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Molecular identification and pathogenicity of *Ralstonia solanacearum* isolates collected from north west of Pakistan

Muhammad Junaid[1*]; Ayesha Bibi[2]; Musharaf Ahmad[3]; Muhammad Ali[4]; Yousaf Noor[5]

[1]MFSC, Peshawar, Department of Agriculture Ext., Govt. of Khyber Pakhtunkhwah, Pakistan; [2]Department of Soil Microbiology, DSPN, ARI Peshawar, Govt. of Khyber Pakhtunkhwah, Pakistan; [3]Department of Plant Pathology, The University of Agriculture, Peshawar, Pakistan; [4]Department of Soil Microbiology, DSPN, ARI Peshawar, Govt. of Khyber Pakhtunkhwah, Pakistan; [5]Cereal Crop Research Institute, Pirsabak, Nowshera, Govt. of Khyber Pakhtunkhwah, Pakistan.

[*]Corresponding author E-mail: mjunaid27@yahoo.com

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Abstract

In the tomato commercial growing districts of Khyber Pakhtunkhwa (KP); a province in the north west of Pakistan, multiple comprehensive surveys were conducted during 2012. The main objectives of the current study were to identify the *Ralstonia solanacearum* (*R. solanacearum*) isolate through its colony characteristics, molecular tools; and to investigate the ability of this pathogen to cause Bacterial wilt (BW) disease, when being inoculated into tomato plant using different inoculation methods. For this purpose, a total of 74 locations covering all over the KP were visited for the presence of tomato plants with BW disease, caused by *R. solanacearum*. The bacterial pathogen was isolated from diseased plant tissues by growing it on the selective 2,3,5-triphenyl-tetrazolium chloride (TTC) medium. Based on colony morphology of *R. solanacearum* on the agar plates; and pathogenicity assays, about 29 isolates were guessed to be *R. solanacearum*. To further confirm the identity of these isolates, a species-specific primers-mediated Polymerase chain reaction (PCR) was carried out. Two specific primers i.e. forward primer: 5'GTCGCCGTCAACTCACTTTCC3', and reverse primer: 5'GTCGCCGTAGCAATGCGGAATCG3', were used for amplification of the 281bp band. Twenty five isolates out of the 29 were genetically confirmed to be *R. solanacearum* based on their amplified 281bp band.

Keywords: Tomato, Bacterial wilt, *Ralstonia solanacearum*, Pathogenicity, PCR

1. Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most important vegetable crops of the world, and is a member of the Solanaceae family (also known as night shade family). There were several important diseases

affecting this well-known and valuable crop. Among which; tomato BW caused by *R. solanacearum* (Smith) was considered to be one of the most destructive diseases, due to its economic impact. As a Gram-negative rod, *R. solanacearum*; expressed a major threat to the production of tomatoes, potatoes, tobacco and other solanaceous crops, in both tropics and temperate zones (Yuliar *et al*., 2015; Aloyce *et al*., 2017). Up to 30% of annual losses in tomatoes and potatoes crops were common in Pakistan (Junaid *et al*., 2018). This pathogenic bacterium has a very wide host range, as more than 200 plant species were attacked by this pathogen.

R. solanacearum is a species complex consisting of groups; races, biovars, bio-types, sub-races and strains. Detection and identification of this bacterial pathogen was routinely carried out on the basis of; biochemical and physiological assays (Alvarez *et al*., 2008; Marco-Noales, *et al*., 2008; Rahman *et al*., 2010) such as Biolog Automated Microplate System (Gebreel *et al*;, 2000), serology using species-specific anti-bodies (Tawfik *et al*., 2008), and molecular techniques (Fegan and Prior., 2005; Abdurahman *et al*., 2017).

Some assays were done to identify the pathogen above or at genus level such as; Gram staining, KOH solubility test, Catalase test, Oxidase test, Production of fluorescent pigment on iron-deficient medium, Lipase activity, and Levan production from sucrose, etc. (Chaudhry and Rashid, 2011; Pawaskar *et al*., 2014; Shahbaz *et al*., 2015). This pathogen could be detected in latent infections and identified using serological methods such as; Enzyme-linked immunosorbent assay ELISA, and Double antibody sandwich DAS-ELISA (Priou *et al*., 2010). However, such methods were limiting due to their lack of specificity and sensitivity. PCR was better than serology in terms of detection and identification of bacterial pathogens including *R. solanacearum*. Effective management of BW disease could be only possible if the pathogen was detected and then identified accurately. The current study focuses on the pathogenicity and molecular identification of *R. solanacearum*.

There exists a lot of controversy regarding the identification of *R. solanacearum* strains in the various parts of the world. In Pakistan; however, scant information is available about the presence of this pathogen. Accurate identification of a local pathogen and knowledge about its ability to cause a specific disease is very important in developing a successful integrated disease management programme. One of the purposes of the present investigation was to identify pathogenic *R. solanacerum* through different techniques including colony characteristics and molecular tools. Moreover, the ability of the pathogen to cause the BW was verified using two different inoculation assays.

2. Material and methods

2.1. Survey and sampling

Tomato growing districts of the Khyber Pakhtunkhwa (KP) province form north west of Pakistan were surveyed throughout the growing period (i.e. April-August, 2012) of the crop. A total of 74 (representing almost all tomato growing areas of KP, Table 1; Fig. 1.) samples were uprooted, collected in paper bags, kept cool, brought to the Clinical Plant Pathology Laboratory, and then processed as soon as possible to avoid the chances of contamination. Stems were cut in two cross sections, followed by suspending the tissues in water to observe bacterial streaming (Tahir *et al*., 2014; Ivey and Lunos, 2015). This method was characteristic for BW and helps in the preliminary identification of bacterial pathogens.

2.2. Isolation of *R. solanacearum* from tomato samples

TTC (2,3,5-triphenyltetrazolium chloride) also known as TZC medium (Champoiseau *et al*., 2009) was used for the isolation of the pathogen. To one liter of semi-cold (55-60^{o}C) sterile nutrient agar (NA)

medium, 5 ml of 1% (w/v) TTC solution was added and mixed well before pouring the medium into plates. Infected stem samples were cut into small pieces; kept in 5-10 ml sterile dist. water for a few minutes, and then crushed using sterilized pistil and mortar to obtain bacterial exudates (ooze). Tissue macerate was left undisturbed for 10-15 min. and then serial dilutions were prepared up to 10^{-7} dilution. The last two dilutions i.e. 10^{-6} and 10^{-7} were streaked on TTC plates to obtain single separate colonies. After 2-3 days incubation at 28-30^{o}C, typical *R. solanacearum* colonies (as reported by Chaudhry and Rashid, 2011; Sagar *et al*., 2013) were selected. Well isolated individual colonies were re-streaked on the TTC medium to purify them. Pure cultures were kept at 4°C for future use.

Table 1: List of districts and locations of the Khyber Pakhtunkhwa (KP), Pakistan, surveyed for Bacterial wilt of tomato

S. no	District	No. of samples	Name of sampling location
1	Abbottabad	2	Abbottabad, Havelian
2	Mansehra	3	Mansehra, Balakot, Oghi
3	Haripur	2	Haripur, Ghazi
4	Battagram	2	Battagram, Allai
5	Torgar	2	Judbah, Hassan Zai
6	Bannu	2	Bannu, Domel
7	Charsadda	3	Charsadda, Shabqadar, Tangi
8	D.I. Khan	5	D.I. Khan, Paharpur, Kulachi, Darban, Paroa
9	Hangu	2	Hangu, Thall
10	Karak	3	Karak, TakhtNasrati, Banda Daud Shah
11	Kohat	2	Kohat, Lachi
12	LakkiMarwat	2	LakkiMarwat, SeraiNaurang
13	Mardan	3	Mardan, Katlang, TakhtBhai
14	Nowshera	3	Nowshera, Pabbi, Jahangira
15	Peshawar	1	Peshawar
16	Tank	1	Tank
17	Swabi	4	Swabi, Lahore, Razzar, Topi
18	Swat	7	Babuzai, Bahrain, Barikot, Charbagh, Khwazakhela, Kabal, Matta
19	Shangla	2	Alpuri, Puran
20	Buner	4	Khudukhail, Mandar, Gagra, Daggar
21	Upper Dir	4	Dir Upper, Barawal, Kalkot, Wari
22	Lower Dir	7	Adenzai, Timergara, Balambat, Munda, Samarbagh, LalQilla, Khall
23	Malakand	2	Batkhela, Dargai
24	Chitral	2	Chitral, Mastuj
25	Upper Kohistan	2	Dassu, Kandia
26	Lower Kohistan	2	Palas, Pattan
	Total no. of samples	74	

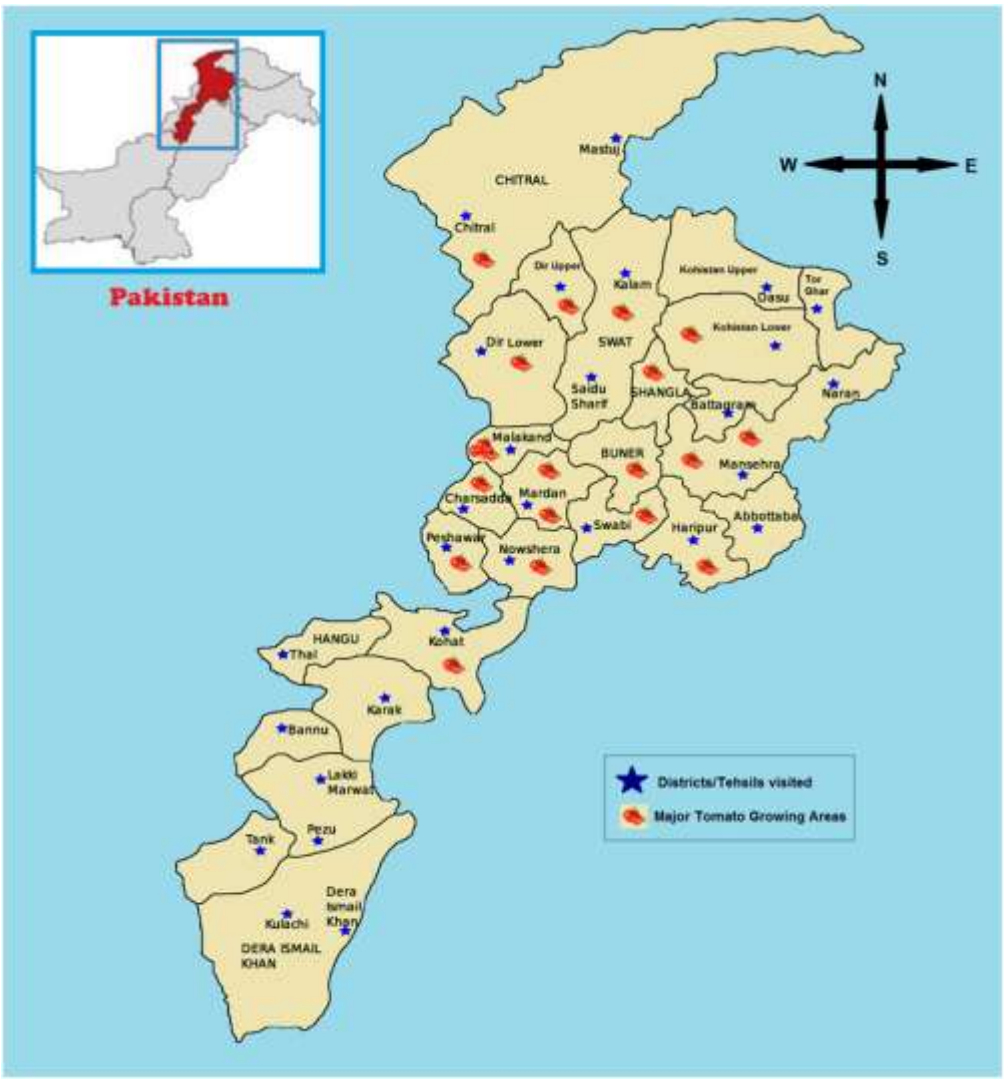

Fig. 1. Map of KP, Pakistan, showing districts (depicted by blue star) surveyed for the determination of *R. solanacearum* infection during the growing season of April- August, 2012.

2.3. Identification of *R. solanacearum* isolates

Bacterial isolates were identified based upon colony morphology on TTC; pathogenicity on host plants, and then confirmed by PCR assay using species-specific primers.

2.3.1. Colony morphology

Colony morphology of *R. solanacearum* isolates on TTC was studied. Color, shape, growth rate, texture and margins of the *R. solanacearum* isolates were recorded. These characteristics were then used for the identification of the pathogen. Speculation about virulent and avirulent colonies were guessed as reported by Tahir *et al*., (2014).

2.3.2. Pathogenicity assay

The objective of the pathogenicity assays was to fulfill Koch's postulates i.e., to make sure that the isolated pathogen was capable of producing wilt disease symptoms on healthy plants similar to those symptoms recorded on the infected tomato samples

from which the pathogen was isolated. More than one pathogenicity assays with different inoculation techniques were used to produce typical disease symptoms on healthy tomato host.

2.3.2.1. Pathogenicity assay I (Root inoculation)

Four weeks old tomato seedlings of susceptible variety (Lyrika) were transplanted into large pots (2 plants/pot), and then inoculated with pure suspension of *R. solanacearum* culture one week later. For preparation of culture suspension; colonies (24 h old) growing on NA medium were washed off the surface with sterile dist. water, centrifuged and then adjusted to a final concentration of 10^7cells\ ml, using Photo-spectrometer (OD=600). Tomato plants were inoculated using two different methods: One set of pots were inoculated by pouring 30 ml of *R. solanacearum* culture suspension containing 10^7cells\ ml into the pots around the root zones of the plants. In the other set of pots, the roots (just below the soil surface) of transplants were slightly pierced with sterile needle in order to produce artificial wounds (in mimicry of natural wounds) before transplantation, and then inoculated by pouring *R. solanacearum* suspension around the root zone immediately after transplanting (Marques *et al*., 2012). Plants exhibiting BW symptoms were recorded.

2.3.2.2. Pathogenicity assay II (Leaf inoculation)

In this case, pathogenicity was carried out by inoculating leaves of relatively younger tomato seedlings as reported by Seleim *et al*., (2014). Pure bacterial culture suspension prepared as stated before. 5-10 susceptible tomato plants at the third true leaf stage were used for inoculation. The leaves were pierced with a sterile needle, and then a sterile cotton swab was used to spread the bacterial inoculum suspension on their surfaces. Another set of plants were also inoculated with the same procedure but without piercing. Inoculated plants were kept at 25-28^{o}C under high relative humidity conditions for 10 days. Symptoms of wilting; chlorosis, or stunting were noticed. The bacterium was re-isolated from infested plants by taking a stem or petiole sections above the inoculation points; placing them in a small volume of sterile dist. water, and then plated on TTC medium. After incubation, plates were observed for typical *R. solanacearum* colonies. Identification of colonies were confirmed through amplification of bacterial DNA, using PCR.

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

To confirm identity of the *R. solanacearum* isolates based upon colony morphology and pathogenicity assays; PCR was performed using species-specific primers i.e. forward primer: 5'GTCGCCGTCAACTCACTTTCC3' and reverse primer: 5'GTCGCCGTAGCAATGCGGAATCG3', which amplified the 281bp fragment in reference to Popoola *et al*., (2015). DNA was extracted with the procedure described by Junaid *et al*., (2018); however, for DNA purification, the Easy-DNA-Extraction-kit (Invitrogen, DeSchelp, the Netherlands) was used. The extracted genomic DNA from each isolate of *R. solanacearum* was amplified using specific primers. PCR master mixture consisted of 1µl of each primer (0.2 µM), 1µl-of Taq-DNA polymerase, 2.25µl buffer (KCl-50 mM+Tris-HCl-2 mM, pH 8.4), 2µl $MgCl_2$ (1mM), 2.5µl (100µM, of each dNTP), which were added to 3µl template DNA (1ng) and HPLC water, in order to make the volume of 25µl used in each PCR tube for amplification. A 30-cycles of PCR were performed for all 29 isolates. PCR conditions were: 95^{o}C for 3 min. (initial denaturation); 94^{o}C for 20 sec. (denaturation); 50^{o}C for 50 sec. (annealing); 72^{o}C for 30 sec. (elongation); 72^{o}C for 5 min. (once at the end). Agarose gel (2%) was used to separate the PCR-amplified DNA bands. Blue tracking (3µl) was added to each PCR-product sample (25µl), and then each sample was carefully uploaded to the wells of the gel. 1Kb size DNA ladder was also used for comparison. Electrophoresis was performed at 200 volts for 30 min. To easily visualize the different bands, the gel was

kept in Ethedium bromide solution (0.5 µg\ ml) for 15 min. The bands were observed under UV light.

3. Results

3.1. Survey and sampling

Surveys conducted in commercial tomato-growing districts of KP revealed that; a total of 74 locations from 26 districts (3-10 fields from each location) were visited, and a total of 74 whole uprooted tomato plants samples exhibiting typical symptoms of BW infection were collected.

3.2. Isolation of *R. solanacearum* from tomato samples

Isolations from apparently infected samples were made on TTC medium. After 48-72 h of incubation at 28-30^{o}C; 29 samples yielded colonies having typical morphology of *R. solanacearum* such as; white elevated colonies, mucoid with pinkish to red centers due to reduction of TTC into insoluble red formazan.

3.3. Identification and characterization of isolates

3.3.1. Colony morphology

All the isolates had large colonies; which were elevated, fluidal and either entirely white or pinkish in color. The virulent colonies were mostly with pinkish to red centers. There were few colonies which were surrounded by bluish border (Table 2).

3.3.2. Pathogenicity assay

The tentative *R. solanacearum* isolates (29) were checked for their ability to cause wilt disease symptoms on tomato seedling using four different inoculation techniques. All isolates produced disease symptoms on using most of the inoculation methods (Table 3). The type of the inoculation method used was found to be important for the success of this pathogenicity assay. When plants were infested through root inoculation method (without piercing with needle); isolates from Mansehra (MAN1); Buner (BNR1), Lower Dir (DLO2) and Swat (SWT1, SWT2) didn't produce the typical wilt symptoms. Moreover; the pathogen from the tissues of these plants neither recovered on TTC medium, nor confirmed through PCR. However; when pathogenicity assay was performed on leaves of young seedlings (with piercing), all the 29 isolates produced wilting symptoms, and then died. Meanwhile; when seedlings leaves were inoculated without piercing, the only plant that survived was the one inoculated with *R. solanacearum* isolate from Mansehra (MAN1).

3.3.3. Molecular identification of *R. solanacearum* isolates

Using species-specific primers, PCR was used to finally confirm the identity of *R. solanacearum* isolates. The 281bp specific band was amplified from 25 isolates, confirming them to be *R. solanacearum* (Fig. 2). However, the template DNA of four isolates (i.e. LMT1, MAN1, DUP1 and NSH2) failed to amplify the 281bp band.

4. Discussion

The selective medium named 2,3,5-triphenyl-tetrazolium chloride (TTC) (Champoiseau *et al*., 2009), was used to isolate *R. solanacearum* from diseased tomato samples. Colonies of the pathogen on this medium were fluidal with pinkish-red centers. Similar results were reported by Rahman *et al*., (2010); Chaudhry and Rashid (2011); Tahir *et al*., (2014). Colony characteristics of *R. solanacearum* were helpful as they differentiated virulent from avirulent isolates. Chaudhry and Rashid, (2011) reported that the colonies of virulent isolates were whitish with pink centers; irregular margin, raised, large in size, and were more fluidal. On the other hand; colonies of avirulent (spontaneous mutant) isolates were deep red with entire margin, and had bluish border.

R. solanacearum generally invades its tomato host through wounds in the roots, colonizes the xylem

Table 2: Identification of *R. solanacearum* isolates according to the morphological and cultural characteristics

S. no.	Origin of the isolate	Isolate code	Field symptoms	Colony morphology	PCR identification
1	Bannu	BAN1	CPW, BS	E, F, I,WP, WR	✓
2	LakkiMarwat	LMT1	PPW	E, F, I, RB	X
3	D.I. Khan	DIK1	PW, BS	E,F, I, W,WP, WR	✓
4	Haripur	HAR1	PPW, BS	E,F, I, WR	✓
5	Mansehra	MAN1	CPW, BS	E,F, I, RB	X
6	Hangu	HAN1	CPW, BS	E,F, I,WP, WR	✓
7	Karak	KAR1	CPW, BS	E,F, I,W,WP, WR	✓
8	Kohat	KOH1	CPW, BS	E,F, I, WR, RB	✓
9	Buner	BNR1	PPW, BS	E,F, I,WP, WR	✓
10	Buner	BNR2	PPW, BS	E,F, I,W,WP, WR	✓
11	Lower Dir	DLO1	PPW, BS	E,F, I, W,WP, WR	✓
12	Lower Dir	DLO2	PPW, BS	E,F, I, W,WP, WR	✓
13	UperDir	DUP1	PPW	E,F, I, RB	X
14	Malakand	MKD1	PW	E,F, I,W, WR	✓
15	Malakand	MKD2	PW	E,F, I, W,WP, WR	✓
16	Shangla	SGL1	CPW, BS	E,F, I, PR,WP, WR	✓
17	Swat	SWT1	CPW, BS	E,F, I, WR	✓
18	Swat	SWT2	CPW, BS	E,F, I, WP, WR	✓
19	Mardan	MDN1	CPW, BS	E,F, I, PR, WR	✓
20	Mardan	MDN2	CPW, BS	E,F, I, W,WP, WR	✓
21	Mardan	MDN3	CPW, BS	E,F, I, W, WR	✓
22	Swabi	SWB1	PPW, BS	E,F, I, WP, WR	✓
23	Charsadda	CHD2	CPW, BS	E,F, I, W,WP, WR	✓
24	Charsadda	CHD1	CPW, BS	E,F, I, W,WP, WR	✓
25	Charsadda	CHD3	CPW, BS	E,F, I, WP, WR	✓
26	Nowshera	NSH1	CPW, BS	E,F, I, PR, WP, WR	✓
27	Nowshera	NSH2	CPW, BS	E,F, I, W, WP	✓
28	Nowshera	NSH3	CPW, BS	E,F, R, RB	X
29	Peshawar	PES1	CPW, BS	E,F, I, WR	✓

Where; CPW= Complete plant wilted, PPW= Partial plant wilted, BS= Bacterial streaming, E=elevated; F=fluidal; PR= pinkish red; R= round; RB= Red with bluish margins; W= White; WP= White with pink center; WR= White with red center.

Table 3: Pathogenicity assay for *R. solanacearum* isolates inoculated to tomato plants using different inoculation methods

S. No.	Origen of the isolates	Isolate code	Pathogenicity tests			
			Pathogenicity assay I Root inoculation		Pathogenicity assay II Leaf inoculation	
			No Piercing	Piercing	No Piercing	Piercing
1	Bannu	BAN1	+	+	+	+
2	LakkiMarwat	LMT1	+	+	+	+
3	D.I. Khan	DIK1	+	+	+	+
4	Haripur	HAR1	+	+	+	+
5	Mansehra	MAN1	-	+	-	+
6	Hangu	HAN1	+	+	+	+
7	Karak	KAR1	+	+	+	+
8	Kohat	KOH1	+	+	+	+
9	Buner	BNR1	-	+	+	+
10	Buner	BNR2	+	+	+	+
11	Lower Dir	DLO1	+	+	+	+
12	Lower Dir	DLO2	-	+	+	+
13	UperDir	DUP1	+	+	+	+
14	Malakand	MKD1	+	+	+	+
15	Malakand	MKD2	+	+	+	+
16	Shangla	SGL1	+	+	+	+
17	Swat	SWT1	-	+	+	+
18	Swat	SWT2	-	+	+	+
19	Mardan	MDN1	+	+	+	+
20	Mardan	MDN2	+	+	+	+
21	Mardan	MDN3	+	+	+	+
22	Swabi	SWB1	+	+	+	+
23	Charsadda	CHD2	+	+	+	+
24	Charsadda	CHD1	+	+	+	+
25	Charsadda	CHD3	+	+	+	+
26	Nowshera	NSH1	+	+	+	+
27	Nowshera	NSH2	+	+	+	+
28	Nowshera	NSH3	+	+	+	+
29	Peshawar	PES1	+	+	+	+

Where; + = Produced disease symptoms (i.e. complete or partial plant wilting and bacterial streaming);
- = No produced disease symptoms

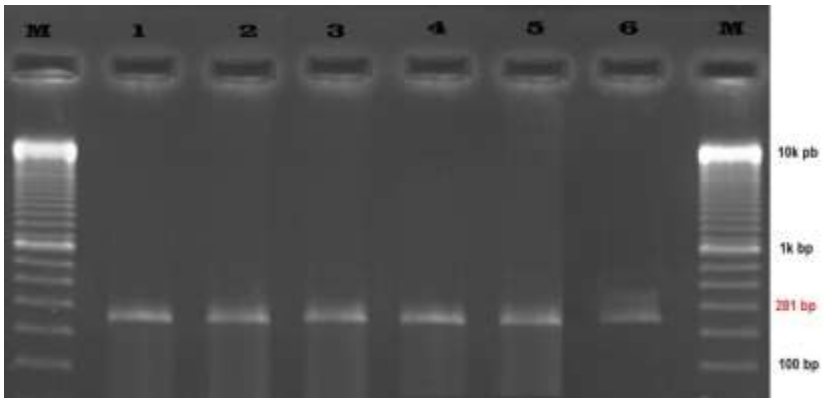

Fig. 2: Amplification of *R. solanacearum* specific 281bp fragment on agarose gel using PCR

vessels, and then spreads rapidly up the stem and throughout the plant. Typical wilting symptoms result from an excessive production of extracellular polysaccharides (EPS) within the vascular system, thus altering water fluxes in the plant (Genin *et al*., 2005). We used two inoculation methods for our pathogenicity assays; root inoculation (pierced verses un-pierced), and leaf inoculation (pierced verses un-pierced), on healthy tomato seedlings. In both assays; the piercing inoculation methods produced better results than the un-pierced one. The reason was that although bacterial pathogens could use natural openings present on plant surfaces for entry into their host tissues; however, they become more successful pathogens if they entered through wounds in the host, as such they will be able to enter the vascular fluid more rapidly. Our previous study on race-determination assay using tobacco plants (Junaid *et al*., 2018), grouped *R. solanacearum* isolates into race 1 (92% isolates), and race 3 (8% isolates). Race identification helps in better characterization of *R. solanacearum* isolates which was a complex species.

Some bacterial strains gave over-lapping results with respect to their colony morphology and pathogenicity tests, which made their identity uncertain. Our all 29 isolates produced typical *R. solanacearum* like colonies on TTC; however, some isolates (i.e. LMT1, KOH1, MAN1, DUP1 and NSH2) also produced deep red colonies with bluish border which were thus considered as avirulent strains according to Chaudhry and Rashid, (2011). This avirulence might be attributed to spontaneous mutation as reported by Meng *et al*., (2011); Schroeder *et al*., (2017). Currently; to confirm the identity of our isolates, we used *R. solanacearum* specific primers (Popoola *et al*., 2015), that amplified the specific 281bp fragment of 25 out 29 of our isolates through PCR (Vanitha and Umesha, 2014). The *R. solanacearum* specific band (i.e, 281bp fragment), could not be amplified when the genomic DNA of the four isolates (i.e., LMT1, MAN1, DUP1 and NSH2) was used as template in the PCR reaction. This indicated that either these isolates might be mutated in their specific gene complementary to the used primers (Meng *et al*., 2011; Schroeder *et al*., 2017), or that they were not *R. solanacearum*. However, it was also possible that they might be other close relatives of R. *solanacearum* (Coenye *et al*., 2003).

Conclusion

Our study reported that TTC medium could specifically distinguish virulent isolates of *R. solanacearum* from avirulent ones. Moreover, the pathogenicity assays could not deceive the researchers as the avirulnt strains of the bacterium did not produce any wilt symptoms. Molecular techniques such as PCR can be considered as the best method for detection or identification of *R.*

solanacearum, but still there is a chance of misleading results due to mutations in the pathogen genetic makeup.

Conflict of interests

The authors declare no conflict of interests.

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