents among species of an organism determining the biological potentialities of the organism which may occur due to environmental conditions as well as hereditary materials (Woolliams *et al*., 2007). In spite of morphological and cultural similarities of *R. solanacearum* isolates of tobacco, variation in the banding pattern of these isolates was observed when these were amplified with ERIC and REP primers. Yang *et al*., 2016 reported that strains of *R. solanacearum* collected from four major tobacco-growing areas in China exhibited different levels of diversity. In our study, isolates from Tangail and Kushtia exhibited low genetic diversity, respectively due to their high genetic identity and least genetic distance. These findings corresponded with those of Rodrigues *et al.*, 2012 who discovered that patterns formed by ERIC primers permitted the sorting of 41 *Ralstonia solanacearum* strains into two primary clusters, with three isolates belonging to one cluster and the rest 38 belonging to the other. These isolates displayed high genetic diversity and were haphazardly distributed in various clusters (Rodrigues *et al*., 2012). Banding pattern produced from tested isolates of *R. solanacearum* from tobacco was divided into three clusters at a 60% level of similarity in this study shown in a dendrogram constructed with UPGMA based on the polymerase chain reaction DNA fingerprint data analyzed with NTSYS software. According to the pathogenic activity of *R. solanacearum* developed by banding patterns of REP, ERIC, and BOXIR primers, the REP-PCR can cluster with 83%, 80%, and 63% similarity co-efficient, respectively which was demonstrated by Hossain *et al.*, 2022. The findings of the study are consistent with the findings of Xu *et al*., 2009 analysis of the genetic variation of Chinese *R. solanacearum* isolates collected from tobacco, which suggested that these strains belonged to phylotype I. *R. solanacearum* can produce varied levels of genetic variation among isolates collected from distinct places, regardless of the hosts that were grouped together using a low level of similarity score (Nishat *et al*., 2015). While studying 97 *R. solanacearum* isolates from different tobacco growing zones of China it was revealed that all the isolated produced typical colonies on TZC medium and all belonged to phytotype I considering tropical strain prevalent in the lowland as well as in moderately elevated areas and highlands (Liu *et al*., 2017). *R. solanacearum* could adapt to different environmental conditions and show genetic diversity according to soil pH, temperature, relative humidity, rainfall, and topographical patterns (Begum *et al*., 2012; Jannat *et al*., 2014). The wide host range of *R. solanacearum* was seen because of the high level of genome variability within the field and clonal isolates of the bacterium (Grover *et al*., 2006).

5. Conclusions

The current study found that the incidence and severity of bacterial wilt varied greatly throughout tobacco-growing regions of Bangladesh. *R. solanacearum's* ability to spread through seeds in tobacco seeds offers new insights into how this bacterial pathogen is spread. Significant genetic variation was found among the biotypes and races of *R. solanacearum* described in this study, according to the rep-PCR genetic diversity analysis. The higher genetic variability among the biotypes and races of *R. solanacearum* was observed in the Brahmaputra and Gangetic alluvium soils compared to the soils of the hill tracts. This is the first in-depth study of tobacco bacterial wilt disease in Bangladesh. The knowledge gained from this study will be useful in developing a long-term plan to reduce financial losses for all solanaceous crop plants.

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Data availability

Data are contained within the article.

Conflict of interest

None to declare.

Authors' contribution

Md. Rashidul Islam: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization; Plabon Saha: Formal analysis, Methodology, Software, Visualization, Writing – original draft; Shiuly Akter: Investigation, Methodology, Software, Visualization, Writing – original draft; Mehedi Hasan: Software, Writing – review & editing; Abu Sina Md Tushar: Writing – review & editing; Muhtarima Jannat: Methodology, Writing – review

& editing; Md. Abdul Haque Mozumder: Writing – review & editing; Abdul Mukaddim: Writing – review & editing; Md. Zahangir Alam: Writing – review & editing. All authors have read and approved the manuscript.

References

Ahmed NN, MR Islam, MA Hossain, MB Meah and MM Hossain, 2013. Determination of races and biovars of *Ralstonia solanacearum* causing bacterial wilt disease of potato. J. Agric. Sci., 5: 86-93.

Ahmed W, J Yang, Y Tan, S Munir, Q Liu, J Zhang, G Ji and Z Zhao, 2022. *Ralstonia solanacearum*, a deadly pathogen: Revisiting the bacterial wilt biocontrol practices in tobacco and other Solanaceae. Rhizosphere, 21: 100479.

Aslam MN, M Tariq, A Muhammad, MJ Asad and MA Hussain, 2015. Incidence and prevalence of bacterial wilt of chili in Punjab, Pakistan. Mycopath., 13: 37-41.

Begum N, MI Haque, T Mukhtar, SM Naqvi and JF Wang, 2012. Status of bacterial wilt caused by *Ralstonia solanacearum* in Pakistan. Pakistan J. Phytopathol., 24: 11-20.

Chandrashekara KN, MK Prasannakumar, M Deepa, A Vani and AN Khan, 2012. Prevalence of races and biotypes of *Ralstonia solanacearum* in India. J. Plant Prot. Res., 52: 26-31.

Chatterjee S, N Mukherjee and DC Khatun, 1997. Status of bacterial wilt in West Bengal J. interacademicia. Department of Plant Pathology, Bidhan Chandra Krishi Viswavidyalaya, India. pp. 97-99.

Denny TP and AC Hayward, 2001. Gram-negative bacteria: *Ralstonia*. APS Press, St. Paul, USA.

Denny T, 2006. Plant pathogenic *Ralstonia* species. In Plant-associated bacteria. Dordrecht: Springer, Netherlands, 573-644.

Elnaggar S, AM Mohamed, A Bakeer and TA Osman, 2018. Current status of bacterial wilt *(Ralstonia solanacearum*) disease in major tomato (*Solanum lycopersicum L*.) growing areas in Egypt. Arch. Agric. Environ. Sci., 3: 399-406.

Fegan M and P Prior, 2005. How complex is the *Ralstonia solanacearum* species complex. Bacterial wilt disease and the *Ralstonia solanacearum* species complex, 1: 449-461.

Genin S, 2010. Molecular traits controlling host range and adaptation to plants in *Ralstonia solanacearum*. New Phytol., 187: 920-928.

Grover A, W Azmi, AV Gadewar, D Pattanayak, PS Naik, GS Shekhawat and SK Chakrabarti, 2006. Genotypic diversity in a localized population of *Ralstonia solanacearum* as revealed by random amplified polymorphic DNA markers. J. Appl. Microbiol., 101: 798-806.

Hossain MM, MM Masud, MI Hossain, MM Haque, MS Uddin, MZ Alam and MR Islam, 2022. Rep-PCR analyses reveal genetic variation of *Ralstonia solanacearum* causing wilt of *Solanaceaous* vegetables in Bangladesh. Curr. Microbiol., 79: 234.

Horita M and K Tsuchiya, 2001. Genetic diversity of Japanese strains of *Ralstonia solanacearum*. Phytopathology, 91: 399–407.

Jannat N, I Hossain, MD Hossain, P Dey and MA Khan, 2014. Genetic Diversity of *Pseudomonas syringae pv. syringae* Causing Leaf Blight of Mango in Bangladesh. Int. J. Agric. Innov. Res., 4: 628-635.

Lin CH, ST Hsu, KC Tzeng and JF Wang, 2009. Detection of race 1 strains of *Ralstonia solanacearum* in field samples in Taiwan using a BIO-PCR method. Eur. J. Plant Pathol., 124: 75-85.

Liu Y, D Wu, Q Liu, S Zhang, Y Tang, G Jiang, S Li and W Ding, 2017. The sequevar distribution of *Ralstonia solanacearum* in tobacco-growing zones of China is structured by elevation. Eur. J. Plant Pathol., 147: 541-551.

Manda RR, VA Addanki and S Srivastava, 2020. Bacterial wilt of solanaceous crops. Int. J. Chem. Stud., 8: 1048-1057.

Nei M, 1972. Genetic distance between populations. Am. Nat., 106: 283-292.

Nishat S, I Hamim, MI Khalil, MA Ali, MA Hossain, MB Meah and MR Islam, 2015. Genetic diversity of the bacterial wilt pathogen *Ralstonia solanacearum* using a RAPD marker. C. R. Biol., 338: 757-767.

Pastrik KH and E Maiss, 2000. Detection of *Ralstonia solanacearum* in potato tubers by polymerase chain reaction. J Phytopathol., 148: 619-626.

Patil VU, J Gopal and BP Singh, 2012. Improvement for bacterial wilt resistance in potato by conventional and biotechnological approaches. Agric. Res. J., 1: 299-316.

Patrice GR, 2008. *R. solanacearum* race 3 biovar 2: detection, exclusion and analysis of a Select Agent Educational modules. The United States Dept. Agric. Nat. Res. Initiative Program, pp. 1-4.

Paudel S, S Dobhal, AM Alvarez and M Arif, 2020. Taxonomy and phylogenetic research on *Ralstonia solanacearum* species complex: a complex pathogen with extraordinary economic consequences. Pathogens, 9: 886.

Rahman MF, MR Islam, T Rahman and MB Meah, 2010. Biochemical characterization of *Ralstonia solanacerum* causing bacterial wilt of brinjal in Bangladesh. Progress. Agric., 21: 9-19.

Razia S, MSM Chowdhury, FM Aminuzzaman, N Sultana and M Islam, 2021. Morphological, pathological, biochemical and molecular characterization of *Ralstonia solanacearum* isolates in Bangladesh. Am. J. Mol. Biol., 11: 142-164.

Rodrigues LM, SA Destefano, MJ Da Silva, GG Costa and AC Maringoni, 2012. Characterization of *Ralstonia solanacearum* strains from Brazil using molecular methods and pathogenicity tests. Plant Pathol. J., 94: 505-516.

Schaad NW, JB Jones and W Chun, 2001. Laboratory guide for the identification of plant pathogenic bacteria. APS press, St. Paul, USA.

Sharma N and DK Sharma, 2014. Incidence and seed transmission of *Ralstonia solanacearum* (Smith) in brinjal (*Solanum melongena* L.). Int. J. Plant Pathol., 5: 63-69.

Tahat MM and K Sijam, 2010. *Ralstoina solanacearum*: The bacterial wilt causal agent. Asian J. Plant Sci., 9: 385.

Williams JG, AR Kubelik, KJ Livak, JA Rafalski and SV Tingey, 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res., 18: 6531-6535.

Woolliams JA, M Toro and K Oldenbroek, 2007. Utilisation and conservation of farm animal genetic resources. Wageningen Academic Publishers Wageningen, The Netherlands, pp. 55-74.

Xu J, ZC Pan, P Prior, JS Xu, Z Zhang, H Zhang, LQ Zhang, LY He and J Feng, 2009. Genetic diversity of *Ralstonia solanacearum* strains from China. Eur. J. Plant Pathol., 125: 641-653.

Yi YJ, RS Liu, HQ Yin, K Luo, EM Liu and XD Liu, 2007. Isolation identification and field control efficacy of an endophytic strain against tobacco bacterial wilt (*Ralstonia solanacarum*). Ying Yong Sheng Tai Xue Bao, 18: 554-558.

Zheng XF, YJ Zhu, B Liu, Y Zhou, JM Che and NQ Lin, 2014. Relationship between *Ralstonia solanacearum* diversity and severity of bacterial wilt disease in tomato fields in China. J Phytopathol., 162: 607-616.

Zhou XJ, J Wang, YW Yang, TC Zhao and BD Gao, 2012. Advances in tobacco bacterial wilt disease. Microbiology/Weishengwuxue Tongbao, 39: 1479-1486.