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16S-rRNA BASED MOLECULAR CHARACTERIZATION OF PLANT PATHOGENIC BACTERIAL SPECIES ASSOCIATED WITH CITRUS IN KHANPUR, PAKISTAN

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Abstract

The percentage of losses attributable to citrus disease brought on by bacterial infections has increased worldwide. However, the mature citrus trees in Pakistan exhibit diseases before and after harvesting. However, the exact disease agents have not yet been discovered. The morphological and molecular characterization of the bacterial pathogens in the current study was complex, as was the evaluation of citrus disease's prevalence, incidence, and severity. The bacterial pathogens were isolated from the infected samples obtained by tissue culture and streaking procedures in the first phase of the study during December. They were then identified based on their colony characteristics. Second, the isolated strains were characterized using the primers 27F (5-CTTCAACTCAAACGCCGGA-3) and 1492R (3-CATCGGCTGTTCGGGAG-5), which were used for the identification of the 16S rDNA region. Thirdly, fresh leaves of two citrus varieties (Succri and Moro blood) were used to investigate the pathogenicity of isolated bacterial strains. The pathotypes of *Xanthomonas* sp. caused the most severe symptoms. This is the first ever study on bacterial pathogens of citrus in Khanpur. This study shows the molecular-based identification of bacterial pathogens causing citrus diseases, which helps to provide recommendations on genetic testing and develop disease management programs, as citrus fruits significantly contribute to household income and foreign exchange in Pakistan.

Key words: Citrus fruits; Canker; Xanthomonas pathotypes; Yield loss; Epidemiology; Genetic diversity

Taxonomy: Molecular Biology, Gene Analysis, Agriculture

Introduction

Citrus is among the woody, evergreen, perennial shrub that is grown for its unusual and non-climacteric fruit (Lee & kadar 2002). Worldwide fruit production reached over six million tons in 2006, with the majority being produced in the sub-tropical regions of Africa (58%), Asia (28%), and the Americas (13%), where there are seasonal variations but little to no cold weather. In tropical areas, small-scale and backyard residences are increasingly prevalent.

Commercial citrus species are members of the genus Citrus, family Rutaceae, and order Geraniales. Mandarins (*Citrus reticulata*) and sweet oranges (*Citrus sinensis*) are two species that are widely farmed for their fruit (Wu *et al*., 2018). Citrus, like most fruits, has few calories, is a rich basis of fiber or carbs, is low in fats and salts, and is a great source of vitamin C. The fruit is also valued for its physiologically active phytochemicals, which are antioxidant nutrients (Huang *et al*., 2007). Citrus phytochemicals may be able to fight cancer, decrease cholesterol, and act as antioxidants (Tian *et al*., 2001). The citrus crop is produced in large quantities and sold as fresh as well as processed fruit byproducts. All fruit portions can be eaten; this is a characteristic of citrus fruits only. The inner mesocarp rind and the outside exocarp are used to extract flavorings and pectin, respectively. The peel is used to extract essential oils for usage in the pharmaceutical and cosmetic industries. Citrus pulp pellets that are made from the pulp and peel after the juice and oils have been extracted are used as animal feed. Products made from seeds include seed oils, dried seed-pressed cakes, and seed meals (Bampidis & Robinson, 2006; Choi, 2006; Bermejo & Cano, 2012).

The accomplishment of long-term disease and pest management measures is essential for citrus output. Citrus is among the few fruit crops susceptible to a variety of deadly diseases that are constantly evolving and can drastically reduce or eliminate citrus output. Citrus hosts a variety of viruses and pests, like many tropical and subtropical crops (Narciso *et al*., 2012). Citrus fruit production for the processed market might not require as strict pest control as citrus fruit production for the fresh fruit market. But maintaining profitable citrus plantations devoted to processing or fresh-fruit markets as well as developing a certification system needs a thorough grasp of the pathogen's biology (Berk, 2016).

Even though Khanpur, Pakistan, the site of our research, is a substantial producer of citrus, which is not only a nutritious fruit but also an economically valuable asset for the country, it is deteriorating due to pathogen invasions and diseases, these diseases have not yet been researched and reported there. An effort has been made in the current study, to summarize the current state of knowledge regarding the primary bacterial pathogens restricting citrus production, the diseases they cause, their diagnosis through the use of molecular markers, toxicity mechanisms, genetic diversity, and the factors influencing the emergence of the causal agents of these diseases. The interactions between pathogens and plants was also discussed. By investigating the epigenetics of citrus diseases in Pakistan, the findings of this study will assist future researchers in locating transgenic lines resistant to HLB and citrus canker. This will also benefit future researchers who are eager to control diseases in an environmentally friendly manner.

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Material and Methods

Study area and sample collection: Citrus orchards situated in the Khanpur, Haripur district Pakistan were chosen for this investigation (Fig. 1). Surveys were conducted in December 2019 and December 2020 between 50 randomly chosen Khanpur orchards, and 15 to 20 samples of leaves and fruits with visible disease symptoms were taken from each of the orchards. To evaluate bacterial leaf and fruit diseases using the criteria developed for disease assessment and subsequent investigation, diseased fruit samples were tagged, brought to the Mycology and Ecotoxicology laboratory, and maintained at laboratory conditions (Akhtar & Alam, 2002; Ahmad, 2018).

Disease Assessment: Depending on the field layout and orchard type for the evaluation of diseases, a random sample strategy was adopted. To record orchard and tree-wise data, a Performa was created and placed in various areas. To assess the prevalence and severity of bacterial infections, surveys were conducted in 50 citrus orchards spread across 50 randomly chosen sites in Khanpur. Blocks of each orchard were randomly selected, and samples were taken from those blocks. Five plants from each block were examined for disease infestation. Several citrus varieties, including Succri, Moro blood, Malta, and Musambi, were investigated. Each orchard's 15–30 plants were carefully examined by visual inspection to detect diseases (Pereira *et al*., 2011). Trees were in the fruiting stage at the time, which made it easier to gather information on diseased fruits. Walking around the circumference of a few chosen trees allowed for a closer look at their trunks, shoots, leaves, and fruits for symptoms of the disease. In addition, leaves and fruits that had peculiar symptoms fell to the ground were seen. All of this data was entered on a main data collecting sheet. To determine differences in incidence and severity, these recorded data were finally analyzed and interpreted. Figure 2 displays a few of the disease signs that have been identified in various orchards.

The following formula was used to determine the disease incidence in each orchard (Fateh *et al*., 2022).

$$\text{Disease incidence \%} = \frac{\text{No. of infected plants}}{\text{Total no. of plants observed}} \times 100$$

The disease severity was assessed using a 0-5 visual rating scale (Kazmi *et al*., 2005), Where 0 represents healthy plants, 1 represents a 1 to 10% decline, 2 represents an 11 to 20% decline, 3 represents a 21 to 30% decline, 4 represents a 31 to 50% decline, and 5 represents a decline of more than 50%.

Percent Disease Index was determined by the formula shown below in each orchard (McKinney, 1923; Verma *et al*., 2022).

$$\text{PDI \%} = \frac{\text{Sum of all numerical ratings}}{\text{Total no. of observations x Maximum disease grade}} \times 100$$

Isolation of bacterial pathogens: The leaf samples were washed first with sterilized distilled water and then sponged with cotton soaked in alcohol under the laminar hood. Then with the aid of a sterile blade infected leaf tissue was removed and 75% ethanol was applied to the removed tissues to surface sterilize them. The tissues were crushed and placed in sterilized Phosphate Buffer Saline (PBS) pH 7.4, test tubes. This suspension was left at room temperature for half an hour. Fruits with lesions were peeled, and a 2 mm portion of the peel was sliced using a sterile scalpel around the lesion. Application of 75% ethanol was used to sterilize these tissues. After that, these sliced tissues were cut into small pieces and immersed in PBS buffer. Then 200 µl of both fruits and leaves PBS suspensions were streaked on Yeast Peptone Glucose Agar (YPGA) media plates (Aritua *et al*., 2006). Different agar plates were streaked with samples from each collection. To prevent contamination, plates were wrapped and tagged with the sample ID. After that, the plates were incubated for 24-48 hours at 28-30°C.

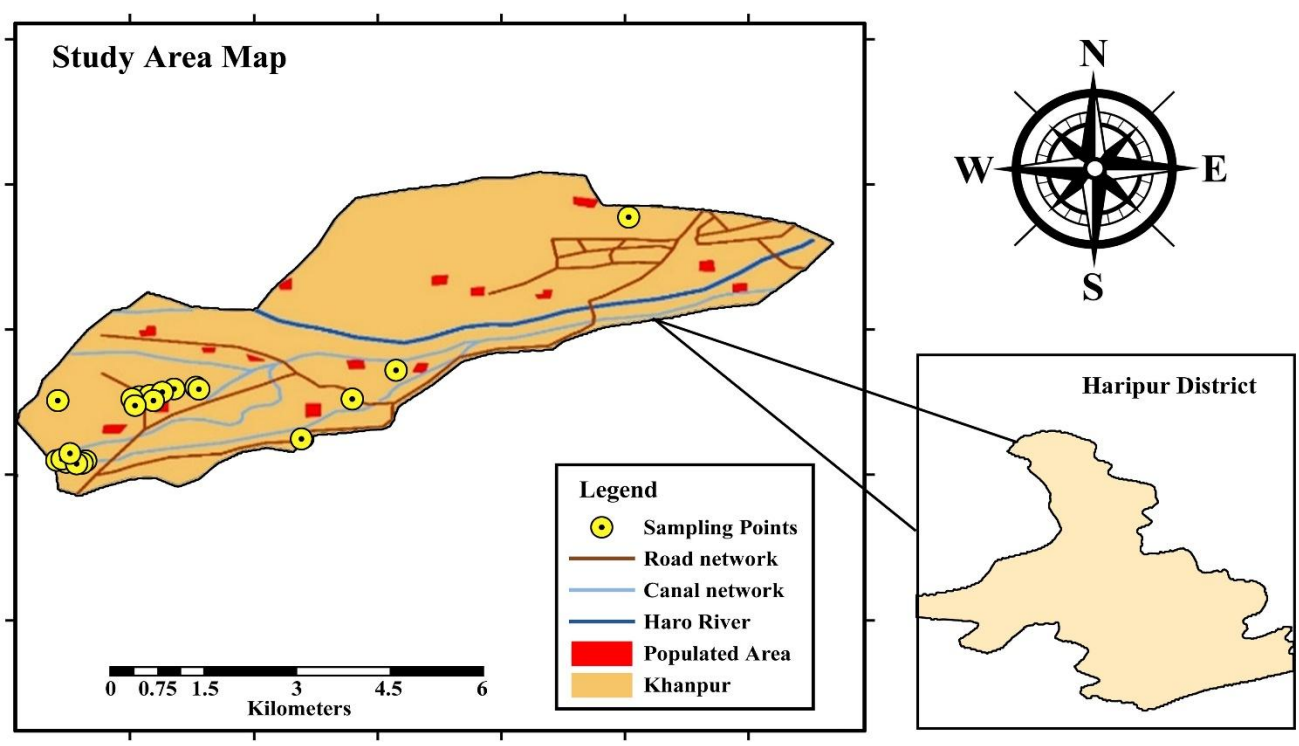

Fig. 1. The geographical coordinates of the sampling sites of Khanpur, Pakistan.

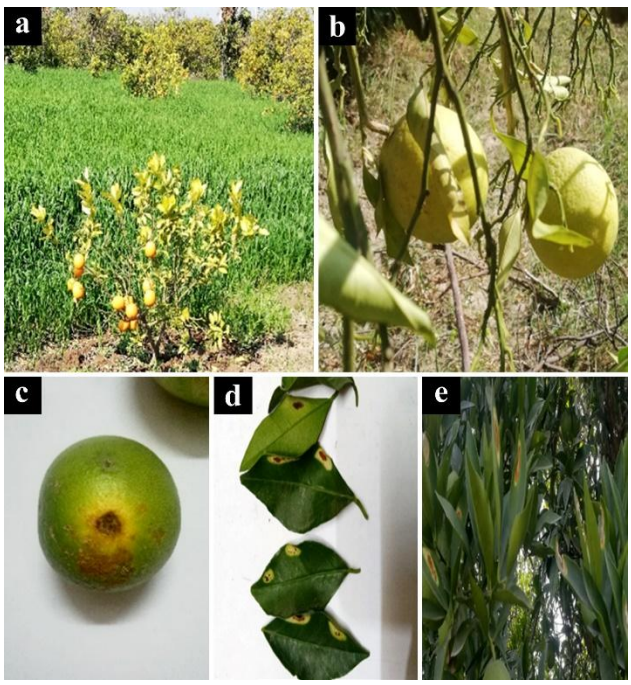

Fig. 2. Different citrus bacterial diseases observed in the orchards: a, b) Citrus Greening, c, d) Citrus canker, & e) Bacterial spot.

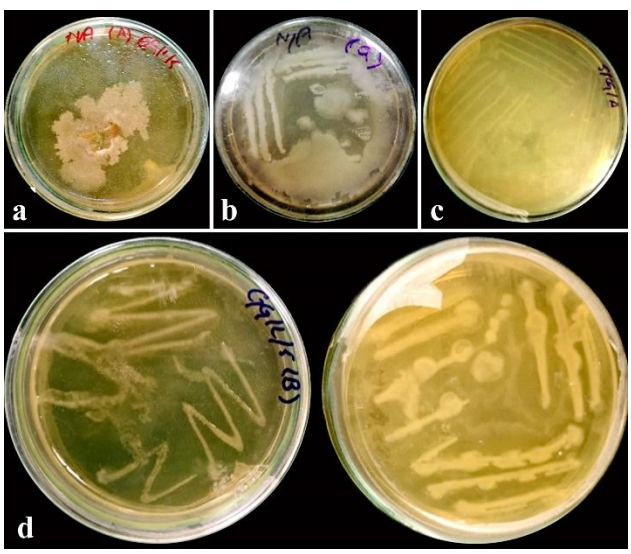

Fig. 3. Colonies of Bacterial pathogens isolated from the diseased fruits and leaf samples (a) Bacterial spot (BS), (b) Citrus Canker (CCF), (c) Citrus Canker (CCL) (d.) Citrus Greening (CGF and CGL).

Molecular identification and characterization of bacterial isolates: After the morphological observation of bacterial isolates on agar plates, the gram staining test was carried out for the screening and differentiating of bacterial isolates. To identify the separated strains, molecular characterization was done utilizing 16S rRNA gene amplification and sequence analysis. Following the protocols of Naseem *et al.*, (2017), from the overnight grown bacterial culture in YPG broth, 1000 µL with an optical density of 0.8 was taken and centrifuged for 10 minutes at 10,000 rpm to extract the bacterial DNA. Then the pellet formed was suspended in 500 µL of CTAB extraction buffer (NaCl 250 mM, Tris-HCl pH 7.5 200 mM, SDS 0.5%, EDTA 25 mM, and 2% PVP), with the supernatant being discarded. After that, the mixture was vortexed and continuously shaken at room temperature for 60 minutes. Then the lysate was centrifuged for five minutes at 8000 rpm. A fresh Eppendorf tube was filled with 450 µL of supernatant and an equivalent volume of isopropanol was added to it and stirred. The mixture was again centrifuged for 10 minutes at 13,000 rpm. The pellet formed was air dried for some time with the supernatant being discarded and was suspended in 50 µL TE Buffer (EDTA 1 mM, Tris-HCl 10 mM). The extracted DNA sample was run on the agarose gel and purified DNA samples were stored at -20ºC for further processing.

PCR amplification: The PCR amplifications and 16S rRNA gene sequencing analysis of 1500 bp fragment were done using the universal primer pair 27F (5-CTTCAACTCAAACGCCGGA-3) and 1492R (3-CATCGGCTGTTCGGGAG-5) (Abu-Obeid *et al*., 2018). In the Labnet MultiGene OptiMax thermal cycler, reactions were conducted. For each 25 µl reaction mixture, reaction buffer, 25 mM $MgCl_2$, dNTPs, forward reverse primers, and Taq polymerase were included (Thermo Scientific). The steps involved are initial denaturation for 5 min at 95°C, which was followed by 30 cycles of final denaturation at 94°C for 2 min, then annealing at 55°C for 2 min, extension for 2 min at 72°C, and a final extension step at 72°C for 7 min (Verma *et al*., 2022). Amplified products were run on 1.2% agarose gels in 1X TBE buffer for 1 hour at a 100 V power source, for identification and visualized in a Gel-Doc Analyzer (InGenius3, Syngene Bio Imaging). A Silica bead gel extraction kit (Thermo Scientific) was used to purify amplified 16S rRNA, and samples were sent to Macrogen Inc. Korea for sequencing analysis.

Molecular and phylogenetic analysis: With the aid of 16S rRNA gene analysis and sequencing, the sequences of isolated bacterial pathogens were obtained. The 16S rRNA gene sequences were constructed and modified using Clustal W Alignment software (Jeon *et al*., 2014), and BLAST (Basic Local Alignment Search tool) analysis was used to compare the genetic similarity of the strains. Following that, the genomes of all bacterial isolates were deposited in Genbank (DDBJ), and accession numbers were acquired for each. The MEGA-11 software was used to create a neighbor-joining phylogenetic tree and assess the evolutionary relationships between the isolated strains (Tamura *et al*., 2013).

Pathogenicity test on citrus leaf: Pathogenicity tests were conducted on fully developed but immature Moro blood and Succri leaves. Leaves were first disinfected in the laminar hood using a 1% sodium hypochlorite solution. Then leaves were arranged on Petri dishes with the discs of filter paper. A sterilized needle punctured each leaf twice, once on the left side and once on the right side of the principal vein on the abaxial side. The right side punctures of each leaf were then infected with 100 µL of the overnight bacterial culture broth. Sterile distilled water was injected into the punctures on the left side as a negative control. To enhance infection, the petri dishes were labeled, put in a polythene bag, and placed in an incubator set at 28 to 30°C for 3 to 4 weeks. The leaves were observed daily to develop the symptoms (Naseem *et al*., 2017).

Table 1. The origin, source, growth, and Gram's Staining of isolated bacterial strains.

Strains ID	Origin	Source	Gram's staining	Growth on YPG
CCL3	Khanpur, KPK	Leaves	-	+++
CCF3	Khanpur, KPK	Fruit	-	++
CCL2	Khanpur, KPK	Leaves	-	++
CCF2	Khanpur, KPK	Fruit	-	+++
CGL3	Khanpur, KPK	Leaves	-	++
CGF3	Khanpur, KPK	Fruit	-	+
CGL5	Khanpur, KPK	Leaves	-	+++
CGF5	Khanpur, KPK	Fruit	-	++
BSL1	Khanpur, KPK	Leaves	-	++
BSF2	Khanpur, KPK	Fruit	-	+

+++ represents active bacterial growth; ++, represents normal growth rate; + represents mild and delayed growth; - represents negative or no growth

Results

Isolation of bacterial pathogens: Citrus plants with diseased leaves and fruits yielded fourteen different bacterial isolates that fit the characteristics of species belonging to the four bacterial genera that are *Xanthomonas, Pseudomonas, Candidatus,* and *Serratia*. These bacterial isolates produced rounded, creamy yellow to off-white, and mucoid colonies (Fig. 3. Table 1) summarizes the origin, source, and morphological screening results of the isolated bacterial strains that were subjected to further analysis.

Disease incidence: The results showed that citrus fruit and leaf diseases are prevailing in all the orchards under study. The result of disease incidence revealed that greening disease had a high incidence in orchard number 8 with a percent disease index (PDI) of 31%, the citrus canker disease had a high incidence in orchard number 3 with a PDI of 18%, and the bacterial spot had a high incidence in orchard number 5 with a PDI of 44%. (Fig. 4) displays the findings of the disease incidence for all orchards.

Molecular characterization: The isolated DNA fragments were run with lambda (λ) DNA standards (25 ng/μ) on the gel to check the concentration of isolated DNA samples. Figure 5 displays the pure DNA bands of the bacterial pathogens isolated from the citrus fruits and leaf diseases.

The amplified products of the 16S rRNA gene having a 1500 bp amplicon were obtained using the primer pair 27F and 1492R, which were custom sequences, and were subjected to BLAST analysis to find the sequence homology. The specific amplification was obtained for the bacterial isolates, including CCF2, CCF3, CCL3, CGF5, CGL5, and BSL1, as shown in (Fig. 6).

With the assistance of BLAST analysis, the nucleotide identities of the 16S rRNA gene sequences of the bacterial strains identified in this study have been compared with one another and with other strains published in the GenBank database to explain the phylogenetic evolutionary relationships between the strains (Fig. 7). After 16S rRNA partial sequence analysis a total of 14 species belonging to the 4 bacterial genera were identified as the major bacterial fruit pathogens of citrus plants in the Khanpur orchards. The species are *Pseudomonas syringae*, *Pseudomonas viridiflava*, *Serratia marcescens*, *Xanthomonas axonopodis* pv. *citri*, and *Candidatus liberibacter*. These species, along with the strains they are showing similarities, are summarized in (Table 2).

Table 2. Bacterial isolates of citrus retrieved from GenBank for the phylogenetic analysis.

Isolate code	Identity	Source	Host	Location	Similar species	Accession numbers	Similarity (%)
Isolate-CB-1	*Candidatus liberibacter*	Leaves	*Citrus sinensis*	KPK, Pakistan	*Candidatus Liberibacter asiaticus* OP108281.1	OQ131081	99
Isolate-CB-2	*Pseudomonas syringae*	Leaves	*Citrus sinensis*	KPK, Pakistan	*Pseudomonas syringae* EU438852.1	OQ131082	99
Isolate-CB-3	*Pseudomonas viridiflava*	Leaves	*Citrus sinensis*	KPK, Pakistan	*Pseudomonas viridiflava* KY604957.1	OQ131083	100
Isolate-CB-4	*Xanthomonas axonopodis*	Fruit	*Citrus sinensis*	KPK, Pakistan	*Xanthomonas axonopodis pv citri* MK818495.1	OQ131084	99
Isolate-CB-5	*Xanthomonas citri*	Fruit	*Citrus sinensis*	KPK, Pakistan	*Xanthomonas axonopodis pv citri* MK818495.1	OQ131085	100
Isolate-CB-6	*Serratia marcescens*	Fruit	*Citrus sinensis*	KPK, Pakistan	*Serratia marcescens* MT263018.1	OQ131086	100
Isolate-CB-7	*Serratia marcescens*	Fruit	*Citrus sinensis*	KPK, Pakistan	*Serratia marcescens* CP055161.1	OQ131087	100
Isolate-CB-8	*Xanthomonas axonopodis*	Leaves	*Citrus sinensis*	KPK, Pakistan	*Xanthomonas axonopodis pv citri* MK818495.1	OQ131088	99
Isolate-CB-9	*Xanthomonas citri*	Fruit	*C. sinensis*	KPK, Pakistan	*Xanthomonas axonopodis pv citri* MK818495.1	OQ131089	100
Isolate-CB-10	*Candidatus liberibacter*	Fruit	*Citrus sinensis*	KPK, Pakistan	*Candidatus liberibacter* OP108281.1	OQ131090	98
Isolate-CB-11	*Pseudomonas syringae*	Fruit	*Citrus sinensis*	KPK, Pakistan	*Pseudomonas syringae* MN905161.1	OQ131091	99
Isolate-CB-12	*Candidatus liberibacter*	Fruit	*Citrus sinensis*	KPK, Pakistan	*Candidatus liberibacter* AB480072.1	OQ131092	100
Isolate-CB-13	*Pseudomonas viridiflava*	Leaves	*Citrus sinensis*	KPK, Pakistan	*Pseudomonas viridiflava* MG972916.1	OQ131093	100
Isolate-CB-14	*Candidatus liberibacter*	Leaves	*Citrus sinensis*	KPK, Pakistan	*Candidatus liberibacter KF712516.1*	OQ131094	100

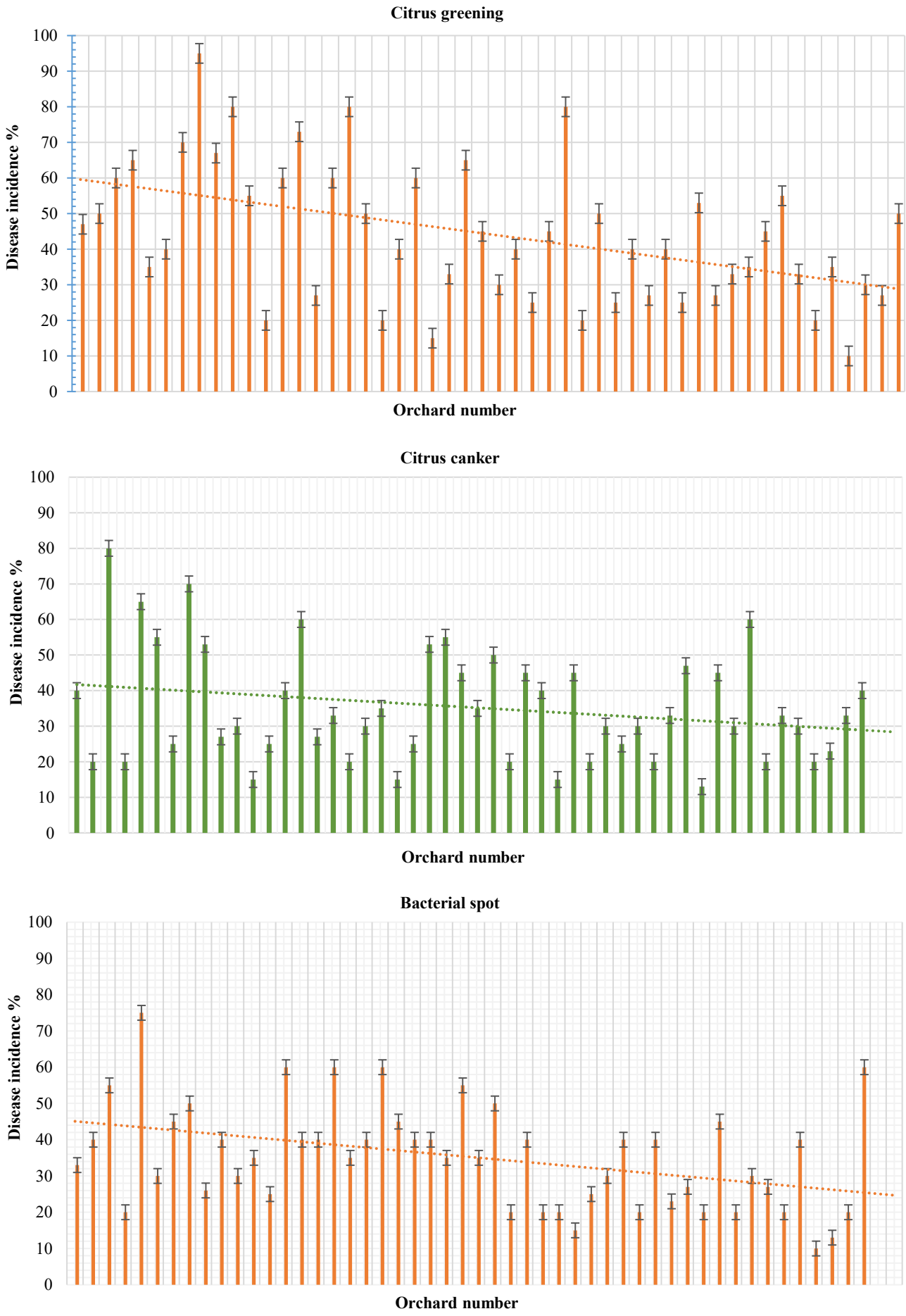

Fig. 4. Disease Incidence of (a.) citrus greening, (b.) canker, and (c.) bacterial spot diseases.

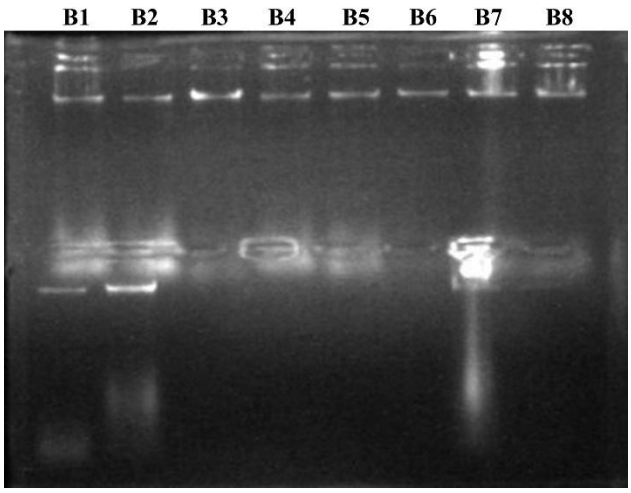

Fig. 5. Pure DNA bands of the isolated bacterial pathogens.

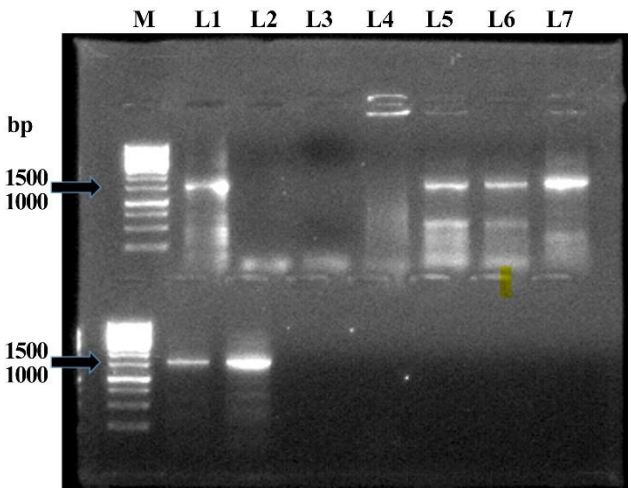

Fig. 6. 16S rRNA gene amplification of bacterial isolates causing Citrus diseases. Lane 1-7 are CCF2, CCL3, CCF3, CGF5, CGL5 and BSL1 respectively. Lane M represents a 1 kb DNA marker.

Pathogenicity test on citrus leaf: The symptoms started to show five days after infection, on the pricking sites in detached leaf pathogenicity tests. Lesions grew larger and changed color from tan to brown after two weeks. After 3 weeks of infection, elevated and spongy lesions typical canker symptoms became apparent. There were canker symptoms for CCL3, CCF3, CCL2, and CCF2. Both CCF3 and CCF2 experienced the development of a light brownish hue at puncture sites, which could not be characterized as a canker-like lesion. The right side of the leaf's abaxial surface, where bacterial broth cultures were infected, had canker signs, whereas the left side showed no symptoms. Results of the bacterial cultures CCF3 and CCF5 tests for citrus leaf pathogenicity are displayed in (Fig. 8).

Discussion

The climate in Pakistan is favorable for the production of all fruits with commercial value. The largest of them all in terms of output and export is citrus. However, the climate in areas that produce citrus is often warm, which favors the growth of disease-causing organisms and phytopathogens. Numerous bacterial diseases have serious economic effects. Citrus canker and HLB, two of the deadliest bacterial plant diseases, are major problems for Pakistan's citrus industry. According to Gottwald *et al*., (2002), burning and uprooting infected groves controlled the illness in some regions of the world, such as America and Brazil but in emerging nations like Pakistan, where the disease has spread to the majority of the region's citrus orchards, such eradication is not practical.

The use of chemicals and antibiotics is one of the other primary methods for managing these diseases; nevertheless, bacterial outbreaks are difficult to manage because of the development of resistance to the target pathogen and the absence of efficient bactericides, along with the fact that their usage is not environmentally friendly (Balogh *et al*., 2010). Applications of aminoglycosides have been observed to give plants systemic acquired resistance against bacterial infections, however, they do not affect the culprit responsible for citrus canker (Jones *et al*., 2012). Others have thought about employing biological remedies like phages to combat phytopathogens. The chemical and biochemical tests used to identify isolated bacterial strains confirmed the characteristics of the pathogens causing citrus canker, and bacterial spot diseases, such as gram-negative, catalase-positive, and oxidase-negative (Vernière *et al*., 1998; Naqvi *et al*., 2022).

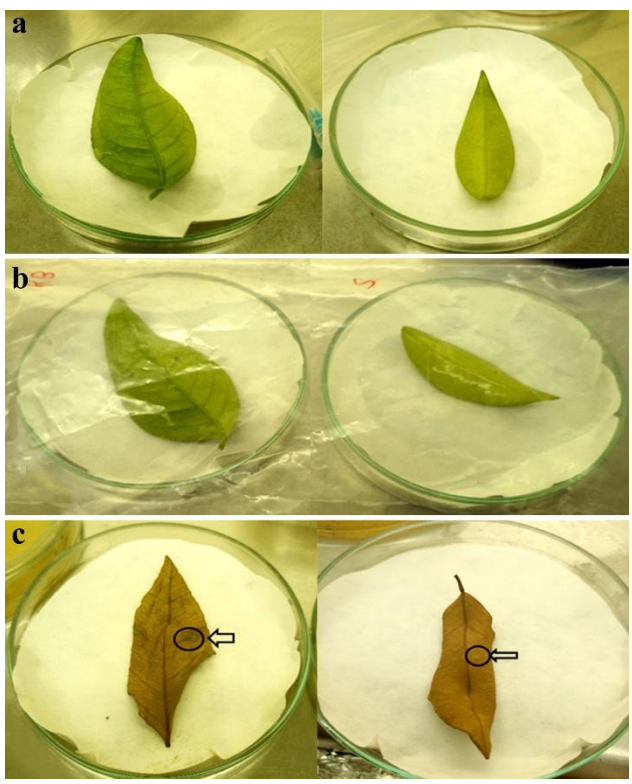

Fig. 8. Pathogenicity test of canker-causing bacterial strains (CCF3, CCF5) on citrus leaves (a.) 1-week post-inoculation (wpi); (b.)2 wpi; (c.) 3 wpi.

For molecular characterization, the chosen bacterial strains with disease potential were accessed. The results of this study showed that the best period to sample for the pathogenic bacteria that cause citrus canker is just before the summer solstice. Bacteria grow rapidly on YPG agar plates in 24 to 36 hours, while the incubation period for the toxicity test lasts up to 3 to 4 weeks. Mild chlorosis is seen on all of the leaves during the first two to three days after

infection. However, only the strains that carried the virulence genes caused lesions on the pricking sides to enlarge after 1 week of infection. Numerous distinct bacterial species cause a wide range of diseases in citrus.

This is the first report on the bacterial strains that are responsible for the citrus canker caused by *Xanthomonas axonopodis*, bacterial spot caused by *Pseudomonas syringae* and *Pseudomonas viridiflava*, and HLB diseases caused by *Candidatus Liberibacter* in Khanpur, Pakistan. However, in earlier investigations as reported by Burhan *et al*., (2007) on the prevalence of citrus canker diseases in Pakistan citrus fruit harvests, only the phenotypic and morphological disease indicators on plants have been evaluated. An effort was made in this study to pinpoint the species that are responsible for the diseases and the consequences they were having on the overall citrus crop production.

The isolated bacterial strains by partial sequence16S rRNA gene analysis found the closest relatives from the *Xanthomonadaceae, Rhizobiaceae,* and *Pseudomonadaceae* families based on clustering (Preecha *et al*., 2018). Following a BLAST similarity search, bacterial strains were chosen for relatedness and similarity based on their origin and plant toxicity to build phylogenetic trees. Based on 16S ribosomal RNA sequencing a neighbor-joining phylogenetic tree was constructed for the bacterial strains CCF3, CCL5, and CCF5 were closely related to the strain *Xanthomonas axonopodis pv citri* MK818495.1 whereas the bacterial strains CGF5 and CGL5 were closely related to the strain *Candidatus Liberibacter asiaticus* OP108281.1. Strain BSL1 is closely related to strain *Pseudomonas syringae* KR922304.1 and strain BSL2 is closely related to *Pseudomonas viridiflava* MG972916.1 strain.

The results of this study are in line with the findings of Monteiro *et al*., (2005) who reported that the citrus canker is caused by *Xanthomonas citri* (*Xanthomonas axonopodis* pv. Citri) belonging to the family *Xanthomonadaceae* of the Gammaproteobacteria. According to Teixeira *et al*., (2005), three Candidatus species have been reported to cause HLB citrus greening diseases in Brazil that are 'Ca. *Liberibacter asiaticus'* (CaLas), 'Ca. Liberibacter africanus' (CaLaf), and 'Ca. Liberibacter americanus' (CaLam). The isolated species Serratia marcescens is closely related to strain MT263018.1 was identified in the current study and is in line with the findings of Hasan *et al*., (2022), who also revealed that the isolates as *Serratia marcescens* strains are responsible for black rot disease of *Citrus sinensis* fruits in Bangladesh that reduces the quantity and quality of the citrus and causes enduring losses to the farmers and fruit industry.

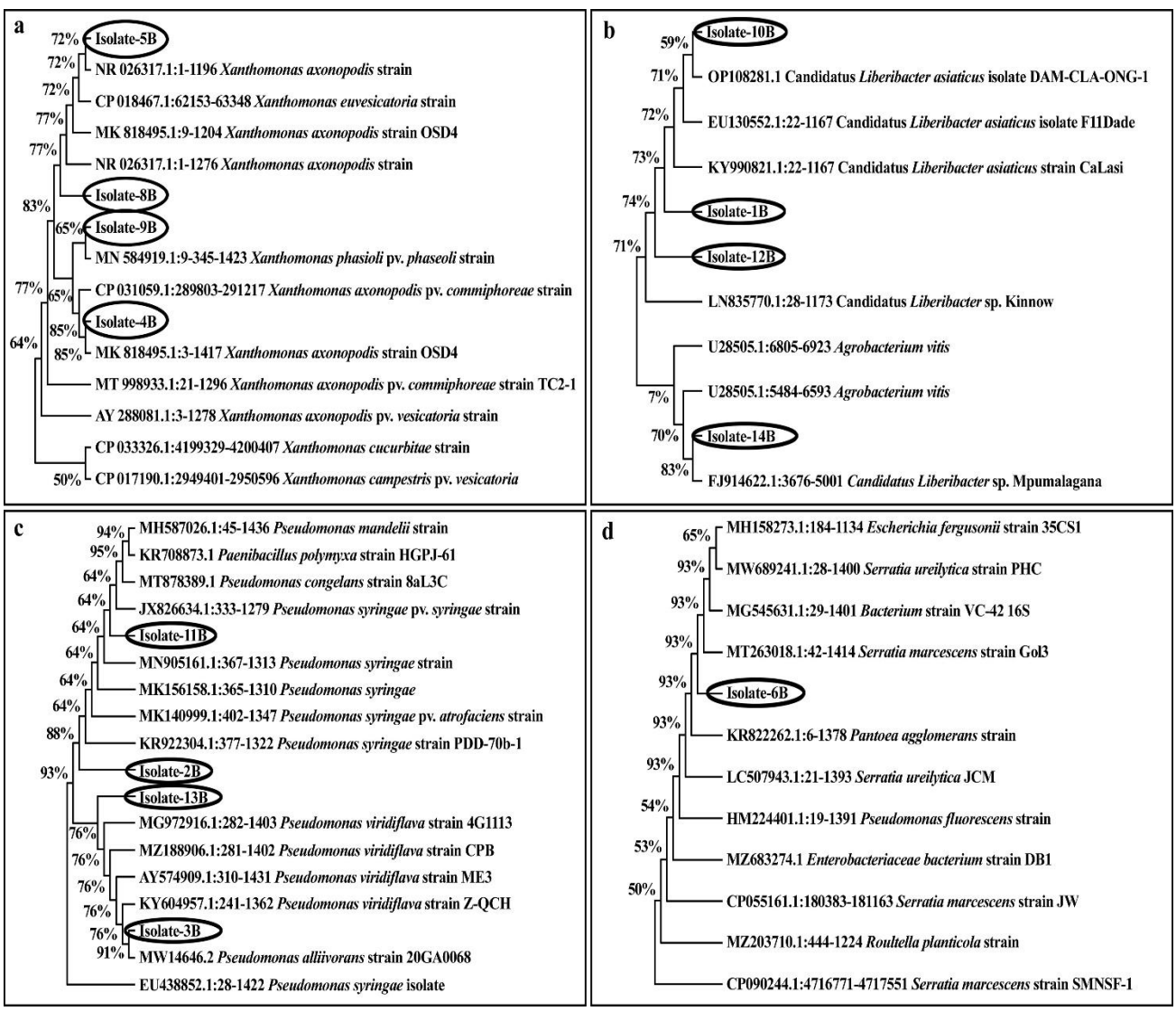

Fig. 7. Neighbor-joining tree showing evolutionary links between bacterial strains that cause citrus diseases using Bootstrap methodology to construct the phylogenetic trees (a). *Xanthomonas axonopodis* (b.) *Candidatus Liberibacter* (c.) *Pseudomonas syringae* and *Pseudomonas viridiflava* (d.) *Serratia marcescens.*

Conclusions

The findings of this research revealed that the citrus crop in Khanpur, Pakistan's is at risk due to bacterial pathogens that are deteriorating this valuable asset of the country. The production of citrus is seriously threatened by the broad-spectrum toxicity of citrus bacterial diseases, the cultivation of vulnerable citrus varieties, and the advent of emerging strains. The pathogens associated with the diseases were identified as *Xanthomonas campestris pv citri*, *Candidatus Liberibacter*, *Pseudomonas syringae*, *Pseudomonas viridiflava*, and *Serratia marcescens* which are responsible for canker, citrus greening, bacterial leaf spots and black rot diseases of *Citrus sinensis* respectively.

In conclusion, the 16S-rRNA-based molecular characterization successfully identified several plant pathogenic bacterial species associated with citrus in Pakistan. This study provides valuable insights into the diversity of bacterial pathogens affecting citrus crops in the region. The identified species, highlight the potential threats to citrus production and underscore the need for effective management strategies. This molecular approach proved to be a reliable and accurate method for identifying and characterizing these pathogens. In the future, the identified bacterial isolates hold significant promise for application in management trials and development of sustainable control measures to mitigate their impact on citrus orchards in Pakistan and improve crop productivity.

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