ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF *XANTHOMONAS AXONOPODIS PV. CITRI* FROM SELECTED SPECIES

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(Received 25th Apr 2023; accepted 1st Aug 2023)

Abstract. Citrus fruits, that belong to the *Rutaceae.* family, have a great economic, nutritional, and therapeutic importance. It has long been a significant source of foreign exchange for Pakistan. Unfortunately, various diseases affect its production and quality, the deadliest of which is known as citrus canker caused by *Xanthomonas axonopodis pv. citri.* The goal of current research was to isolate and characterize the strains causing citrus canker on the basis of phenotypical and genotypical features. Fruits with possible canker lesions were used for the isolation from the different Citrus Orchards in Khanpur, District Haripur, Pakistan. Citrus fruits, both symptomatic and asymptomatic, were sampled and evaluated during the survey. Only diseased citrus fruits yielded *Xanthomonas*-like bacterial strains were isolated and identified by carrying out different biochemical tests including catalase, Kovac's oxidase and KOH test. The isolated strains were further identified through polymerase chain reaction (PCR) by using specific ITS primers. Total of eight bacterial isolates were identified to be *Xanthomonas* spp. through biochemical tests and molecular results revelled the 2A and 2B bacterial isolates as., *Xanthomonas citri* subsp. *citri. (Xcc)*. Thus, it was concluded that isolated strains were pathogenic bacteria, that is an actual cause of bacterial canker in commercial citrus fruits. Our findings suggested that diseased citrus fruits, could act as a source of dispersal for citrus canker in areas like Pakistan.
Keywords: *citrus canker, Xanthomonas axonopodis pv. citri, phenotypical and genotypical characterization*

Introduction

Citrus is a member of the *Rutaceae* family of trees and shrubs. Citrus is thought to have originated in the southern Himalayas, China's northeast, and India (Gmitter and Hu, 1990). Citrus fruits, such as oranges, lemons, and grapefruits, are an important crop in Pakistan with multiple economic, nutritional, medicinal, culinary, and employment benefits. It is one of the top fruits exported by the country. The export of citrus contributes significantly to Pakistan's economy. Citrus fruits are a rich source of vitamin C, which is essential for human health. They also contain other vitamins and minerals, including potassium, folate, and thiamine (Neves et al., 2010). It has been used for medicinal purposes for centuries. They are believed to have anti-inflammatory, anti-cancer, and anti-viral properties, among others. Citrus fruits are widely used in Pakistani cuisine, particularly in the preparation of drinks, desserts, and chutneys (Buono et al., 2023).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2201_665679

Asiatic citrus canker (ACC) is one of the most challenging citrus diseases in the world. *Xanthomonas pv. citri*, causes, citrus canker. and is very infectious. Although an infestation can wipe out entire orchard crops, the illness is not harmful to humans or animals. Citrus canker is a severe issue in Asia and South America, limiting citrus supply. When the disease strikes during the early phases of plant development, it causes significant losses (Iqbal et al., 2007). Grapefruit, sweet orange, and lime are all affected by canker in strongly infested locations. There is no cure, and resistance cannot be bred into the population. This is especially true in areas prone to tropical storms. Hundreds of million of dollars are expended each year on disease inhibition, quarantine, suppression, and regulatory programmes around the world. The trade of fruit coining from contaminated zones is no doubt the most devastating consequence of citrus canker outbreaks which has great impact on business (Saikia et al., 2021; Yasuhara-Bell et al., 2023).

Early spring is when bacterial cells proliferate and spread through various sources, including wind-driven rain, air streams, and natural world activities. Long-distance transmission is primarily caused by infected plants, seedlings, and berries, and commercial shipments of sick fruit. Contaminated clothing, tools, and materials used in harvesting and post-harvest handling can also spread the disease. Fruits are sensitive for longer periods, causing multiple infections and varying wounds. Late autumn contamination is generally latent, with the virus becoming active in the succeeding season. One of the most essential activities in the disease decrease is the prevention of primary infection on fresh shoots. Chemical control tactics aren't completely successful when the environment is conducive to the spread of disease. The most effective bactericidal sprays for safeguarding plants and fruits are copper-based products (Borde/aux, mixture, copp/er, hydroxide, basic copp/er , chloride, copp/er oxychloride, and tri-basic, copper sulphate) (Koshariya et al., 2023). These materials can help to minimize the spread of disease, but they won't be able to eliminate existing diseases. Copper use in large quantities may generate phytotoxicity issues in treated groves (Zhao et al., 2023; Chaube et al., 1992; Shouket et al., 2023). Koller et al. (2006) proposed at high inoculum concentrations, copper treatments were ineffective in reducing citrus canker; but, at low inoculum absorptions, both Bordeaux, mixture and copper oxy-chloride were beneficial in controlling the illness. Only 13.4 percent of citrus orchard strains that had never been exposed to copper sprays thrived. The tolerance of the bacterium to copper was improved by mixing mancozeb with copper. As a result, the use of mancozeb in combination with copper for controlling citrus canker bacteria should be reconsidered (Meneguim et al., 2007). Foliar sprays of copper. hydroxide was the most active in directing canker (800 ppm). Windbreaks, together with the treatment of copper bactericide are considered as effective citrus canker management techniques (Gottwald and Timmer, 1995).

Citrus canker was first recorded in the Punjab district of British India in 1940 Shehzadi and Naz (2019), and was described as the most damaging agricultural disease. Citrus canker was initially discovered in Punjab, followed by Andhra Pradesh, Kamataka, Madhya Pradesh, Rajistan, and Pradesh. Citrus canker affected lime and many other citrus cultivars, causing serious damage. Commercial acid lime growers also suffered significant financial losses as a result of this disease. Citrus canker is a severe issue for all citrus growers (Madhavi et al., 2000). This disease also infects nursery plants of Kinnow and Mandarin in Punjab (Sohi and Sandhu, 1968). Florida has a long history of citrus farming. This disease was first identified in Florida in 1912. Infected seedlings

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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supplied from Japan brought the illness to the southeast United States. During 1933, diseased trees were eliminated in Florida and other nearby states (Schubert et al., 2001). The disease's symptoms can be seen on all above ground components, including leaves, twigs, and fruits. Lesions appear in cycles, timed to coincide with phases of dense drizzle, high temperatures, and development spurts (*Figure 1*).

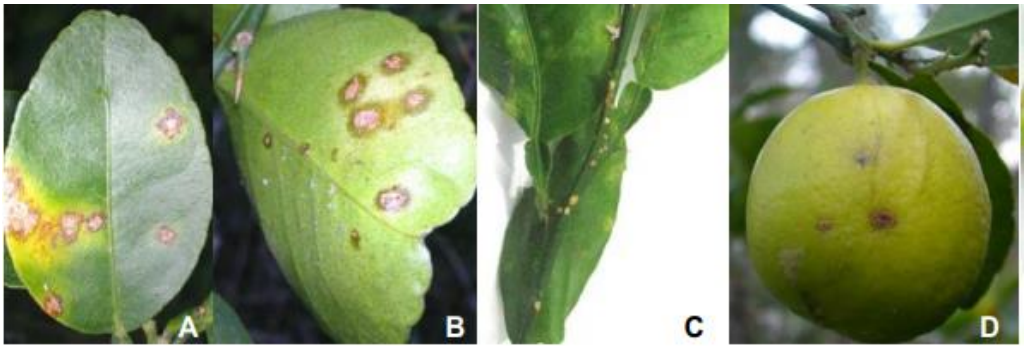

Figure 1. *Citrus cv. produces many forms of lesions on various plant sections. (A) Yellow halo, and elevated wounds on superior, side of leaf; (B) Uneven raised. lesions on underside; (C) Twig lacerations; (D) Fruit injuries (Sourced from Timothy R. Gottwald) (Sharma and Sharma, 2009)*

Citrus fruit is primarily grown in Pakistan's four provinces of Punjab, Khyber Pakhtunkhwa (KPK), Sindh, and Baluchistan. According to the Pakistan Horticulture Development and Export Company, Punjab produces more than 90% of the nation's total production of kinnow; KPK is known for producing the majority of the nation's oranges (Naseem et al., 2016). There is no cure for citrus canker disease that is both effective and efficient. However, this disease may well be treated by using some basic strategies to reduce the spread of the disease as soon as it is introduced into the field (Gambley et al., 2009). The bacterial infection known as citrus canker disease, which affects citrus plants, can be treated chemically. To control the spread and severity of the disease, these methods often involve the use of various chemical agents, such as copper-based fungicides and bactericides. When used prior to infection, copper-based solutions can reduce bacterial populations on plant surfaces and serve as a preventative intervention. They function by halting the pathogen's growth and preventing it from spreading. To reduce environmental damage and avoid the emergence of copper-resistant strains, it is crucial to use these compounds sparingly and by suggested criteria (Pereira et al., 2021). Integrated pest management techniques, which combine chemical treatments with other preventative measures, are also essential for long-term disease management in citrus orchards (Smith et al., 2021).

Citrus canker control approaches rely on chemicals, however there are numerous major bottlenecks. One of the key issues is the emergence of bacterial resistance, which can happen when chemicals are used continuously and carelessly over time and eventually lose their efficacy. These practices also degrade ecosystems, pollute water sources, harm helpful insects, and have negative environmental effects. Chemical treatments that lack selectivity can potentially harm beneficial bacteria, disrupting ecological processes that occur naturally (Atiq et al., 2023). The environment may become contaminated with persistent substances, endangering human health and the safety of food. Additionally, citrus producers, especially smaller ones, may be burdened by the cost of frequent

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applications (Schubert et al., 2001). Regulations limiting the use of specific chemicals further reduce control possibilities. Additionally, it is difficult to treat infections that have already spread because these chemical therapies work better as preventives than as remedies. Effectiveness depends on appropriate timing and accurate administration, yet these might be challenging to accomplish. There is a rising interest in creating alternative and integrated strategies, such as cultural practices, biological control, resistant citrus varieties, and enhanced orchard cleanliness, to manage citrus canker effectively while minimizing downsides to get around these limits. A growing number of people are interested in creating alternative and integrated strategies for managing citrus canker as a result of these constraints and bottlenecks. These could involve the sole biological control techniques which will increase the production of citrus production throughout Pakistan (*Figure 2*).

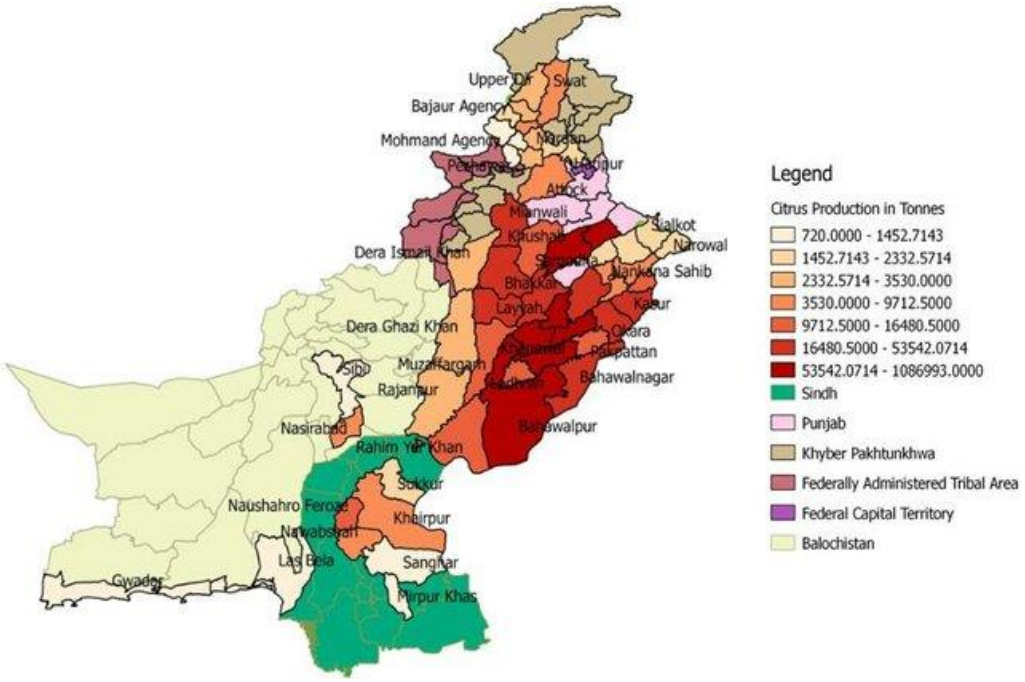

Figure 2. *Khyber Pakhtunkhwa (KP), Punjab, Balochistan, and Sindh provinces of Pakistan have significant citrus-producing districts (Naseem et al., 2016)*

Thus, by keeping in mind the importance of citrus crops and biological control, the current study was designed to isolate, phenotypical and genotypical characterization of the bacterial population in citrus orchards at Khanpur, District Haripur, Pakistan.

Material and Methods

Sample collection and isolation

Citrus fruits with and without symptoms (citrus cankerlike, corky wounds) were randomly gathered from citrus orchards in Khanpur, Haripur District (15 cankered and 15 healthy. fruits were obtained in total). Within two days, samples placed in sterile bags were sent to laboratory for isolation. The samples were disinfected for 5 minutes in 75 % ethanol and then evaporated to dryness. Canker lesions and 3 mm of the peel around them

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were removed with a sterilized scalpel and immersed in sterile distilled water for 15 minutes at room temperature (SDW). The macerate was then placed on a plate of fresh yeast extract, (10 g/l), dextrose, (20 g/l), calcium carbonate (20 g/l), and agar (15 g/l) medium and incubated at 28 °C for 3 to 4 days. After isolat.ion characteristic yellow isolates were purif/ied by sub-culturing single colonies of each isolate onto specific YDC (Calcium carbonate 2%, Dextrose 1%, Agar 2%). media according to the method used by (Schaad et al., 2001). The pure yellow isolates were resuspended in sterile distilled water and kept at 4°C.

Biochemical characterization

To identify and classify various bacterial species based on biochemical profiling, the catalase, starch hydrolysis, and oxidase assays are frequently utilized in the identification of bacterial pathogens. A series of biochemical tests that were performed on all pure bacterial isolates. Gram reaction, KOH, catalase, simmon citrate and oxidase activities, and starch hydrolysis were all investigated. Isolates were inoculated and cultivated on a Y.DC, agar plate at 28 °C for 4.8. hours for all assays (Schaad et al., 2001).

Gram staining

Principle

The capability of the bacterial cell wall to retain the crystal violet dye following solvent treatment is the core principle of gramme staining. Glycoproteins are more abundant in Gram-positive bacteria, whereas lipids are much more abundant in Gram-negative bacteria. The crystal violet dye is taken up by all bacteria initially, but the solvent dissolves the lipid layer of Gram-negative bacteria. Gram negatives lose their predominant stain as the lipid layer weakens. Solvent, on the other hand, dehydrates and closes pores in gram-positive cell walls, preventing the spread of the violet-iodine combination and leaving bacteria colorless (Tripathi and Sapra, 2021).

Procedure

Crystal violet, Lugol iodine, Acetone, and Safranin counterstain were used to make Gram reagent. Isolates were created by smearing pure culture on a transparent slide with distilled water using a sterile needle. Using sterile water, a drop of crystal violet was administered, swirled in, and allowed to dry for 30 seconds before being rinsed. A droplet of Lugol's Iodine was added after SDW wash, following the lasting wash with acetone. Then, a drop of safaranin was added, held for 30 seconds, and then eroded clean as a final step. The mounts were dried on blotting paper, stained with a drop of Canada balsam, and examined under a microscope at 100X (Hussain et al., 2010).

KOH test

Principle

The potassium hydroxide test (KOH test) is used to identify microorganisms that are Gram negative. KOH dissolves the thin peptidoglycan layer in Gram negative bacteria's cell walls but has no effect on Gram positive bacteria's cell walls. The Gram-negative cell wall is disintegrated, letting the cell to lyse and liberate its subjects, together with the DNA. Consequently, a viscid chromosomal substance is released, making the suspension thick. This solution sticks to the loop when touched (Mubeen et al., 2015).

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Procedure

A solution of 3% potassium hydroxide was placed on the glass slide, and then a 24-hour-old culture was mixed with a sterile needle and swirled for a few seconds until being checked for thin threads. The bacterium is gram-negative if the loop is visible when the needle is raised (Mubeen et al., 2015).

Catalase test

Principle

This test proves the existence of catalase, an enzyme that catalyses the diffusion of oxygen from hydrogen peroxide (H_2O_2). The enzyme catalase aids in the breakdown of hydrogen peroxide into oxygen and water. When a tiny inoculum is exposed to hydrogen peroxide, the presence of the enzyme is confirmed by the rapid production of oxygen bubbles. A lack of or insufficient bubble formation is a sign of catalase insufficiency (Ali et al., 2017).

Procedure

On a clean slide, a single colony was placed, and hydrogen peroxide was carefully smeared over it. Catalase production was tested by introducing H_2O_2 (3 percent v/v.) to a bacterial culture and looking for bubbles of free oxygen, which showed the existence of catalase. On a clean slide, a single colony was placed, and hydrogen peroxide was carefully smeared over it. The presence of catalase was evaluated by adding H_2O_2 (3 percent v/v) to a bacterial culture, and the presence of catalase, was shown by free oxygen gas bubbles (Ali et al., 2017).

Starch hydrolysis test

Principle

The enzymes amylase and oligo-1,6-glucosidase are secreted into the environment when bacteria are grown on starch agar. Clear spots surrounding bacterial growth indicate that the organism has hydrolysed starch (García et al., 2019).

Procedure

The starch agar medium was made (potato starch was used) and poured into the Petri plate, allowing it to harden before streaking the test bacteria and incubating it for 96 hours at 28 ^{0}C in an inverted manner. Following the incubation period, a dropper was used to soak the area of the plates with iodine. solution for 30 seconds, and the change in color of the medium around the growth line was observed (Mubeen et al., 2015).

Kovac's oxidase test

Principle

The oxidase test looks for a cytochrome oxidase system in bacteria that catalyses the transfer of electrons from donors to a redox dye, tetramethyl-p-phenylene-diamine. The dye has been switched to a rich purple color. Internal oxidase enzymes are produced by cyanochrome-containing organisms. The oxidation of cytochrome c is catalysed by this oxidase enzyme. The respiratory chains of oxidase-positive organisms contain cytochrome c, which causes the reagent to turn blue/purple (Guo and Grosser, 2002).

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Procedure

Fresh bacterium culture was utilized for the test, with 1% Kovac, reagent placed in the center of Whatman filter paper. No.1 and a platinum needle utilized to gently rub the bacterium on the filter paper. Within 30 to 60 seconds, the results were visible (Guo and Grosser, 2002).

Molecular characterization

DNA extraction

For DNA extraction, colonies were grown on NA media for 48 hours at 28 °C, and genomic DNA was extracted using a straight forward heating procedure. A loop full of a pure isolate was suspended in 500 mL DW, heated to 95° for 10 minutes, chilled on ice, then centrifuged for 3 minutes at 13,000 g. The supernatant was kept frozen at 20°C until it was required (Izadiyan and Taghavi, 2020).

Polymerase chain reaction (PCR) assay

The phylogenetic correlation between all of the isolated bacteria and the distinct strains reported at the National Center for Biotechnology Information (NCBI) was determined through amplification and sequencing of the internal transcribed spacer region as described by Smith et al. (2021), from the isolated strains using specific and generic primers (*Table 1*). A Perkin–Elmer GeneAmp PCR System 2400 PCR cycle was used for amplification. The reaction mixture system consisted of 10x buffer 2.5 ul, Taq DNA polymerase 1.25 U, dNTP 0.8 mM, ITS-F or ITS-R 0.5 mM, DNA template 1.0 ul, and H_2O 16.6 ul (25 ul). Initial denaturation at 94°C for 10 minutes, earlier denaturation at 94°C for 1 minute, annealing for 1 minute, extension at 72°C for 2 minutes, and final extension at 72°C for 10 minutes were used in the PCR amplification. A total of 32 cycles of PCR were performed (Adriko et al., 2014). Finally, the PCR products were separated using a 1% agarose gel electrophoresis (Adriko et al., 2014). While TIANgel Midi Purification kit (TIAGEN BIOTECH, Beijing, China) was used to obtain gel bands with the necessary product, and Genescript Company, Nanjing, China performed the sequencing.

Table 1. *Primers used for molecular identification (Adriko et al., 2014)*

Primer	Sequence 5′- 3′	Amplicon Size	Annealing temperatures	Target species & Pathovars
C-ITS-X2b	GGCGGG.GACTTCGAGTCCCTAA	254 bp	68 °C	*Xanthomonas* spp.
C-ITS-X2j	GGCGGGGACTTCGAGTTCCTAA			
C-ITS-X2a	CGGGGACCTCGAGTCCCTA			
C-ITS-X2k	GCGGGGACTTAGAGTCCCTA			

Sequencing and phylogenetic analysis

After sequencing the retrieved sequences were analyzed by using NCBI BLAST analysis (http://blastt.ncbi.nlm.nih.gov/Bla.st.cgi) to incorporate the sequences obtained previously for the development of the phylogenetic tree. Sequences having a 98 percent similarity to currently existent sequences were judged to be the same species. Furthermore, numerous alignments were done using Cluu,stal X 1.83, and MrEGA 4.00 was used to construct the phylogenetic tree (Tamura et al., 2013).

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Results

Cultural, and morphological, characteristics, of X. axonopodis pv. citri isolates

Total of eight *Xanthomonas*-like colonies were isolated from damaged fruits taken at random from citrus orchards from Khanpur, District Haripur, Pakistan. The phenotypic characteristics of all bacterial isolates were consistent with those found in the genus *Xanthomona.* The isolated colony shape was filiform in all of the test isolates, with a rounded elevation and an entire colony edge (*Table 2*). Many workers had previously reported similar outcomes (Ibrahim et al., 2019). On YDC, all isolates had brilliant yellow, mucoid, and round colonies (*Figure 3*). They were recognized as members of the genus *Xanthomonas* based on phenotypic tests. There were no *Xanthomonad*-like colonies seen in symptomless fruits.

Table 2. *Characteristics of X. axonopodis pv. citri isolates*

S.No	Isolates	Colour	Colony Shape	Elevation	Margin	Cell Shape
01	Xac 2a	Yellow	Filiform	Convex	Entire	Rods
02	Xac 2b	Yellow	Filiform	Convex	Entire	Rods
03	Xac 3a	Light Yellow	Filiform	Convex	Entire	Rods
04	Xac 3b	Yellow	Filiform	Convex	Entire	Rods
05	Xac 4a	Yellow	Filiform	Convex	Entire	Rods
06	Xac 4b	Yellow	Filiform	Convex	Entire	Rods
07	Xac 5a	Yellow	Filiform	Convex	Entire	Rods
08	Xac 5b	Yellow	Filiform	Convex	Entire	Rods

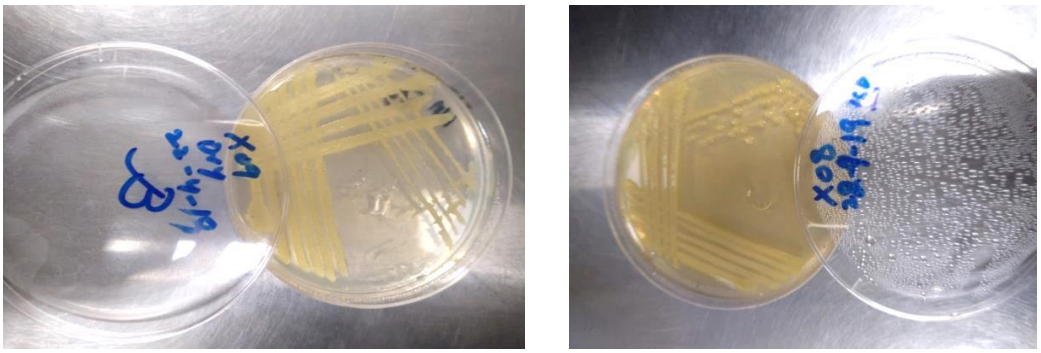

Figure 3. *Showing Xanthomonas axonopodis pv. citri cultured on YDC agar plates*

Biochemical characterization

The biochemical characteristics of *Xanthomonas axonopodis pv. citri* were investigated using a variety of chemical tests, including Gram staining, potassium hydroxide (KOH) solubility testing, catalase testing, starch hydrolysis testing, and oxidase testing. Gram's reaction and oxidase tests reveal that all isolates are negative, however Catalase, KOH, Simmon Citrate, and Starch hydrolysis tests reveal that they are all positive. The following are the features of Gram-Negative bacterium and the findings obtained (*Table 3*).

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Table 3. *Biochemical characteristics of Gram-negative bacteria*

Tests	Reaction	Appearance
Gram Reaction	Negative	Small Rods, Pink Colored Colony
KOH Test	Positive	Thread. Like Slime
Catalase Test	Positive	Gas Bubbles Formation
Starch Hydrolysis Test	Positive	Clear Zone in Iodine Stained Medium
Kovac's Oxidase Test	Negative	No Colour

Gram staining

X. axonopodis pv. citri Gram-stained mounts revealed that the trial bacteria did not preserve the purple/violet hue of the chief stain (Crystal violet), but that cell looked pink due to counterstaining with the dye safaranin. As a result, the test bacteria were gram negative with rods, a hallmark of plant harmful bacteria.

Potassium hydroxide (KOH) test

Gram-negative bacteria have moderately flimsy cell walls that are bordered by an external membrane; hence the formation of slime threads or loops indicates that they are Gram-negative. Exposure to 3 percent KOH easily disrupts this, releasing the viscous DNA. Gram-positive bacteria, on the other hand, have a thicker, more solid cell wall that can withstand the disruptive effects of KOH. The test bacteria demonstrated a positive reaction to the KOH test in this investigation (*Table 3*).

Catalase test

After treating the test bacterium with some drops of 3% H_2O_2 on a glass slide, it formed gas bubbles, indicating a positive catalase test (*Table 3*).

Starch hydrolysis

The test bacterium grown on starch agar swamped with Lugol's iodine created a colorless area around bacterial growth (*Table 3*), indicating that it passed the starch hydrolysis test. Exoenzyme amylase hydrolyzes starch and breaks it down into dextrins, maltose, and glucose/alpha amylase in the test bacteria. Many workers had previously reported similar outcomes.

Kovac's oxidase test

The crux of Kovac's oxidase test is the presence of cytochro.me oxide, which is a saprophytic bacterium characteristic. The bacterium was positive to test whether the purple color formed between 30-60 seconds. Our study's isolates were all oxidase negative (*Table 3*).

Molecular identification

Total genomic DNA was isolated from all bacterial isolates and a 254-bp PCR product was produced using gDNA as a template (*Figure 4*), as described.in the Materials and Methods section.

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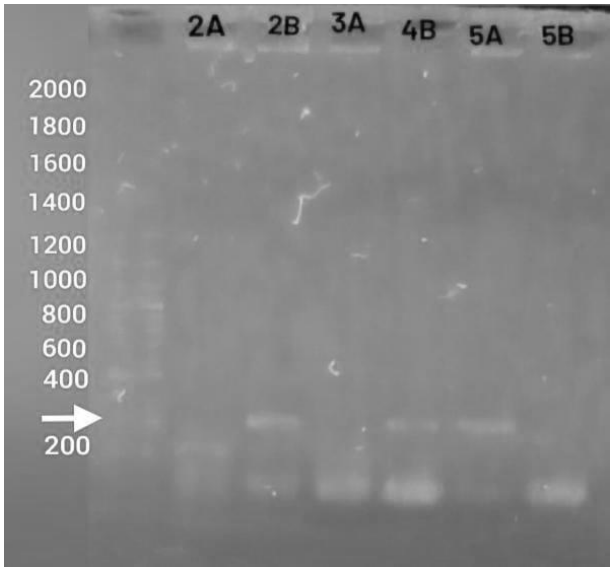

Figure 4. *Detection of X. axonopodis pv. citri strains using specific primers lane 1: 1 kb DNA ladder, Lane 2A,2B, 3A, 4B, 5A, & 5B, are the amplified PCR products of bacterial isolates*

Phylogenetic analysis

The ITS sequences of all the bacterial isolates were aligned and a phylogenetic tree was generated, as shown in "Material' and Methods." Many phylogenetically related bacterial species, when the ITS of the isolates were compared to those in the NCBI database, they were determined to be similar to the bacterial isolates discovered in this analysis (*Figure 5*). The topology of the phylograms established that the bacterial isolates (2A & 2B) used in this study were allocated to different subspecies of *Xanthomonas citri*.

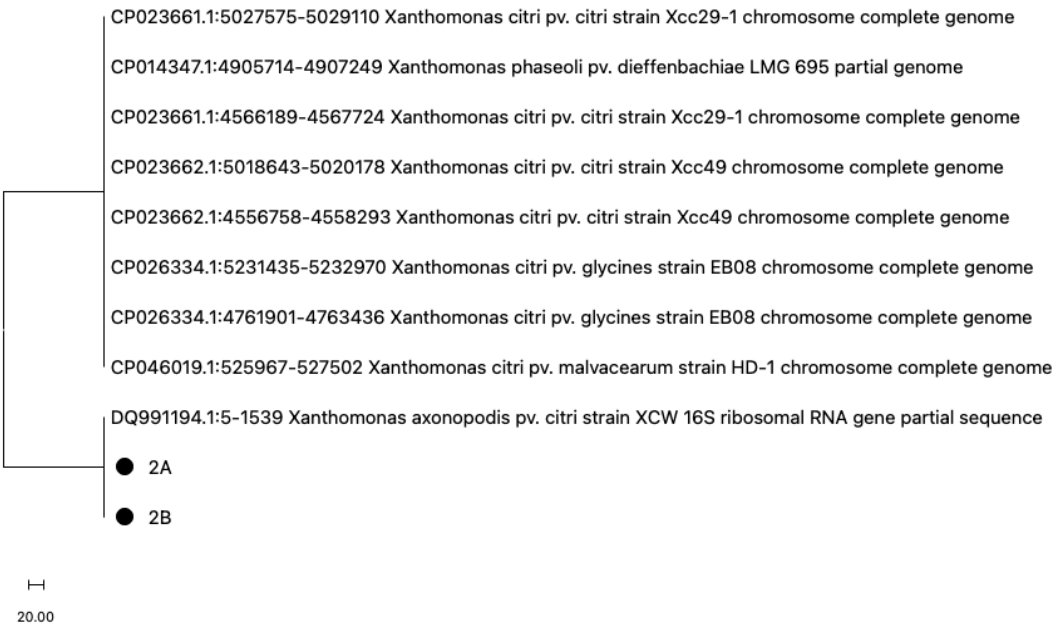

Figure 5. *Phylogenetic tree of isolated strains by using neighbour joining method with GeneBank reference strains*

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Discussion

Citrus is a popular fruit crop with a diverse range of species and a reputation for delicacy. Its cultivation covers a huge region, and as a result, it ranks first in Pakistan's fruit production. Unfortunately, citrus output has been harmed by a variety of microbial diseases, the most serious of which is citrus canker caused by *Xanthomonas axonopodis pv. citri*. Every year, this disease causes massive crop losses, and it is most prevalent in the presence of citrus leaf miner (*Phyllocnistic citrella*) and favorable weather parameters. Citrus leaf miners develop feeding channels in the leaves, which help the infection spread. Citrus stands first in area and production among the world's tree fruits. In Pakistan, citrus fruits are the most important fruit crops grown on the area of 160,000 hectares with production of 1.5 MMT annually. Citrus fruit is grown in all four provinces of Pakistan, but Punjab produces over 95% of the crop because of its greater population, favorable growing conditions with the sufficient water availability. Citrus is divided into different groups sweet oranges, Mandarine, Grapefruit, Lemon and Lime which are being grown commercially.

Citrus fruits come in a variety of sizes, vibrant colors, and incredible scents. They provide many health benefits, from boosting your immune system to reducing your risk of heart disease. Citrus fruits are high in phytonutrients, including flavonoids. According to several studies, flavonoids may help prevent the development of certain types of cancer. Pakistan is one of the world's leading citrus-producing nations. Long-term droughts, on the other hand, have resulted in a recent agricultural water problem. As a result, citrus production is declining, while demand for some citrus fruits, such as sweet oranges and tangerines, is rising.

In this investigation, phenotypic, genetic, and pathogenicity assays were used to characterize Xcc. isolates from local citrus fruits. Despite the fact that the *Xanthomonas citri* (Xcc.) patho. types (A, A*, and A.W) have comparable symptoms and genetic features, their host range allows them to be separated (Ngoc et al., 2010). According to pathogenicity tests, the Xcc. isolates used in this study can infect sweet oranges, and lemons. Our eight isolates were classified as Xcc. pathotype A based on their host range as mentioned in previous study. Citrus bacterial canker has also been found in Saudi Arabia, Oman, and Iran, our neighbouring countries, which were causing different level of canker diseases (Ibrahim et al., 2019). In addition, *Xanthomonas citri*, other pathogens that can cause the citrus canker-like symptoms in citrus fruits (orange) include *X. citri* subsp. aurantifolii, Citrus bacterial spot, *Pseudomonas syringae*, *Alternaria alternata*, and *Phytophthora* spp., as well environmental stress factors (Gottwald et al., 2002). We used culture methods in our lab to investigate if Xcc. might thrive on the surface of seemingly healthy fruits (orange). Our (limited) findings revealed that presumably healthy fruits lacked recognizable Xcc. isolates, indicating that they were unlikely to be a source of inoculum transmission, Xcc. can multiply rapidly in fruit, stem, and foliar lesions, but it has a short lifespan outside of the plant (Shiotani et al., 2009). Furthermore, washing fruits with water, chlorine, and detergent, as well as treating fruits with sodium orthophenyl phenate (SOPP) in the packaging process before export, are the most effective methods for eradicating viable surface populations of bacteria from apparently healthy fruits. The surface tension of the water is reduced when detergent is used, allowing chlorine to reach the bacterial cell surface more easily. This method can also be used to clean the fruit surface of dirt, sooty mold, and scale insects (Gottwald et al., 2009). Because Xcc. was successfully isolated from citrus fruit lesions observed in orchards, thus indicating that the packinghouse procedures and post-harvest treatments will be

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DOI: http://dx.doi.org/10.15666/aeer/2201_665679

insufficient to remove the pathogen from canker lesions. Currently, there is no way to completely remove Xcc. from cankered fruits. A major source of concern is the spread of Xcc. to new locations in citrus-growing countries (Schans, 2011). Our findings back up previous study that demonstrated that Xcc. isolates may survive in canker lesions of harvested fruits throughout transit or storage, and that shipping fruit at a cool temperature (4–15 °C) had little effect on bacterium survival (Golmohammadi et al., 2007). The presence of living bacteria within lesions, according to the findings, could pose a danger of disease transmission via infected fruit. Using high bootstrap values, both neighbour joining trees suggested that isolates from the current investigation were indisputably belong to the Xcc. cluster. Our isolates were classified into one cluster (A pathotype) by the neighbour joining-atpD tree, which was unique from the A*/Aw pathotypes. Using an Xcc. strain collection from around the world (Ngoc et al., 2010). In our opinion, AtpD barcoding has promising potential as a pathotype-level discriminating tool for Xcc. Citrus is a high-yielding crop that is mostly grown in Pakistan (Koupaei et al., 2014). Citrus / trees are grown in streets, private and public / gardens, as well as in citrus growing districts across the country, posing a danger of transferring pathotype A isolates to a liable host via contaminated fruits. Because of Pakistan's commercially significant citrus output, strict border restrictions and a more intensive inspection programme are required. In marketplaces and citrus-producing areas where these markets exist, a secondary inspection survey should be conducted.

Conclusion

This study was focused on the isolation and characterization of *Xanthomonas axonopodis pv. citri* that causes the citrus canker disease in citrus plants. Initially, the sampling was carried out from the citrus orchards of Khanpur in District Haripur, Pakistan. The different subspecies of *Xanthomonas citri* were isolated and identified by using biochemical and molecular techniques. Our findings reveal that the major cause of citrus cancer is *Xanthomonas spp.* The presence of living bacteria within lesions, according to the research, could pose a danger of disease transmission through infected fruit. That could be controlled by using chemical methods, but bacteria have proven to be rather more recalcitrant to such chemical treatments as compared to the fungal pathogens. Thus, there is a need for biological control by using bacteriophages that have proven to have the potential to huddle effective disease regulation as part of the unified management approach.

Acknowledgements. The authors greatly acknowledge and express their gratitude to the Researchers Supporting Project number (RSP2024R335), King Saud University, Riyadh, Saudi Arabia.

Funding statement. The current research work is supported by a Research Grant provided by Higher Education Commission Pakistan through NRPU-HEC Project No. No: 10066/KPK/ NRPU/R&D/HEC/ 2017.

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