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POLYPHASIC CHARACTERIZATION OF *RALSTONIA PSEUDOSOLANACEARUM* ASSOCIATED WITH BACTERIAL WILT OF BRINJAL IN EASTERN INDIA

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ABSTRACT
Bacterial wilt caused by *Ralstonia solanacearum* species complex RSCC is a major constraint to brinjal production in India. Bacterial wilt samples of brinjal were collected from Cuttack, Odisha, India and the pathogen was isolated, identified and characterized. The infected plant had sudden wilting of green leaves, vascular browning and oozing when emerged in sterile water. The pathogen was isolated and named as ODRS19. The distinct colony characteristics were observed by growing the isolate ODRS19 on TZC, Nutrient Agar, and Kings B media. Biochemical characterisation by VITEK®2 Automated microbial identification System confirmed that this isolate belongs to RSCC 16S rRNA gene sequencing identifies the isolate ODRS19 as *Ralstonia pseudosolanacearum*. It was further classified as Race I based on the host range studies and Biovar III based on carbohydrate utilization patterns. The results of phylotype multiplex PCR indicated the specific 280 and 144 bp bands confirming this as phylotype I. Comprehensive characterization of this isolate helps to understand the characteristics of the pathogen in eastern India and will aid in formulating accurate diagnostic and management techniques for bacterial wilt of brinjal.

KEYWORDS: Bacterial wilt, *Ralstonia*, Brinjal, Biovar, Race, Phylotype

INTRODUCTION
Solanum melongena, also referred to as aubergine, eggplant or brinjal, is a less expensive vegetable crop, which is produced globally and has the potential to offer considerable nutritional value that is vitamins, phenolics and antioxidants[3]. Different abiotic and biotic factors severely impact productivity of brinjal and one of such is wilting. One of the most destructive infections of brinjal is bacterial wilt that is caused by soil-borne pathogen *Ralstonia solanacearum*[18]. The disease is called green wilt because the leaves of the affected plant are still green when they start to exhibit the signs of wilting[13]. Bacterial wilt disease has been reported to cause yield loss of around 10 to 90 percent[22] and in severe cases can cause up to 80-90 percent[15] of yield loss.

It is a soil borne pathogen mostly prevalent in the tropics, subtropics and warm temperate parts of the world[29]. It infects over 200 species and 50 families of plants and is rated second on a list of the top 10 most scientifically and economically important bacterial plant pathogens[14,18]. *Ralstonia solanacearum* is present in a species complex which includes several phylotypes, sequevars, and strains that are differentiated in respect to geographical origin, host range, virulence, and ecological adaptation[7]. Considerable variability has been reported among isolates in terms of colony morphology, biochemical characteristics, pathogenicity, and molecular profiles, which significantly influence disease severity and epidemiology in different agroclimatic regions[1]. This genetic and pathogenic diversity allows the pathogen to adapt to various environmental conditions, as well as to various host plants, which makes it extremely difficult to manage the disease.

Characterization of the individual isolate is essential to understand the pathogen diversity, virulence potential virulence and hosts-pathogen interaction. Detailed characterization of isolates provides critical information for accurate pathogen identification, monitoring strain distribution, and developing targeted disease management strategies, including resistant cultivar development and biological control approaches[18]. Understanding the variability and pathogenic potential of individual isolates also contributes to improved epidemiological surveillance and supports the development of sustainable and region-specific management strategies for bacterial wilt[21].

The present study aimed to isolate, identify, and comprehensively characterize a virulent *Ralstonia pseudosolanacearum* isolate associated with bacterial wilt of brinjal using morphological, biochemical, molecular and pathogenicity analyses.

MATERIAL AND METHODS
Collection of samples and isolation of pathogen
Brinjal Plants showing bacterial wilt symptoms were collected from farmers field, Gatiroutpatana, Cuttack, Odisha (20° 18′ 36.648″ N, 84° 52′ 42.114″ E). and brought to laboratory. The diseased plants were thoroughly dipped in tap water,

cut several inches above the root area on the stem, dipped in clear sterile water and tested whether they were oozing. Bacterial ooze of the cut end of the samples was streaked on Nutrient Agar (NA) plates. The plates were subsequently incubated at 28 ^{0}C for 24 h. Following isolation, a purification of bacterial isolates was done through streaking of one colony of each of the isolates on triphenyl tetrazolium chloride (TZC) medium[10]. The isolates were subculture in TZC slants and incubated at 28 ^{0}C for 48 h.

Pathogenicity Test

One loop full of isolated bacterial colonies was added to nutrient broth and allowed to grow in a shaking incubator maintained at 28ºC and 150 rpm for 48 h. Brinjal seedlings (VNR 212) were prepared in a polyhouse and 4 weeks old seedlings were transplanted into pots containing sterile soil. Seedling roots were injured before transplantation. Freshly grown inoculum of bacterial isolates for 48 h at 28 ± 2°C in nutrient broth with an inoculum load of 10^8 CFU/ml was poured around the root zone using the soil drenching method. Three replicates were maintained in different pots. The control was maintained by soil drenching with sterilised double distilled water The development of symptoms was monitored weekly for 1 month. The pathogen was reisolated to satisfy Koch's postulates.

Morphological and Cultural characterization

One loop full of bacterial colonies from the pure culture were streaked on to Triphenyl Tetrazolium Chloride (TTC/TZC) agar medium, Nutrient Agar (NA) medium and Kings B Medium and incubated at 28 ± 2°C for 48–72 h. Colony morphology including size, shape, margin, texture, colony colour, elevation and pigmentation was observed. Bacterial cell morphology and Gram reaction were determined by Gram staining and microscopic examination under oil immersion (Kelman, 1954)

Biochemical Characterization

The biochemical analysis was done using the VITEK®2 Automated microbial identification System (bioMérieux, France). Isolated colonies were streaked on nutrient agar plates and incubated at the right temperature with 24 -48 h to obtain pure cultures. Gram staining was done[28] and based on the gram reaction suitable VITEK identification card (Gram Negative) was chosen. The pure culture of bacteria was inoculated into 3 ml of sterile VITEK saline (0.45% NaCl) and swirled to achieve a suspension of a uniform colony. A densiCheck turbidity meter was used to adjust the inoculum density to 0.50-0.63 McFarland. VITEK 2 bacterial suspension was subsequently loaded into VITEK 2 cassettes tubes and then VITEK 2 Compact system was loaded using the cassettes and the sample was automatedly filled, incubated, and identified. The system analysed the biochemical reaction and identified the isolate.

Molecular Characterization

Genomic DNA was extracted from pure cultures of isolated bacteria grown in nutrient broth using the Qiagen Dneasy® Ultraclean® (Catalog number: 12224-50) Microbial by following the manufacturer's protocol (https://www.qiagen.com/us/Resources/). For amplification of 16s rDNA gene, universal primer 27F (5'-AGAGTTTGATCCTGGTCAG-3') and 1492R (5'-TACGGTTACCTTGTTACGACTT-3' were used[17]. PCR was performed using a thermal cycler (Applied Biosystems MiniAmp Plus, USA). The reaction mixture (25 µl) was prepared according to the protocol described by Fujiyoshi *et al*. (2020)[8], with minor adjustments. The reaction mixture included 2.5 µl of 10X PCR buffer with $MgCl_2$ already added, 1 µl of each primer, 0.5 µl of dNTPs, 0.3 µl of Taq DNA polymerase, and the remaining volume was filled with molecular grade nuclease-free ddH_2O to reach 25 µl. The PCR protocol consisted of initial denaturation at 95°C for 3 min, followed by 25 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 15 s, and extension at 72°C for 30 s and a final extension at 72°C for 5 min[31]. Amplified products were analysed by gel electrophoresis on a 1% agarose gel. Amplified 16S rDNA gene products were purified using the PureLink Quick PCR Purification Kit (Invitrogen, USA; Catalog number: K310001) following the manufacturer's protocol and quantified using a Nanodrop spectrophotometer (Eppendorf, Hamburg, Germany). The PCR products were subjected to sanger sequencing, and the obtained sequence was subjected to a BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The 16S rDNA gene sequence was deposited in the GenBank database (https://submit.ncbi.nlm.nih.gov/subs/genbank/) and accession numbers were obtained. Phylogenetic trees were constructed with MEGA 12.0 software (https://megasoftware.net/active_download) using the neighbour-joining method.

Races identification

The races of *Ralstonia pseudosolanacearum* was identified by pathogenicity test on wide host range[5]. The races of *R. pseudosolanacearum* isolates were identified by pathogenicity test on wide host range. One month old seedlings of tomato, brinjal, chilli, ginger, banana and mulberry were inoculated by soil inoculation method. The incubated plants were then kept until symptoms development.

Determination of biovars

The *R. pseudosolanacearum* isolates were differentiated into biovars based on their ability to utilise disaccharides (sucrose, lactose and maltose) and sugar alcohols (mannitol, sorbitol and dulcitol), as described by Hayward (1964)[11] and He *et al.* (1983)[12]. Approximately 2 ml of the melted medium (Table 1) was dispensed into a test tube. The inoculum was prepared by adding several loopfuls of bacteria from 24 h old cultures to distilled water to make a suspension containing approximately 10^8 CFU/ml. Then, 20 µl of the bacterial suspension was added to the test tube and incubated at 28ºC. The tubes were then examined 3 days after inoculation for changes in pH based on colour change (Table 2).

Table 1. Composition of media (pH: 7.0-7.1) for biovar determination

Chemical	Amount
$NH_4H_2PO_4$	1.0 g
$MgSO_4.7H_2O$	0.2 g
KCl	0.2 g
Difco Bacto Peptone	1.0 g
Agar	3.0 g
Bromothymol blue	80.0 mg
Sugar	1%
Distilled water	1000 ml

Table 2. Biovar determination was based on the following (He *et al*., 1983)

Substrate	Biovars					
	1	2	3	4	5	6
Cellobiose	-	+	+	-	+	+
Maltose	-	+	+	-	+	+
Lactose	-	+	+	-	+	+
Dulcitol	-	-	+	+	-	-
Sorbitol	-	-	+	+	-	+
Mannitoal	-	-	+	+	+	+

- : Negative reaction
+ : Positive reaction

Phylotype determination

A phylotype-specific multiplex PCR (PmxPCR) was performed to determine the phylotype affiliation of all strains (Fegan and Prior, 2005). A set of four phylotype-specific forward primers (Nmult:21: 1F, Nmult:21:2F, Nmult:23:AF, Nmult:22: InF, and Nmult:22: RR) with a unique and conserved reverse primer (Nmult:22: RR) targeted in the 16S-23S ITS region was used for the study (Table 3). The mixture contained 12.5µl of 2×PCR buffer, 0.6µl of each forward primer (10 µmol/l), 2.4 µl of reverse primer (Nmult:22: RR), 0.4 µl of OLI1 and Y2 primers and 2µl template mix (about 50 ng/µl). The final volume was made up to 25 µl using PCR-grade water. The PCR reaction was prepared and subjected to thermocycling at the following temperatures: 94°C for 15 s, 59°C for 30 s, and 72°C for 30 s and a final extension of 72ºC for 10 min. PCR products were resolved using agarose 1.5% (wt/vol) gel electrophoresis. Amplicon sizes were estimated by comparison to a 100-bp DNA ladder.

Table 3. List of primers and amplicon size for Pmx-PCR used in phylotyping of *Ralstonia solanacearum* species complex

Primers	Sequence (5'-3')	Specific to phylotype	Amplicon
Nmult21:1F	CGTTGATGAGGCGCGCAATTT	Forward primer for phylotype I	144 bp
Nmult21:2F	AAGTTATGGACGGTGGAAGTC	Forward primer for phylotype II	372 bp
Nmult23:AF	ATTACSAGAGCAATCGAAAGATT	Forward primer for phylotype III	91 bp
Nmult22: InF	ATTGCCAAGGACGAGGAAGTA	Forward primer for phylotype IV	213 bp
Nmult22: RR	TCGCTTGACCTATAAACGAGTA	Reverse primer for all phylotypes	-
OLI1	GGGGGTAGCTTGCTACCTGCC	Species-specific forward primer	288 bp
Y2	CCCA CTGCTGCCTCCCGTAGGAGT	Species-specific reverse primer	-

RESULTS

Collection of samples and isolation of pathogen

The infected brinjal plants showed sudden wilting of green leaves without yellowing (Figure 1), vascular brown discolouration of the stem and the presence of a milky-white bacterial ooze was observed when the cut stem segments were immersed in sterile water, identifying the usual signs of bacterial wilt caused by *Ralstonia sp.* The causal pathogen was isolated on nutrient agar media and designated as ODRS19.

Figure 1: Wilting symptom of brinjal plant affected with *R. pseudosolanaearum*.

Morphological and Cultural characterization

The isolated bacterial pathogen (ODRS19) exhibited red pigmented colonies in the TZC plate. The colonies were circular with smooth edges, glistening, opaque and homogenous in colour, medium to big in size. The same pathogen produced creamy white to translucent colonies on the nutrient agar medium with. The colonies were circular, small round with whole margins and convex elevation, smooth surface and mucoid texture. On Kings B Medium, the isolate produced circular to irregular shaped colonies that were cream to off-white in colour with margins that were complete to undulating with smooth and glittering surface (Figure 2).

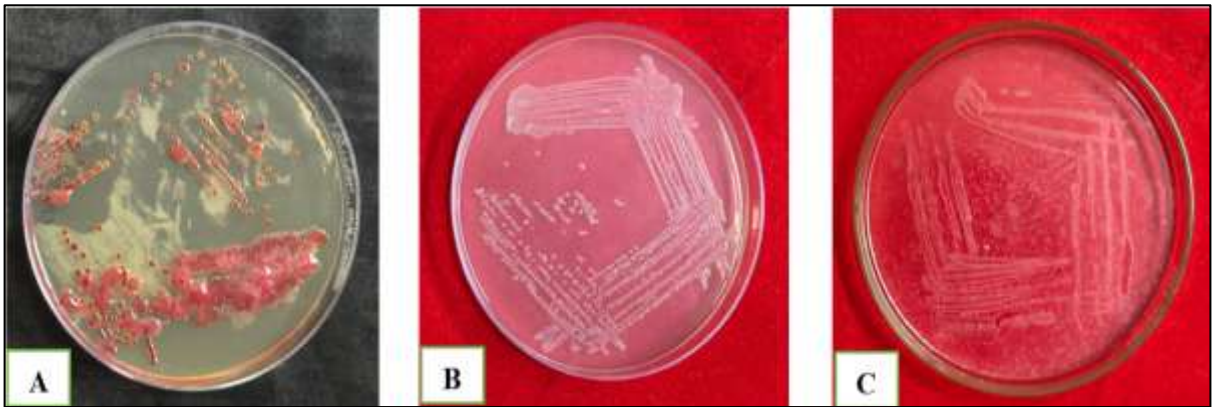

Figure 2: Growth of ODRS19 (*Ralstonia pseudosolanacearum*) on: (A) TZC media, (B) Nutrient Agar media, (C) King B media.

Biochemical Characterisation

The biochemical characterization of isolate ODRS19 established positive reactions to several important enzymatic activities, such as pyrrolydonyl arylamidase, 2 -galactosidase, urease, tyrosine arylamidase, ornithine decarboxylase and lysine decarboxylase. The isolate fermented a number of carbohydrates including glucose, maltose, mannitol, palatinoFse and sucrose and fermented citrate, succinate, lactate and malate. It tested negative for production of hydrogen sulfide, lipase activity and the use of substrates adonitol, sorbitol and trehalose were negative (Table 4).

Table 4: Biochemical characterization of isolated pathogenic bacteria ODRS19 isolates using VITEK®2 automated microbial identification System

Sl. No.	Biochemical details	Abbreviation	ODRS19
1	Ala-Phe-Pro-Arylamidase	APPA	-
2	Adonitol	ADO	-
3	L-Pyrrolydonyl-Arylamidase	PyrA	+
4	L-Arabitol	IARL	+
5	D-Cellobiose	dCEL	+
6	Beta-Galactosidase	BGAL	+
7	H_2S Production	H_2S	-
8	Beta-N-Acetyl-Glucosaminidase	BNAG	-
9	Glutamyl Arylamidase pNA	AGLTp	-
10	D-Glucose	dGLU	+
11	Gamma-Glutamyl-Transferase	GGT	+
12	Fermentation/Glucose	OFF	+
13	Beta-Glucosidase	BGLU	-
14	D-Maltose	dMAL	+
15	D-Mannitol	dMAN	+
16	D-Mannose	dMNE	-
17	BETA-Xylosidase	BXYL	+
18	BETA-Alanine Arylamidase pNA	BAIap	-
19	L-Proline Arylamidase	ProA	-
20	Lipase	LIP	-
21	Palatinose	PLE	+
22	Tyrosine Arylamidase	TyrA	+
23	Urease	URE	+
24	D-sorbitol	dSOR	-
25	Saccharose/Sucrose	SAC	+
26	D-Tagatose	dTAG	-
27	D-Trehalose	dTRE	-

28	Citrate (Sodium)	CIT	+
29	Malonate	MNT	-
30	5-Keto-D-Gluconate	5KG	-
31	L-Lactate Alkalinization	ILATk	+
32	Alpha-Glucosidase	AGLU	-
33	Succinate Alkalinization	SUCT	+
35	Phosphatase	PHOS	-
36	Glycine Arylamidase	GlyA	+
37	Ornithine Decarboxylase	ODC	+
38	Lysine Decarboxylase	LDC	+
39	Decarboxylase Base	ODEC	+
40	L-Histidine Assimilation	IHISa	-
41	Coumarate	CMT	-
42	Beta-Glucuronidase	BGUR	-
43	O/129 Resistance (comp. vibrio.)	O129R	-
44	Glu-Gly-Arg-Arylamidase	GGAA	-
45	L-Malate Assimilation	IMLTa	+
46	Ellman	ELLM	-
47	L-Lactate Assimilation	ILATa	-

Molecular Characterization

PCR amplification of isolates ODRS19 of *R. pseudosolanaearum* using specific universal primers 27F/1492R yielded 1500 bp product encoding 16S rRNA confirming that the ODRS19 isolates belonged to *R. pseudosolanacearum* (Figure 3). ODRS19 showed 97.13 % similarity of 16S rRNA gene sequence to *Ralstonia pseudosolanacearum* (NCBI accession no. PX107868) with the reference genome. Phylogenetic analysis of the partial 16S rRNA sequences of *Ralstonia pseudosolanacearum* ODRS19 isolates with reference sequences of *Ralstonia* and related species (Figure 4) with a bootstrap value of 1000 to support the typology of the tree with long branch length from the nearest clusters.

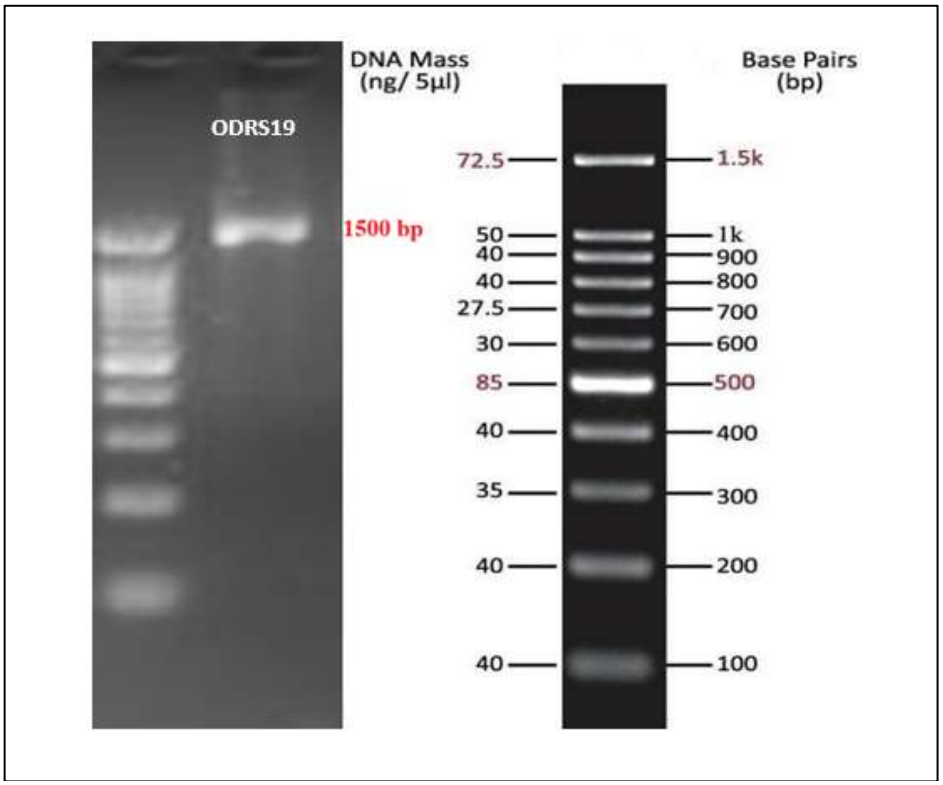

Figure 3: Gel electrophoresis photograph of successful amplification of bacterial 16S rRNA gene of (1,2, and 3) *Ralstonia pseudosolanacearum* ODRS19 with a 1Kb bp DNA ladder as size reference. Bands at ~ 1500 bp confirmed successful amplification of target sequences

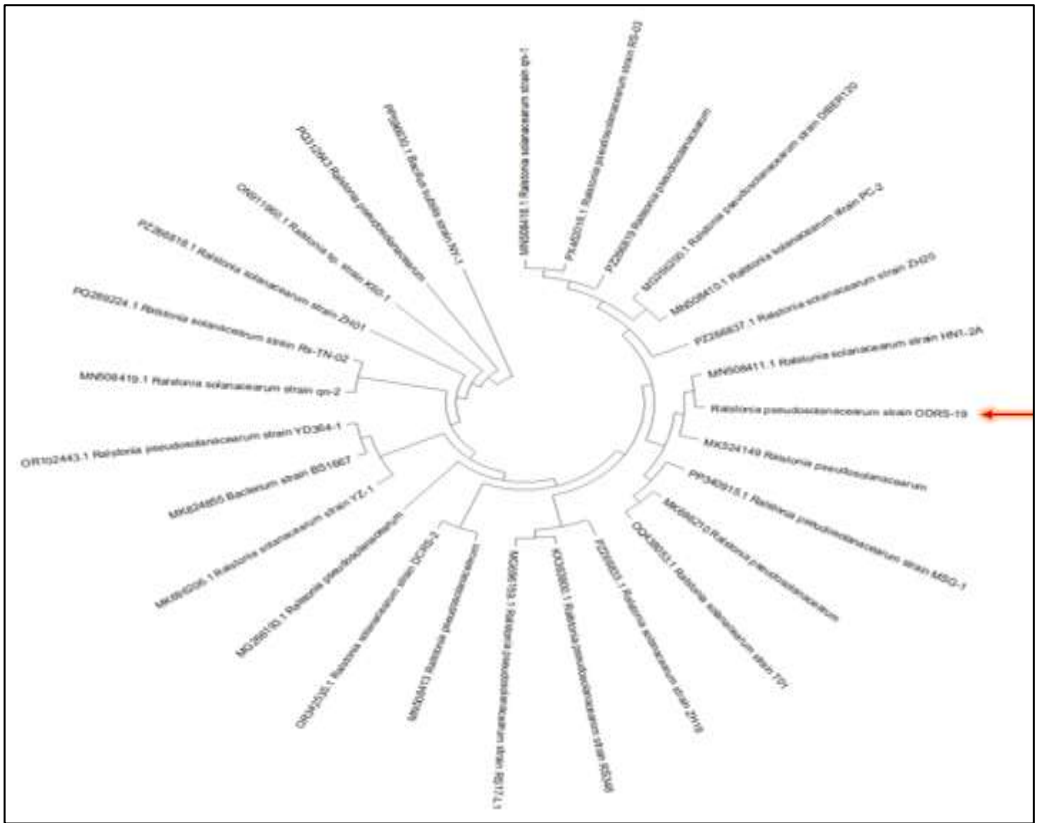

Figure 4: Phylogenetic tree based on 16S rRNA sequence data, showing the position of *R. pseudosolanacearum* ODRS19 (NCBI accession no. PX107868) within the genus *Ralstonia*.

Race Determination

The isolate ODRS19 produced typical wilt symptoms on brinjal, chilli and tomato but failed to infect ginger, banana and mulberry indicating that the isolates belong to Race I of *R. pseudosolanacearum*.

Biovar Determination

The isolate ODRS19 oxidized disaccharides (lactose, maltose and cellobiose) but failed to oxidize sugar alcohols (mannitol, sorbitol and dulcitol), indicating that the isolate belongs to Biovar III of *R. pseudosolanacearum* (Figure 5).

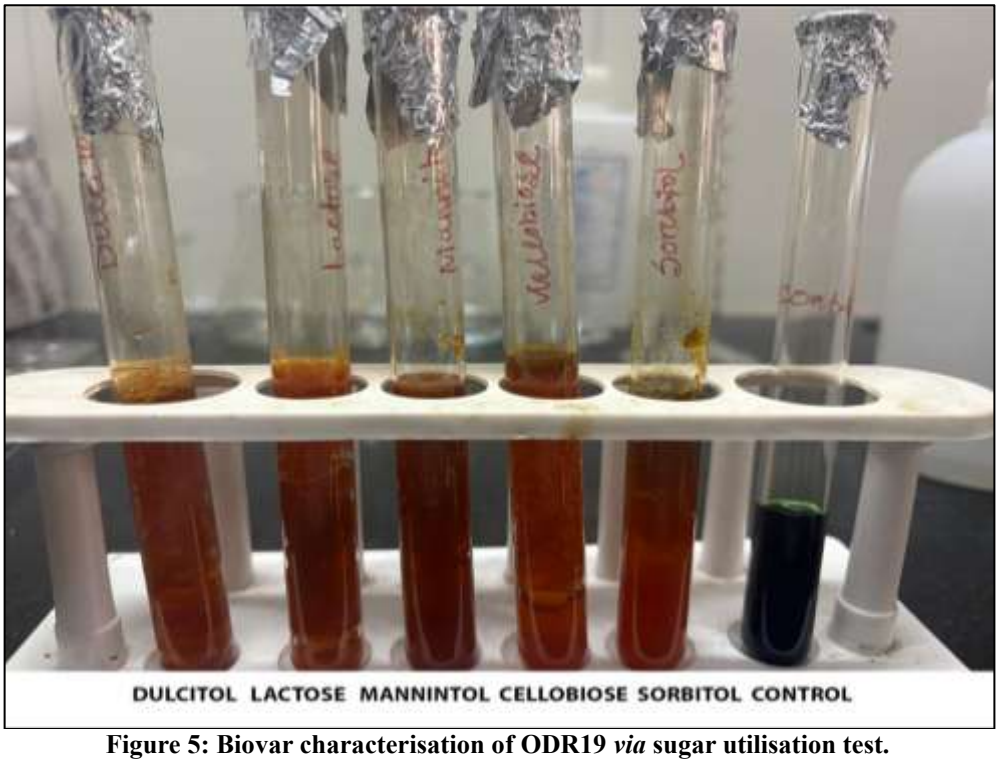

Figure 5: Biovar characterisation of ODR19 *via* sugar utilisation test.

Phylotype determination

A phylotype-specific multiplex PCR (Pmx-PCR) was performed to determine the phylotype affiliation of all strains. A set of four phylotype-specific forward primers (Nmult:21:1F, Nmult:21:2F, Nmult:22:InF and Nmult:23:AF), with a unique conserved reverse primer (Nmult:22:RR:) and *R. solanacearum* specific primers (759R and 760F) (Table 5), these primers targeted in the 16S-23S intergenic spacer region (internal transcribed spacer). The Pmx-PCR of isolated *R. pseudosolanacearum* ODRS19 yielded 144 bp amplicon and 280 bp amplicon (Fig. 5). It revealed that the isolate ODRS19 was *R.* pseudo*solanacearum* and belonged to phylotype I.

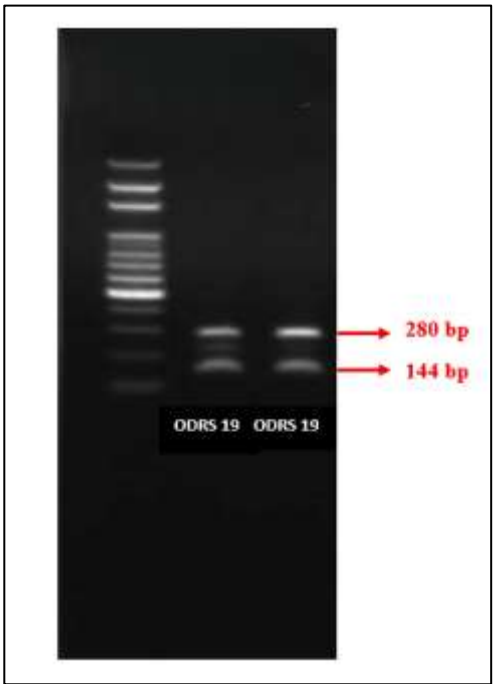

Figure 5. Gel electrophoresis photograph of successful amplification at 280bp and 144 bp

DISCUSSION

Bacterial wilt caused by RSSC is a major problem in all brinjal growing areas of India in recent years, posing a significant constraint to brinjal production and can cause about 10% to 90% of yield loss depending upon the environmental condition and growth stage of the crop[20]. The collected bacterial wilt samples exhibited characteristics symptoms like wilting of green leaves without yellowing and the vascular bundles showed discolouration and there was appearance of ooze when the stems were cut and dipped in sterile water. These findings are line with the findings of Valdez-Morales *et al*. (2023)[30] who reported sudden wilting of brinjal plants along with vascular discoloration and oozing caused due to *Ralstonia pseudosolanacearum*.

Ralstonia pseudosolanacearum produces irregular, mucoid colonies with a cream-white appearance and pink centers on the TZC medium as reported by Saini *et al.* (2025)[23] and forms cream to milky white, raised colonies with glossy texture with irregular to circular margins as reported in EPPO bulletin (2018)[6]. Similarly in this study the isolated *R. pseudosolanacearum* ODRS19 produced circular red to pink colonies with smooth margin and mucoid texture and creamy white to translucent colonies on nutrient agar medium and off-white circular to glistening colonies on Kings B Medium.

The biochemical characterisation of *R. pseudosolanacearum* isolate by VITEK®2 Automated System that revealed that the isolate was as a member of the *Ralstonia solanacearum* species complex based on its Gram-negative reaction, oxidase and catalase positivity, and oxidative utilization of carbohydrates including D-glucose, D-mannose, D-fructose, and N-acetyl-D-glucosamine. The isolate tested negative for urease, indole production, and hydrogen sulfide formation. The biochemical profile corresponded to descriptions reported for *Ralstonia pseudosolanacearum* by Zou *et al*. (2025)[32]. The strain ODRS19 was identified as *Ralstonia pseudosolanacearum* based on 16S rDNA gene sequence. *R. pseudosolanacearum* has been reported in india previously from Tamilnadu by Selastin Antony *et al*. (2015)[24], in Himachal Pradesh by Saini *et al*. (2025)[23] and from Southwest Indian Ocean Region by Cellier *et al*. (2023)[4].

The race determination was done for the isolated *R. pseudosolanacearum* ODRS19 based on the host range studies as described by He *et al*. (1983) and all the isolates produces symptoms on brinjal, chilli and tomato but failed to produce any symptom in ginger, banana and mulberry, thus suggesting the isolate belong to race I (Buddenhagen and Kelman , 1964). The isolate was found to be biovar III which is mostly prevelant biovar of *Ralstonia* species in India particularly eastern India as reported by Dinesh *et al*. (2010).

CONCLUSION

The present study successfully isolated and characterized a bacterial pathogen that causes wilt in brinjal and named it ODRS19. The isolate possessed typical wilt symptoms, typical colony morphology, and a typical biochemical profile that was similar to the *Ralstonia solanacearum* species complex. Identification of ODRS19 was confirmed by 16S rDNA sequencing as *Ralstonia pseudosolanacearum* Host range studies identified it as Race I and carbohydrate utilization tests

found it as Biovar III and multiplex PCR revealed it to be phylotype I. These findings provide essential information on the pathogen causing bacterial wilt and is essential for accurate diagnosis and effective management of bacterial wilt of brinjal.

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