



RESEARCH ARTICLE

Association of *Pantoea agglomerans* with citrus canker of lemon in the northern region of West Bengal

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Received: 27 April 2026; Accepted: 16 June 2026; Available online: Version 1.0: 20 July 2026

Cite this article: Ritwik S, Surajit K, Prateek MB, Sekhar B, Nilesh B, Manoj KD, Puspendu D, Samrat S, Kuntal B, Soham M. Association of *Pantoea agglomerans* with citrus canker of lemon in the northern region of West Bengal. Plant Science Today. 2026; 13(sp5): 1-11.

<https://doi.org/10.14719/pst.15278>

Abstract

Lemon is valued for its nutritional and therapeutic benefits. Its production in the northern region of West Bengal is severely affected by citrus canker, one of the most destructive bacterial diseases of citrus. The isolation of different bacterial isolates for the present investigation was performed during 2024. Whereas the virulence study was conducted across two cropping seasons (2024–25) to identify the virulent bacterial isolates associated with citrus canker under the local conditions of North Bengal. Twenty bacterial isolates were obtained and they were evaluated through pathogenicity test. Among the isolates, five showed consistent virulence under artificial inoculation conditions. Isolate no. 4 (UBKVXAC4) was found to be most virulent, showing maximum disease severity index (39.43 % in first year and 40.05 % in second year) and disease increase percentage (64.57 % and 67.79 % in first and second year). Molecular characterisation using 16S rRNA gene sequencing identified three isolates as *Xanthomonas axonopodis* pv. *citri*, while two were identified as *Pantoea agglomerans*, suggesting a potential involvement in the development of canker-like symptoms. Further confirmation using Xac-specific primers targeting the *hrpW* gene showed amplification only in *X. axonopodis* pv. *citri* isolates, whereas no amplification was detected in the *P. agglomerans* isolates. The findings indicate that, besides the primary citrus canker pathogen, *P. agglomerans* may also be associated with canker-like symptoms in lemon under the agro-climatic conditions of northern West Bengal. This study highlights the diversity of bacterial communities associated with citrus canker and provides useful information for future disease management and resistance screening programmes.

Keywords: citrus canker; lemon; *Pantoea agglomerans*; disease severity index; *Xanthomonas axonopodis* pv. *citri*

Introduction

Citrus (*Citrus* sp.) is commonly considered the world's leading tree-fruit crop, is the most important fruit crop under the family Rutaceae. It is believed that most of the citrus species originated in the tropical and sub-tropical regions, specifically in India, China and the region between these two countries, more particularly in Northeastern region of the country. Citrus comprises a highly diverse group of species. It is widely available and popular worldwide, adding to human diets. Among the species in the acid group of citrus, lemon [*Citrus limon* (L.) Osbeck] occupies an important place for its excellent flavour and nutritive quality. It is a species of the family Rutaceae native primarily to Northeast India (Assam), Northern Myanmar, Asia and other regions of Asia, including China. They are rich sources of vitamin C, folic acid, dietary fiber and several antioxidant compounds that promote human health. Regular consumption of lemon has been associated with reduced risk of various health disorders, including

cardiovascular diseases, certain cancers and birth defects (1). The fruits are rich in vitamin C, organic acids, minerals, antioxidants and essential oils, making them highly valuable for fresh consumption as well as for processing industries involved in beverages, pharmaceuticals, cosmetics and food preservation. In India, lemon and other citrus fruits occupy an important position in the horticultural sector and contribute significantly to farmers' income generation, rural employment and agro-based industries. Owing to its high market demand, regular bearing habit and year-round economic return, lemon is considered an important cash crop in several citrus-growing states of India, including West Bengal (2). According to the Food and Agriculture Organization (FAO), India produced approximately 3.8 million tonnes of lemons and limes in 2024, making it the largest global producer of the crop (3). According to the National Horticulture Board (NHB) data published through the Agricultural and Processed Food Products Export Development Authority (APEDA), West Bengal produced about 94920 tonnes of citrus fruits, with major cultivation

concentrated in the northern districts of the state under humid Terai condition (4), specific data regarding lemon production remains limited. Global lemon production is severely constrained by several biotic and abiotic stresses.

The Northern region of West Bengal is one of the major centers of citrus diversity, hosting numerous indigenous and cultivated citrus species. These warm and humid climatic conditions favour extensive citrus cultivation but also create an ideal environment for disease development. Acid lime, Assam lemon and other citrus species are extensively cultivated in districts such as Cooch Behar, Jalpaiguri, Alipurduar, Uttar Dinajpur and Dakshin Dinajpur. Despite the high economic and horticultural importance of lemon orchards, their current productivity is under serious threat due to multiple challenges, among which citrus canker ranks as the most destructive bacterial disease affecting lemon trees. Citrus canker is one of the most destructive bacterial diseases of citrus, causing severe damage to leaves, twigs and fruits, ultimately leading to premature fruit drop, decline in fruit quality and significant reduction in orchard productivity. Under favorable warm and humid conditions, the disease may cause yield losses ranging from 10–50 % and in severe epidemics, losses may exceed 70 % in susceptible citrus cultivars (5). Citrus canker is highly prevalent in this region, significantly affecting yield and fruit quality across different citrus species. This disease is predominantly caused by *Xanthomonas axonopodis* pv. *citri* (Xac) and is widely distributed across major lemon-producing regions worldwide. The widespread occurrence of the disease highlights the need for effective management strategies to sustain citrus production in the region.

The pathological significance of citrus canker extends beyond yield reduction because the disease spreads rapidly through wind-driven rain, contaminated nursery materials and mechanical injuries, making its management extremely difficult in disease-endemic regions. In addition, the continuous emergence of variable bacterial strains and the association of secondary bacterial communities with canker lesions complicate accurate pathogen identification and disease management strategies. Several studies have emphasised the importance of molecular characterisation for understanding pathogen diversity, virulence variability and epidemiological behaviour of citrus canker pathogens (6). Recently, bacterial species belonging to the genus *Pantoea* have also been reported from citrus-associated lesions and diseased plant tissues, suggesting possible opportunistic or associative interactions within citrus disease complexes.

Citrus canker has been classified into five distinct forms, designated as canker “A”, “B”, “C”, “D” and “E”, based on differences in host specificity, virulence and geographical distribution (7). The most widespread and destructive form is canker A. It is also referred as the A-strain or Asiatic canker, which was initially reported from Florida and considered as the most aggressive among the known isolates of citrus canker (6, 8). This strain is most commonly associated with citrus canker disease in scientific literature. In India, the presence of different strains (pathotypes) of the pathogen was first reported by previous researchers (9).

Variations in pathogenicity and expansion of host range are often associated with alterations in virulence-related genes, arising from mechanisms such as horizontal gene transfer, intragenomic recombination, or spontaneous mutations.

Pantoea agglomerans is normally found naturally in plants (10). Several species within the genus *Pantoea* are recognised as plant pathogens. Among them, *P. ananatis* is known to infect a wide range of economically important crops, including pineapple, maize, onion and sudangrass (10). Several studies have explored the pathogenic characteristics of *Pantoea* species across a broad spectrum of plant hosts. Within citrus ecosystems, *Pantoea* is often reported as the most prevalent and dominant saprophytic bacterium associated with citrus canker lesions. Earlier research indicates that interactions between *Xanthomonas* and *Pantoea* can affect disease dynamics, with *Pantoea* contributing to a reduction in the population of *Xanthomonas* (11). Overall, evidence from various plant systems suggests that *Pantoea* has the ability to acquire virulence-associated genes from established plant pathogens, thereby enabling it to exhibit pathogenic behavior (12).

However, various investigators have reported the occurrence of numerous citrus canker pathogen isolates along with their variability in pathogenicity, virulence and molecular characteristics across different citrus-growing regions, nationally and internationally (7, 8). Recent studies have also highlighted the association of non-traditional bacterial species, particularly *P. agglomerans*, with plant disease complexes and their potential to acquire pathogenic traits through horizontal gene transfer. In citrus ecosystems, *Pantoea* species are frequently associated with canker lesions and may influence disease development through interactions with *Xanthomonas* sp. However, information regarding the diversity and molecular characterisation of citrus canker-associated bacterial isolates from the northern region of West Bengal remains limited. Therefore, the present investigation was undertaken to isolate, characterise and evaluate the virulence of citrus canker-associated bacterial isolates from this region, with special emphasis on identifying aggressive pathogens and understanding the possible association of *P. agglomerans* with citrus canker symptoms.

Materials and Methods

Study area

The study area is geographically located at 26.396205° N latitude and 89.385903° E longitude. This study region is characterised by the presence of per humid climate, with a high annual average rainfall exceeding 3000 mm and temperatures between 10 °C and 38 °C.

Collection and isolation of twenty different citrus canker associated bacterial isolates

Infected leaf samples from different citrus species (Different host species mentioned in Table 1) with citrus canker symptoms were collected from several areas, mainly the northern region of West Bengal, such as five from Alipurduar, six from Cooch Behar, three from Dakshin Dinajpur, two from Uttar Dinajpur, two from Jalpaiguri and two from Malda districts. These infected parts were used for isolation of the bacteria following the procedure of Schaad *et al.* on nutrient agar media (13). The bacterial fluidal lines were observed on nutrient media after 2 to 3 days after incubating at 28 ± 2 °C. Different biochemical procedures, like - Gram staining (14) and KOH test (15) was performed further for better clarity. Koch's postulate (16) was also performed by inoculating leaves of a whole plant by injection method with a syringe full of bacterial broth (1×10^8 CFU/mL) (17).

Table 1. Result of pathogenicity test of citrus canker associated bacterial isolates used in this experiment

Isolate (I)*	Place	Host	Isolation year	Test of pathogenicity
I1	Solsolabari, Alipurduar	Assam Lemon	2024	Positive
I2	Damsibad, Alipurduar	Acid Lime	2024	Positive
I3	Majhian, Dakshin Dinajpur	Acid Lime	2024	Positive
I4	Pundibari, Cooch Behar	Acid Lime	2024	Positive
I5	Panbari, Alipurduar	Acid Lime	2024	Positive
I6	Salkumar More, Jalpaiguri	Mandarin	2024	Positive
I7	Kaliyaganj, Uttar Dinajpur	Lemon	2024	Positive
I8	Mathabhanga, Cooch Behar	Acid Lime	2024	Positive
I9	Dinhata, Cooch Behar	Acid Lime	2024	Positive
I10	Sonapur, Cooch Behar	Acid Lime	2024	Positive
I11	Gopalganj, Malda	Acid Lime	2024	Negative
I12	Aiho, Malda	Acid Lime	2024	Negative
I13	Tapan, Dakshin Dinajpur	Acid Lime	2024	Negative
I14	Buniadpur, Dakshin Dinajpur	Acid Lime	2024	Negative
I15	Falakata, Alipurduar	Lemon	2024	Negative
I16	Maynaguri, Jalpaiguri	Lemon	2024	Negative
I17	Raiganj, Uttar Dinajpur	Acid Lime	2024	Negative
I18	Sitalkuchi, Cooch Behar	Lemon	2024	Negative
I19	Nurpur, Alipurduar	Acid Lime	2024	Negative
I20	Sahebganj, Dinhata, Cooch Behar	Lemon	2024	Negative

*Pictures of the culture plates of Isolate 1 to 5 are presented in Fig. 1

Pathogenicity test

Healthy six-month-old lemon plants maintained under insect proof conditions were used for the pathogenicity assay. Prior to inoculation, all glassware, syringes and inoculation materials were sterilised properly to maintain aseptic conditions throughout the experiment. Twenty isolates were inoculated (1×10^8 CFU mL⁻¹) into individual lemon plants using the injection method using sterile syringes, while control plants were injected with sterile distilled water only. After inoculation, the plants were maintained under controlled conditions (Temperature- 25 °C to 30 °C, relative humidity - 80 to 85 %, leaf wetness period- 24 to 72 hr) and observed regularly for symptom development (Well-developed canker lesions that are rough, corky and raised) for three weeks. Development of typical citrus canker symptoms was recorded. Only the isolates, which yielded positive results in the pathogenicity tests, were taken for further studies.

Virulence study

The collections of the isolates for the present investigation were performed during 2024 and virulence evaluation was conducted across two cropping seasons (2024–25). This study was conducted under insect proof conditions using healthy six-month-old potted lemon plants of uniform size and vigour. The experiment was laid out in a completely randomised design (CRD) with 11 treatments [10 treatments (T1 to T10) were the positive pathogenicity test result shown isolates (I1 to I10) and one control treatment (TC) without any isolate] and 3 replications, where each replication consisted of three plants, resulting in a total of nine plants per treatment. Ten bacterial isolates that showed positive pathogenicity responses were used as treatments along with one uninoculated control. Bacterial inoculum was prepared freshly from 48 hr old actively growing cultures, suspended in sterile distilled water and standardised to approximately 1×10^8 CFU/mL⁻¹ using turbidity adjustment. Separate inoculum preparations were used for each experimental year to maintain culture viability and consistency. For each replication three plants were used.

Individual lemon plants were inoculated with positive pathogenicity test result given isolates through the above-said inoculation technique. The observations (By observing percentage leaf area affected by the canker lesion) of disease severity index (DSI) after inoculation (20 days after inoculation) and disease increase

percentage after inoculation were taken and calculated by formulas and rating scale (Table 2) given by Bock *et al.* (18). The disease severity index (DSI) and disease increase percentage were calculated on a per-plant basis from the mean severity of infected leaves.

Disease severity index (DSI)=

$$\frac{\text{Sum (class frequency} \times \text{score of rating class)}}{(\text{total number of plants}) \times (\text{maximal disease index})} \times 100$$

Disease increase percentage =

$$\frac{\text{DSI after inoculation} - \text{DSI before inoculation}}{\text{DSI before inoculation}} \times 100$$

Table 2. Disease rating scale for assessment of citrus canker severity in lemon plants

Grades	Disease severity % (Percentage leaf area affected by the canker lesion)
0	0
1	0 ± 3
2	3 ± 6
3	6 ± 12
4	12 ± 25
5	25 ± 50
6	50 ± 75
7	75 ± 87
8	87 ± 94
9	94 ± 97
10	97 ± 100
11	100

DNA extraction from different virulent bacterial isolates

Extraction of total genomic DNA from all the virulent bacterial isolates was performed using a DNA isolation kit (Nucleo-pore gDNA Fungal Bacterial Mini Kit [50] Cat. # NP-7006D). Following extraction, the amount, purity and integrity of the DNA were assessed using agarose gel electrophoresis, ethidium bromide staining and ultraviolet (UV) light visualisation.

Polymerase chain reaction amplification

The 16S rRNA (ribosomal ribonucleic acid) gene was detected by polymerase chain reaction (PCR) amplification using primers specific to the 16S rRNA gene, 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT) as the forward and reverse primer pair, respectively. A total of 25 µL reaction mixture was prepared for each sample. The composition of PCR reaction mixture was mentioned in Table 3.

Table 3. Composition of polymerase chain reaction (PCR) reaction mixture

Substances	Amount
Forward primer	1.00 µL
Reverse primer	1.00 µL
Nuclease free water	8.50 µL
Green taq master mix	12.50 µL
Template DNA	2 µL
Total	25 µL

After that PCR was set at the specific conditions (initial denaturation of 3 min at 94 °C, denaturation of 30 s at 94 °C, annealing for 45 sec at 50 °C, extension for 1 min 50 s at 72 °C for and final extension for 10 min at 72 °C for 35 cycles). Then gel electrophoresis was performed to check the presence of specific gene and amplified product size (19).

Identification of the bacteria and sequence analysis

The region (specified to bacterial 16S rRNA gene) of the five isolates was amplified using a common primer pair. The amplified products were subsequently sequenced using the BigDye® Terminator (BDT) v3.1 Cycle Sequencing Kit on an Applied Biosystems™ MiniAmp™ Plus Thermal Cycler. Sequence analysis was performed on a Genetic Analyzer (ABI 3730xl) employing the Sanger dideoxy chain termination method. Then the sequences were compared with reference sequences from GenBank using BLASTN (Basic local alignment search tool for nucleotide sequences) programme in the NCBI website (National Center for Biotechnology Information) and aligned using CLUSTAL W programme using MEGA 11 software.

Confirmation about bacteria by extracting DNA directly from leaf samples and running specific primer

DNA extraction was performed by the CTAB (Cetyltrimethylammonium bromide) method (20) directly from the infected leaf samples for the virulent isolates. Detection of specific gene (hrpw gene) was performed by PCR amplification, which was set up using Xac-specific primer pair- XACF (5'-CGTCGCAATACGATTGGAAC-3') and XACR (5'-CGGAGGCATTGTCGAAGGAA-3') as forward and reverse primer pair, respectively. A total of 11 µL reaction mixture was prepared for each sample. Composition of the PCR reaction mixture was as below table (Table 4).

After that PCR was set at the amplification conditions (initial denaturation of 5 min at 94 °C, denaturation of 15 sec at 94 °C, annealing of 30 sec at 60 °C, extension of 45 sec at 72 °C and final extension of 7 min at 72 °C for 35 cycles). Next gel electrophoresis was performed to check the presence of specific gene and amplified product size.

Table 4. Composition of polymerase chain reaction (PCR) reaction mixture

Substance	Amount
Master mix	7 µL
Forward primer	1 µL
Reverse primer	1 µL
Template DNA	2 µL
Total	11 µL

Confirmation about the pathogenic association of *Pantoea agglomerans*

Pathogenicity re-confirmation was carried out following Koch's postulates to verify the pathogenic association of *P. agglomerans*. Pure cultures of the *P. agglomerans* isolates were re-isolated from previously infected lemon tissues and cultured on nutrient agar medium under aseptic conditions. Fresh bacterial suspensions were prepared from 48 hr old cultures and adjusted to approximately 1×10^9 CFU ml⁻¹ using sterile distilled water. Healthy six-month-old lemon plants maintained under insect proof conditions were inoculated through the injection method using sterile syringes, while control plants received sterile distilled water only. The inoculated plants were maintained under controlled conditions and observed regularly for symptom development for three weeks. Bacteria were subsequently re-isolated from symptomatic tissues and compared with the original isolates based on colony morphology, Gram reaction and molecular characteristics to confirm their association with the disease.

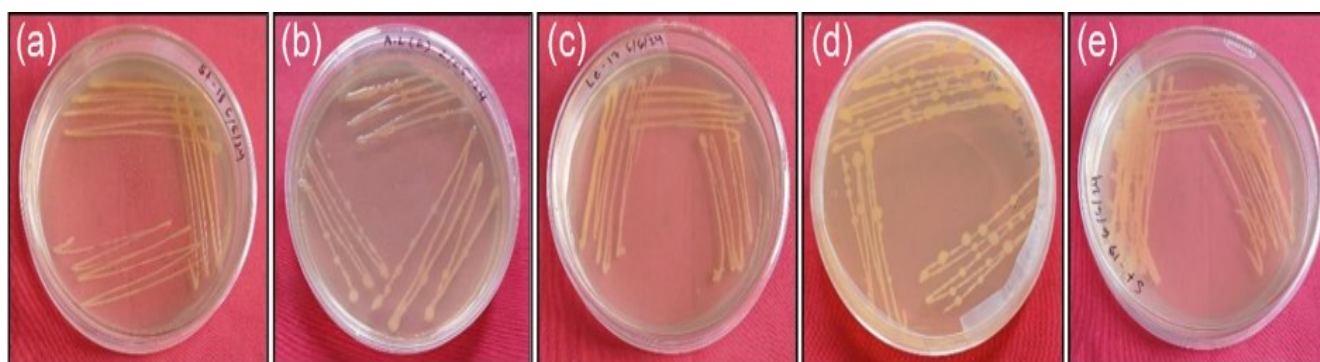
Statistical analysis

Data regarding virulence study was normalised using angular transformation. Duncan's new multiple range test (DNMRT) was performed to compare differences in the means of various observational units at $\alpha = 0.05$ level. Statistical analyses were performed using R statistical software, version 4.4.1 (21).

Results

Isolation and identification of the test pathogen

Standard isolation method was followed for isolation of bacterial pathogens. The pure culture of each pathogen was observed and identified based on their colony characteristics. Bacterial streaks typically appeared creamy or light yellow (Fig. 1). This distinctive yellow pigment is a key identifier for the *Xanthomonas* genus. They were generally described as mucoid or slimy. Further, all the virulent bacterial isolates showed pink to red coloration under Gram staining, indicating that they were Gram-negative in nature. Similarly, in the KOH solubility test, all isolates produced a distinct viscous thread within a few seconds after mixing with 3 % KOH

**Fig. 1.** Cultures of five virulent isolates (a) Isolate 1; (b) Isolate 2; (c) Isolate 3; (d) Isolate 4; (e) Isolate 5.

solution, confirming positive KOH reactions. The results of both tests collectively supported the Gram-negative nature of the isolates, which is characteristic of *Xanthomonas* spp. and associated bacterial pathogens.

Pathogenicity test

Individual lemon plants were inoculated with twenty isolates through injection inoculation technique. Then the plants were kept under observation for three weeks. Visual symptoms were recorded and examined. Koch's postulate was also performed (Fig. 2). The results and observations are mentioned below (Table 1). In the present investigation, out of twenty isolates, isolates 1–10 yielded positive results, whereas isolates 11–20 were negative in pathogenicity test (Table 1). Therefore, the experiment was proceeded with from isolate 1 to isolate 10 for further studies.

Evaluation of virulent isolates

It was observed that after inoculation, treatment no. 4 (T4) showed the maximum disease severity index of 39.43 % in first year and 40.05 % in second year, followed by treatment no. 1, 2, 3 and 5 (T1, T2, T3, T5) and they showed no significant difference among them in both years. The rest of the treatments (T6 to T10 and TC) were statistically at par with each other in both years (Table 5). Regarding the percentage of disease increase, similar trends were observed in both the years. Treatment 4 showed significantly higher disease increase of 64.57 % and 67.79 % in first and second years, respectively. Treatment 1, 2, 3 and 5 were found to be other virulent isolates in both the years from the observation of disease increase percentage (Table 5). Therefore, isolate 1 to 5 were found to be virulent and isolate 4 was discovered to be the most pathogenic of all. It was discovered that the remaining isolates were considerably less pathogenic (Fig. 3 and 4). So, further studies proceeded with these five virulent isolates.

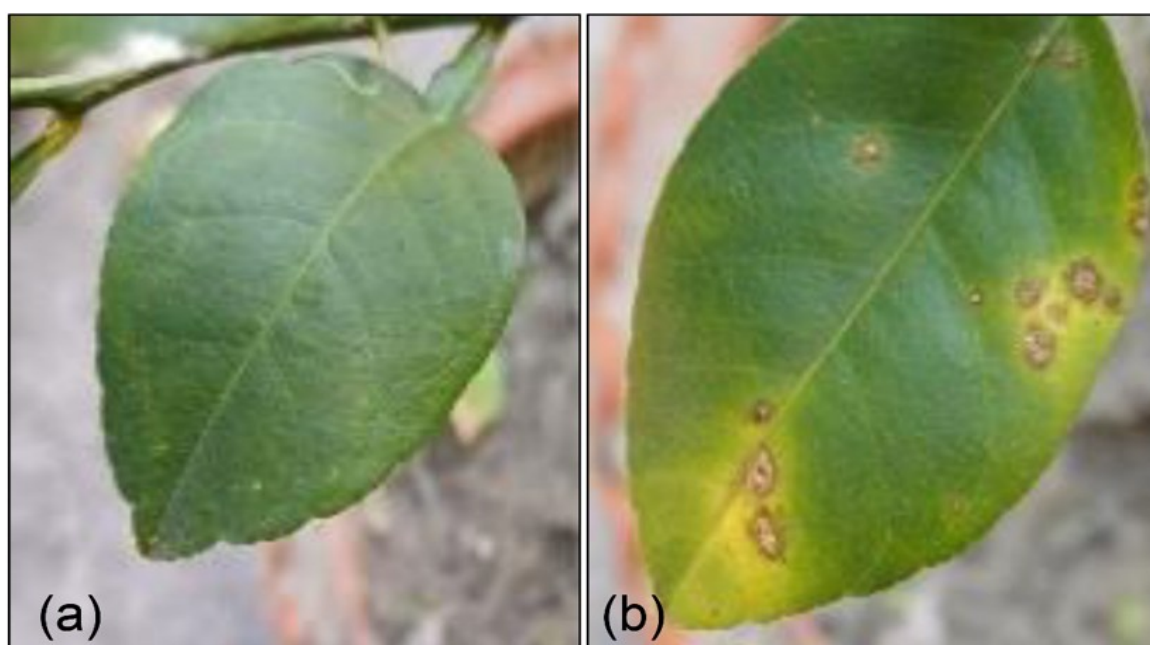


Fig. 2. Development of citrus canker symptoms on lemon leaves following artificial inoculation: (a) healthy leaf before inoculation; (b) same leaf showing characteristic canker lesions after inoculation

Table 5. Evaluation of virulence among the isolates

Treatments	Disease severity index after inoculation (1 st year) (%)	Disease severity index after inoculation (2 nd year) (%)	Disease increase percentage after inoculation (1 st year) (%)	Disease increase percentage after inoculation (2 nd year) (%)
T1	33.35 (35.27) ^b	29.68 (33.00) ^b	66.68 (54.75) ^b	48.17 (43.95) ^b
T2	33.10 (35.12) ^b	29.51 (32.90) ^b	38.92 (38.59) ^d	22.02 (27.98) ^d
T3	32.00 (34.45) ^b	28.71 (32.39) ^b	49.93 (44.96) ^c	34.30 (35.82) ^c
T4	40.45 (39.43) ^a	41.41 (40.05) ^a	81.39 (64.57) ^a	85.58 (67.79) ^a
T5	31.89 (34.38) ^b	28.33 (32.14) ^b	64.73 (53.57) ^b	24.03 (29.31) ^d
T6	23.71 (29.06) ^c	23.39 (28.92) ^c	6.36 (14.28) ^e	4.76 (12.58) ^e
T7	23.71 (29.06) ^c	21.68 (27.73) ^c	6.36 (14.28) ^e	6.19 (14.35) ^e
T8	23.48 (28.98) ^c	23.74 (29.10) ^c	5.27 (13.12) ^e	6.77 (15.06) ^e
T9	23.45 (28.90) ^c	21.66 (27.72) ^c	5.31 (13.12) ^e	6.09 (14.23) ^e
T10	24.75 (29.83) ^c	21.50 (27.61) ^c	3.80 (11.23) ^e	5.41 (13.34) ^e
TC	23.68 (29.06) ^c	22.52 (28.32) ^c	6.36 (14.45) ^e	5.60 (13.65) ^e
SE(m) (±)	1.158	0.851	1.508	1.072
CV (%)	6.239	4.77	8.527	7.093
CD (0.05)	3.396	2.496	4.422	3.146

CV (%): coefficient of variance, SE(m) (±): standard error of mean, CD (0.05): critical difference at 5 % level of significance; TC: treatment control. Superscript letters (Duncan letters) are used to indicate significant differences among the observations. Angular transformation was followed by disease severity index after inoculation and disease increase percentage after inoculation.

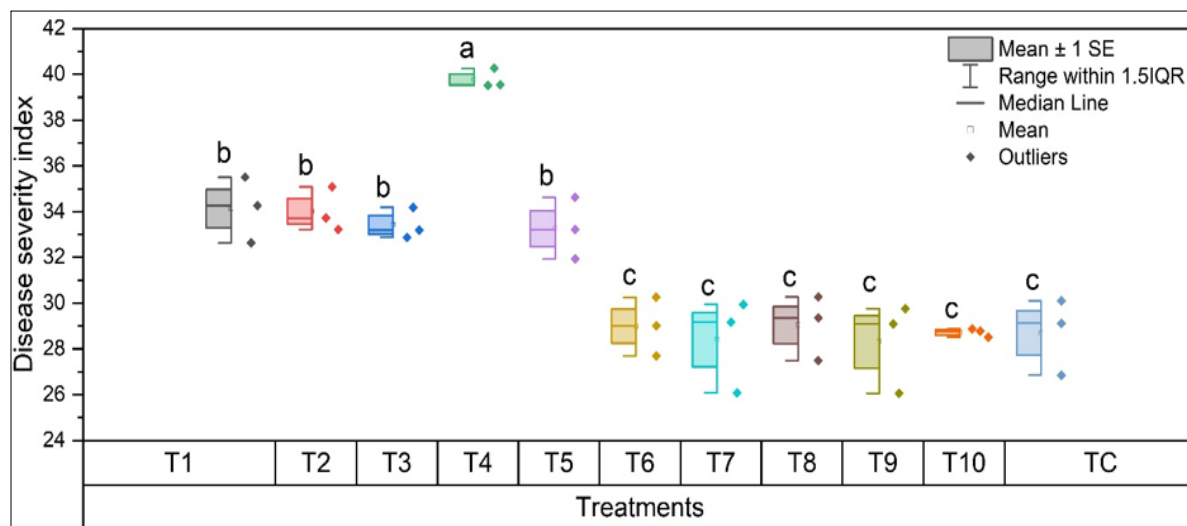


Fig. 3. Disease severity index after inoculation for evaluation of virulent isolates (Pooled).

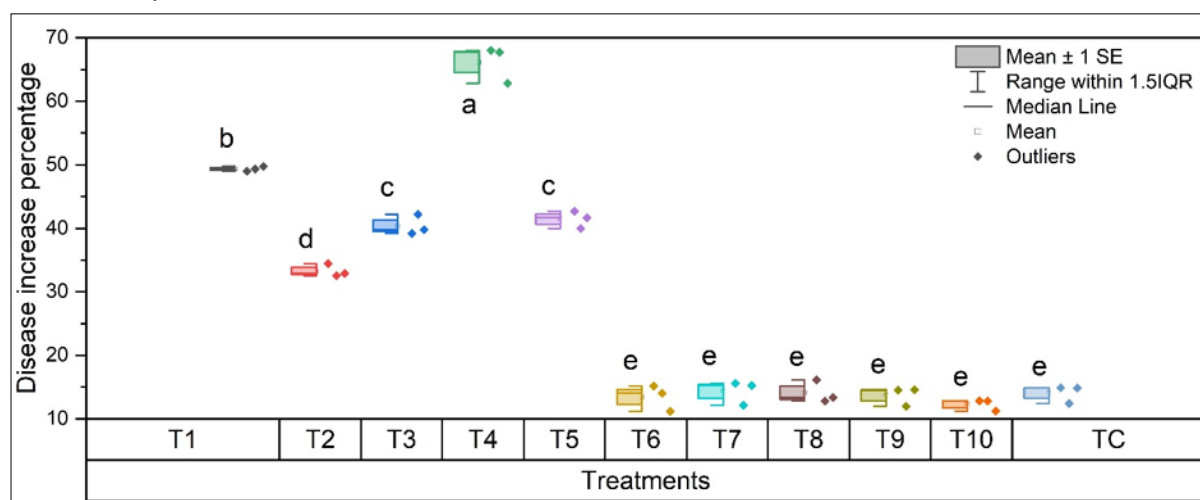


Fig. 4. Disease increase percentage after inoculation for evaluation of virulent isolates (Pooled).

Molecular characterisation of the virulent isolates

Amplification of the 16S rRNA gene was carried out using PCR with bacterial-specific primers, 27F (AGAGTTTGATCMTGGCTCAG) as the forward primer and 1492R (TACGGYTACCTTGTTACGACTT) as the reverse primer. The resulting products of PCR were resolved on agarose gel (1 %) and the fragments of DNA were visualised under a UV transilluminator (Fig. 5). Amplification with this primer set yielded an approximately 1500 bp fragment, confirming the presence of the bacterial 16S rRNA gene in the tested isolates.

After analysis of our sequences through NCBI it was found that the three out of five virulent isolates were *Xac* and two out of them were *P. agglomerans* (Table 6). While it is established that citrus canker disease is caused by *Xac*, but two out of five isolates were found to be *P. agglomerans*. Therefore, for confirmation, the specific primer run was carried out. The *hrpW* gene was amplified through PCR reaction using specific primer pair, XACF (5'-CGTCGCAATACGATTGGAAC-3') and XACR

(5'-CGGAGGCATTGTCTGAAGGAA-3') as forward and reverse primers and the PCR amplicons were resolved on agarose gel (1 %) and the DNA fragments were visualised using a UV transilluminator. Amplification with the *hrpW*-specific primer set produced a 561 bp fragment in the bacterial isolates; however, no amplification was observed in isolates 2 and 5, as clearly depicted in Fig. 6. Based on these results, it can be inferred that *P. agglomerans* was also associated with citrus canker disease.

Further, the phylogenetic analysis based on 16S rRNA gene sequences grouped the virulent isolates into two distinct clusters corresponding to *Xac* and *P. agglomerans*. Isolates UBKVXAC1, UBKVXAC3 and UBKVXAC4 showed close phylogenetic relationship with reference *Xanthomonas citri* strains from GenBank, whereas isolates UBKVPA2 and UBKVPA5 clustered with reported *P. agglomerans* strains, confirming their molecular identity (Fig. 7).

Table 6. Characterisation of the virulent isolates

Virulent isolates	Host	Similarity	Sequenced product length	NCBI accession number
I1 (UBKVXAC1)	Assam Lemon	<i>Xanthomonas axonopodis</i> pv. <i>citri</i> (99.60 %)	1277 bp	PX488750
I2 (UBKVPA2)	Acid Lime	<i>Pantoea agglomerans</i> (100 %)	1286 bp	PV565697
I3 (UBKVXAC3)	Acid Lime	<i>Xanthomonas axonopodis</i> pv. <i>citri</i> (99.86 %)	1288 bp	PX550601
I4 (UBKVXAC4)	Acid Lime	<i>Xanthomonas axonopodis</i> pv. <i>citri</i> (98.73 %)	1275 bp	PX550600
I5 (UBKVPA5)	Acid Lime	<i>Pantoea agglomerans</i> (98.58 %)	1251 bp	PV565700

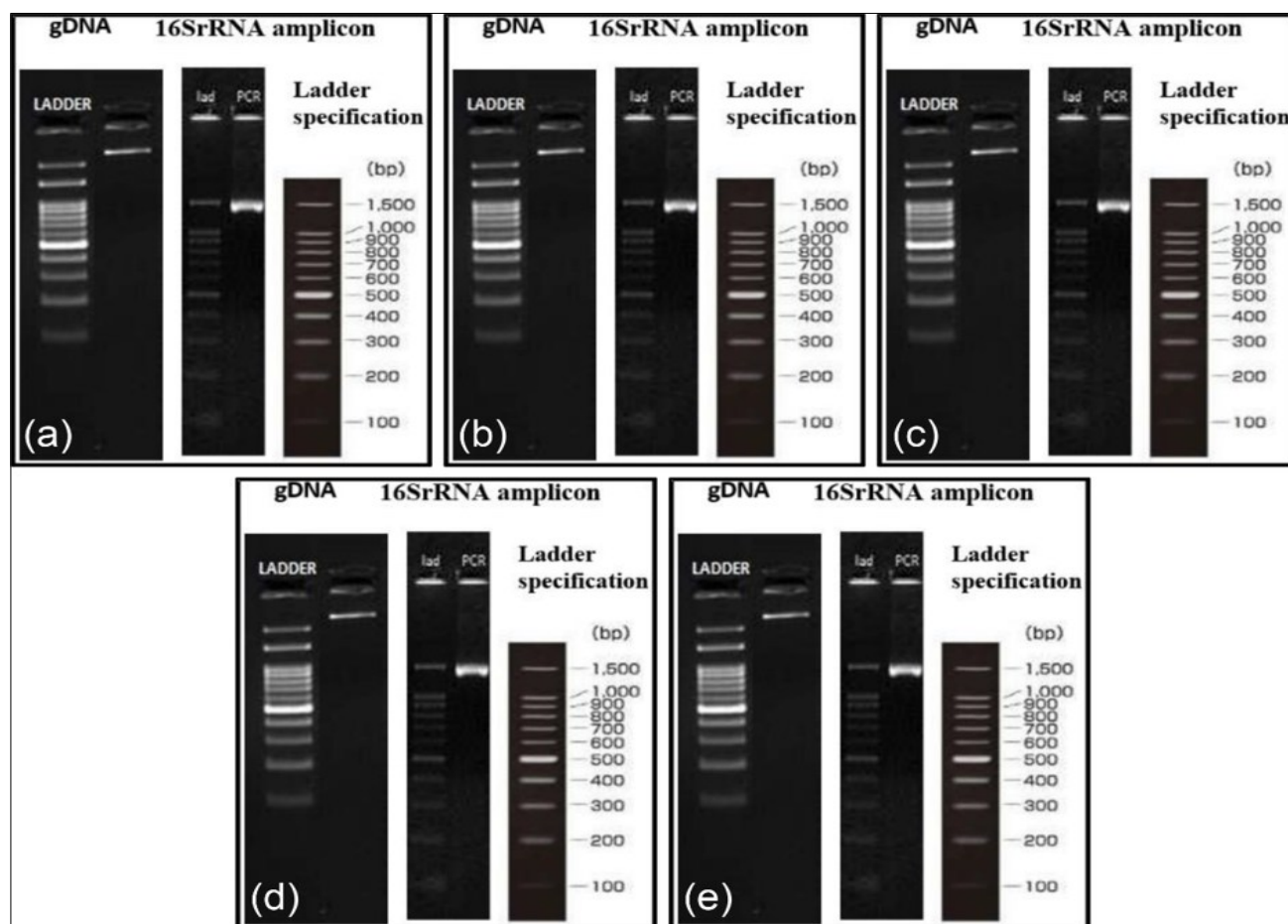


Fig. 5. Agarose gel electrophoresis showing amplification of the 16S rRNA gene (~1500 bp) from five virulent citrus canker-associated bacterial isolates using universal primers 27F and 1492R. Lane M: 1 kb DNA ladder; Lane a: Isolate 1 (*Xanthomonas axonopodis* pv. *citri*); Lane b: Isolate 2 (*Pantoea agglomerans*); Lane c: Isolate 3 (*Xanthomonas axonopodis* pv. *citri*); Lane d: Isolate 4 (*Xanthomonas axonopodis* pv. *citri*); Lane e: Isolate 5 (*Pantoea agglomerans*). Amplification of the expected ~1500 bp fragment confirmed successful amplification of the bacterial 16S rRNA gene in all isolates.

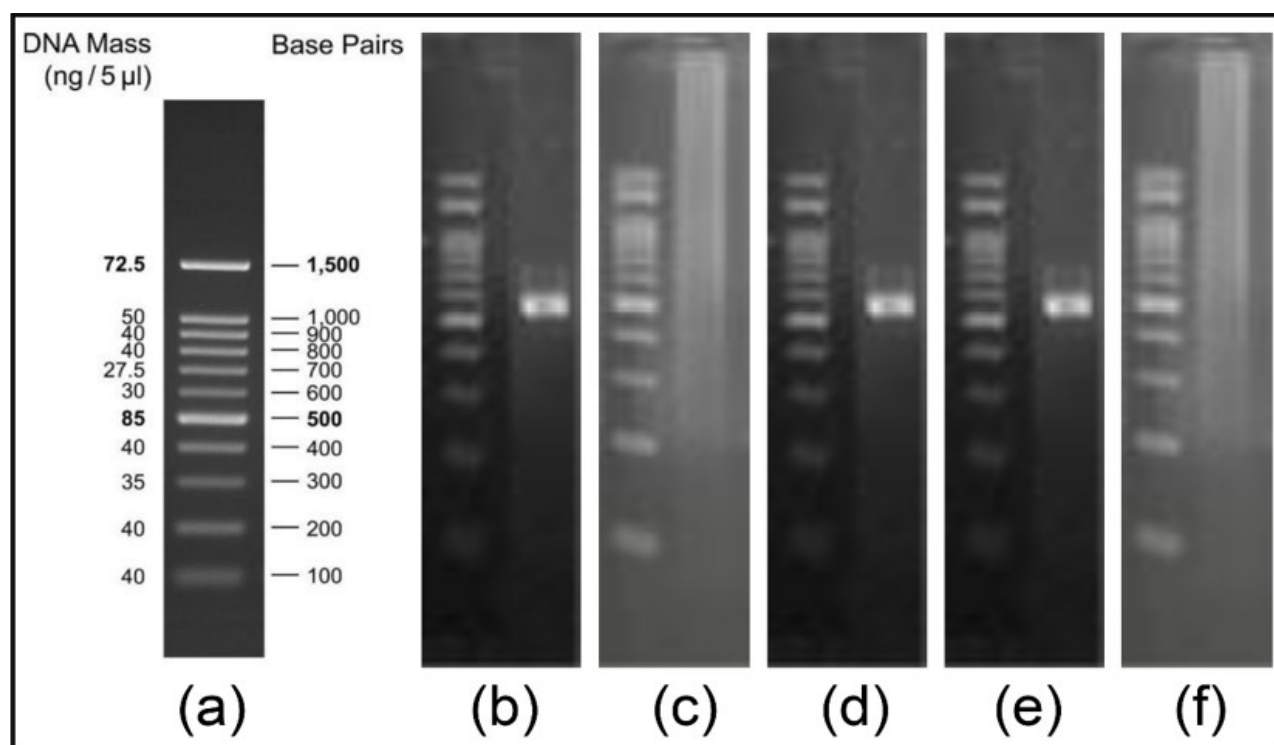


Fig. 6. Agarose gel electrophoresis showing amplification of the *hrpW* gene (~561 bp) using Xac-specific primers (XACF/XACR) in virulent citrus canker-associated bacterial isolates. Lane a: 100 bp DNA ladder; Lane b: Isolate 1 (*Xanthomonas axonopodis* pv. *citri*); Lane c: Isolate 2 (*Pantoea agglomerans*); Lane d: Isolate 3 (*Xanthomonas axonopodis* pv. *citri*); Lane e: Isolate 4 (*Xanthomonas axonopodis* pv. *citri*); Lane f: Isolate 5 (*Pantoea agglomerans*). The expected 561 bp amplification product was observed only in the *Xanthomonas axonopodis* pv. *citri* isolates (Lanes b, d and e), whereas no amplification was detected in the *Pantoea agglomerans* isolates (Lanes c and f), confirming the specificity of the primer set for Xac isolates.

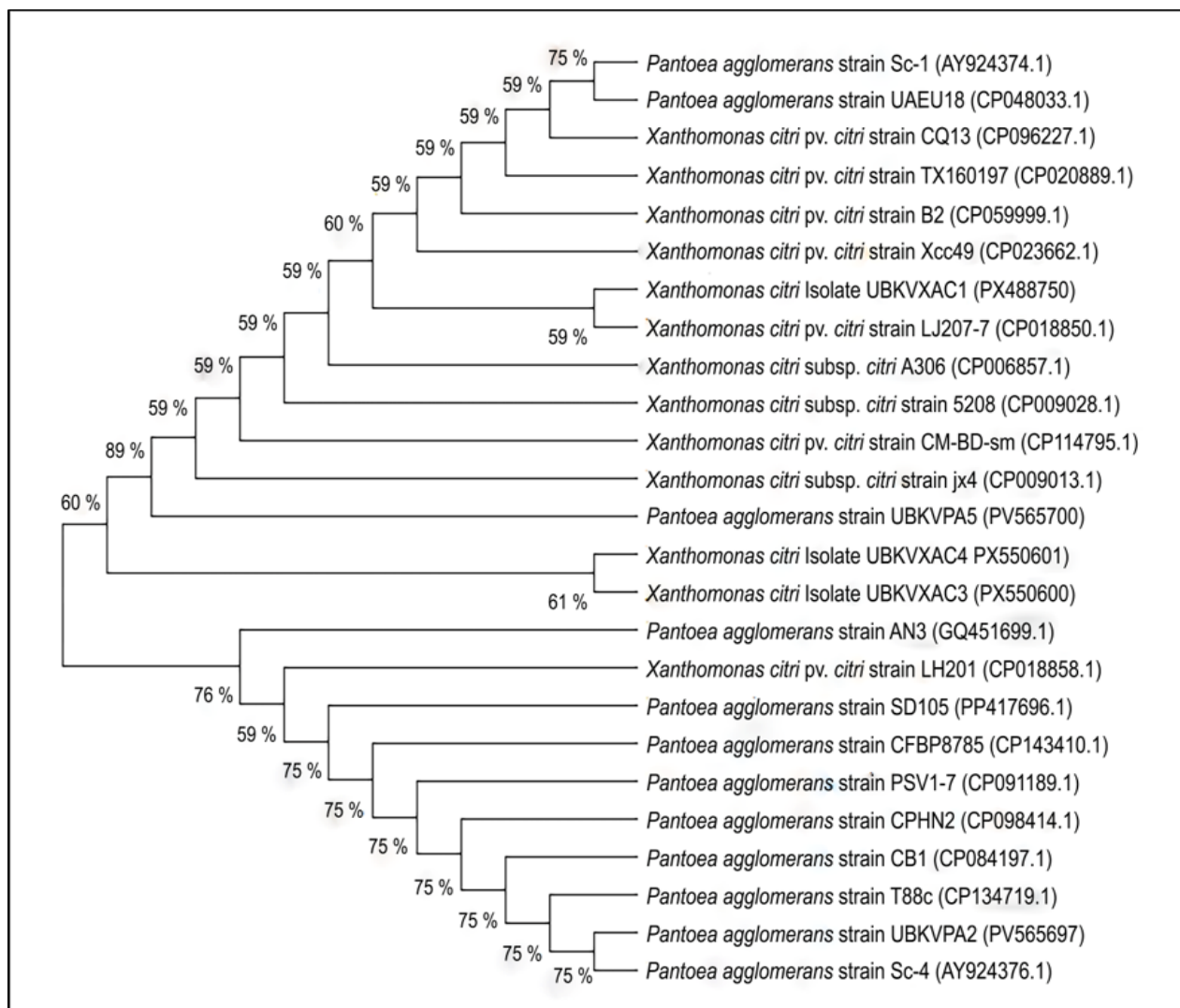


Fig. 7. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences. This analysis involved 25 nucleotide sequences. Evolutionary analyses were conducted in MEGA11.

Pathogenic association of *Pantoea agglomerans*

The *P. agglomerans* isolates produced mild canker-like symptoms on inoculated lemon leaves under greenhouse conditions after inoculation. Re-isolation from symptomatic tissues yielded bacterial colonies with morphological characteristics like the original isolates. The re-isolated bacteria also exhibited Gram-negative reaction and showed high sequence similarity with *P. agglomerans* during molecular analysis. Although these observations indicated the consistent association of *P. agglomerans* with symptomatic citrus tissues, the symptoms produced were comparatively less severe than those caused by Xac. Therefore, the findings suggest that *P. agglomerans* may act as an associated or opportunistic bacterium within citrus canker lesions and additional studies on virulence determinants are required to establish its definitive pathogenic role in citrus canker development.

Discussion

Identification of the pathogen morphologically is the preliminary approach for further studies. Creamy yellow or light yellow streaking lines were observed, which is due to the production of xanthan exopolysaccharide (EPS), which is an important virulence factor. Preliminary identification of the isolates was further supported

through conventional biochemical characterisation. The bacterial isolates were found to be Gram-negative under Gram staining observation. All virulent isolates showed positive reactions in KOH test also. These morphological and biochemical characteristics collectively supported the preliminary identification of the isolates as citrus canker-associated bacteria prior to molecular characterisation. Similar findings were reported by previous researchers (22, 23).

The pathogenicity test demonstrated considerable variation among the citrus canker-associated bacterial isolates in their ability to induce disease symptoms under local conditions. The isolates that failed to produce symptoms may have lost pathogenic fitness or may possess lower adaptability to the prevailing environmental conditions and host tissues. Similar variability in aggressiveness among citrus-associated *Xanthomonas* isolates has been reported previously. Previous researchers observed differences in aggressiveness among isolates of *Xanthomonas campestris* pv. *citrumelo* associated with citrus bacterial spot in Florida nurseries (24). Although the pathogen studied by those authors belongs to a different pathovar and disease system than Xac causing citrus canker in the present study, the findings nevertheless support the broader concept that pathogenic variability and host adaptation are common characteristics within citrus-associated *Xanthomonas* populations.

The virulence study further revealed substantial differences in disease severity and disease increase percentage among the isolates, indicating variability in their pathogenic potential. Among all the isolates, isolate 4 consistently exhibited the highest disease severity and disease progression during both years of investigation, suggesting superior adaptation to the host and local agro-climatic conditions. The enhanced virulence of I4 may be associated with increased production of extracellular polysaccharides, greater bacterial multiplication within host tissues, or more efficient secretion of virulence-associated proteins involved in host colonisation and lesion development. Environmental conditions prevailing in the humid Terai region of West Bengal may also favor the expression of pathogenicity-related genes in highly aggressive isolates. Unexpectedly, the uninoculated control treatment exhibited low to moderate disease severity values during both years of the experiment. This observation may be attributed to latent or naturally occurring background infection present under the humid agro-climatic conditions of the Terai region of West Bengal, where citrus canker is highly prevalent throughout the year. Despite maintaining insect proof conditions and adopting aseptic precautions, incidental contamination through aerosols, irrigation splashes, handling operations, or pre-existing epiphytic bacterial populations on plant surfaces cannot be completely ruled out. Similar background infection in control plants under high natural disease pressure has occasionally been reported in citrus pathology studies conducted in disease-endemic regions.

However, although the control plants exhibited measurable disease severity, the disease progression and severity recorded in the inoculated treatment, particularly in I4, were significantly higher than the control, indicating the distinct pathogenic potential of the tested isolates. Therefore, the relative differences among treatments remain biologically meaningful for evaluating virulence. The comparatively lower disease increase percentage observed in T1 during the second year may be attributed to environmental variation, differential host response, or fluctuations in the physiological fitness of the bacterial isolate between experimental seasons. In contrast, T4 consistently exhibited high virulence across both years, indicating greater stability in pathogenic aggressiveness and better adaptation to the prevailing environmental conditions.

The higher virulence exhibited by I4 may be attributed to its identity as Xac, the established causal agent of citrus canker, possessing specialised virulence factors such as the type III secretion system, extracellular polysaccharide production and efficient host tissue colonisation ability. In contrast, isolates 2 and 5 identified as *P. agglomerans* produced comparatively moderate symptoms, suggesting a weaker or opportunistic association with diseased tissues rather than strong pathogenic specialisation.

Previous researchers tested the pathogenicity and virulence in their study on management of citrus canker bacterium by using botanicals and chemicals in the same way (25). They also found virulence difference among the isolates. Same type of work was also performed by previous researchers (26). They did a comparative study of the strains Xac isolated from citrus canker in Japan and cancris B in Argentina. They found that citrus canker A organism was more virulent than citrus canker B organism, as it was found in the experiment, highlighting the heterogeneity within the pathogen population.

Molecular characterisation is a crucial approach for identifying DNA-level variations and evaluating genetic diversity

among different strains. Molecular characterisation was performed in our present investigation to confirm the identification of the isolates. Molecular phylogenetic and evolutionary relationship analyses were reported by earlier researchers who characterised citrus canker-causing strains using 16S rRNA gene sequencing with universal bacterial primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTGTACGACTT-3') (27). Whereas, in phylogenetic tree the clustering of the isolates into separate *Xanthomonas* and *Pantoea* groups indicates considerable bacterial diversity within citrus canker-associated lesions in North Bengal. The close association of the present isolates with previously reported reference strains further supports the reliability of the molecular identification and suggests possible evolutionary relatedness with globally distributed citrus-associated bacterial populations.

After analysis it was found that three out of five virulent isolates were Xac and two out of them were *P. agglomerans*. These findings suggest that *P. agglomerans* may coexist within canker lesions and potentially influence disease dynamics. Therefore, for confirmation the specific primer run was carried out and it was confirmed. Further, a *Pantoea* specific virulence assay was performed and from the re-confirmation of pathogenicity and successful re-isolation of *P. agglomerans* from symptomatic tissues suggest its consistent association with citrus canker-affected lemon plants under local conditions. Morphologically, symptoms produced by the *Pantoea* isolates differed slightly from the typical citrus canker lesions caused by *Xanthomonas*. The lesions induced by I4 were more prominent, raised, corky and surrounded by distinct chlorotic halos, which are characteristic symptoms of citrus canker. Conversely, lesions associated with I2 and I5 were comparatively smaller, less corky and showed reduced halo development, indicating lower aggressiveness and possible secondary colonisation behaviour. Similar findings were reported in previous studies, which demonstrated that *Pantoea* spp. possess the ability to acquire pathogenic traits and behave as opportunistic plant-associated bacteria under favourable environmental conditions (10). The detection and re-isolation of *P. agglomerans* from citrus canker lesions suggest its close association with diseased citrus tissues rather than confirming a direct causative role in citrus canker development. The bacterium may behave as an opportunistic or commensal organism that colonizes pre-existing lesions created by Xac, particularly under the warm and humid environmental conditions prevailing in North Bengal.

Association of *P. agglomerans* with citrus canker lesions has also been reported in earlier investigations, suggesting that the bacterium may occur as a secondary coloniser or resident microbial associate within diseased citrus tissues. Previous researchers reported interactions between *X. citri* and *Erwinia herbicola* (currently classified under *Pantoea* spp.) in citrus tissues, indicating possible ecological association between these bacteria (11). Similarly, another studies states that *Pantoea* species can survive epiphytically and opportunistically colonise plant lesions under favorable environmental conditions (10). These findings support the present observation of *P. agglomerans* association with citrus canker lesions in North Bengal.

Numerous investigations have examined the pathogenic behaviour of *Pantoea* species across a wide range of plant hosts and have identified several factors that may contribute to disease development. In many reports, *Pantoea* has been found in association with well-known plant pathogenic bacteria, including

Pseudomonas savastanoi (28). In citrus ecosystems, *Pantoea* is frequently described as the most abundant and dominant saprophytic bacterium inhabiting citrus canker lesions. Previous studies have shown that interactions between *Xanthomonas* and *Pantoea* can influence disease progression, with *Pantoea* reducing the population density of *Xanthomonas* and thereby modulating canker development under certain conditions (29). At the same time, its frequent occurrence alongside pathogenic bacteria raises the possibility of its involvement in disease development.

A plausible explanation for the emergence of pathogenic traits in *Pantoea* lies in horizontal gene transfer, a process through which bacteria acquire genetic material from other organisms. This mechanism enables the acquisition of virulence-associated genes from established pathogens, potentially converting non-pathogenic or saprophytic bacteria into opportunistic pathogens. Environmental factors are known to influence the efficiency of such gene transfer events, thereby contributing to the evolution and diversification of bacterial pathogenicity (12, 29). Collectively, reports on *Pantoea* pathogenicity across diverse plant systems support the notion that this bacterium can obtain virulence-related genes from established plant pathogens, enabling it to express pathogenic traits.

Conclusion

Citrus canker remains a serious bacterial disease affecting lemon cultivation under the agro-climatic conditions of northern West Bengal, causing considerable damage to plant health and productivity. The present study aimed to molecularly characterize citrus canker-associated bacterial isolates and evaluate their virulence under local conditions. Molecular analysis of the virulent isolates identified three isolates as *Xanthomonas axonopodis* pv. *citri* (Xac), the established causal agent of citrus canker, while two isolates were identified as *Pantoea agglomerans*. The detection and re-isolation of *P. agglomerans* from symptomatic citrus tissues indicate its close association with citrus canker lesions in this region. To the best of our knowledge, this represents the association of *P. agglomerans* with citrus canker-affected lemon plants from the northern region of West Bengal. However, further investigations involving detailed virulence characterisation and pathogenicity studies are required to establish its precise role in disease development. Overall, the findings of this study improve our understanding of the bacterial diversity associated with citrus canker and may contribute to the development of more effective disease management strategies in citrus-growing regions.

Further, future research should focus on detailed pathogenicity confirmation and virulence characterisation of *P. agglomerans* to determine its precise role in citrus canker disease development. Comparative genomic studies, characterisation of virulence-associated genes and investigation of microbial interactions between *P. agglomerans* and Xac under field conditions may provide deeper insight into the ecology and epidemiology of citrus canker-associated bacterial communities. Additionally, large-scale surveys across different citrus-growing regions and evaluation of host resistance against highly virulent isolates would further strengthen disease management strategies.

Acknowledgements

The authors are grateful to the Department of Science and Technology, Government of India, for providing financial support through the INSPIRE Fellowship. They also sincerely thank the Department of Plant Pathology for providing the necessary facilities for the successful completion of this research. The authors further express their gratitude to Uttar Banga Krishi Viswavidyalaya for providing access to laboratory and field facilities.

Authors' contributions

RS performed the experiment and wrote the manuscript. SK designed and supervised the experiment. PMB supervised the experiment and checked the manuscript. SB provided the materials required. NB provided the materials required and supervised the collection method. MKD performed the statistical analysis. PD provided the materials required. SS edited the manuscript. KB provided the materials required and edited the manuscript. SM edited the manuscript. All authors have read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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