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MORPHOLOGICAL, BIOCHEMICAL, AND MOLECULAR CHARACTERIZATION OF *XANTHOMONAS CITRI* SUBSP. *CITRI*, CAUSE OF CITRUS CANKER DISEASE IN PAKISTAN

MUHAMMAD NAUMAN[1], SIDRA MUSHTAQ[1], MUHAMMAD FAHAD KHAN[2], AMJAD ALI[3], SYED ATIF HASAN NAQVI*[1], ZIA UL HAQ[1], MUHAMMAD ASIF ZULFIQAR[4], ATEEQ UR REHMAN[1], MAHMOUD MOUSTAFA[5] AND UMMAD UD DIN UMAR[1*]

[1]Department of Plant Pathology, Faculty of Agricultural Sciences and Technology, Bahauddin Zakariya University, Multan, Pakistan
[2]Department of Plant Protection, Faculty of Agricultural Sciences, Ghazi University, Dera Ghazi Khan, Pakistan
[3]Faculty of Agricultural Sciences and Technology, Department of Plant Protection, Sivas University of Science and Technology, 58140 Sivas, Turkey
[4]PARC Research and Training Station, Bahauddin Zakariya University, Multan, Pakistan Agriculture Research Council, Islamabad
[5]Department of Biology, Faculty of Science, King Khalid University, Abha, Saudi Arabia
[*]Corresponding author's email: atifnaqvi@bzu.edu.pk; ummad.umar@bzu.edu.pk

Abstract

Citrus is being cultivated throughout the globe and its production is increasing every year. Pakistan is ranked at 13[th]number for citrus production. Worldwide, Pakistan is largest exporter and producer of Kinnow. Current study includes the survey for collection of citrus canker infected samples from different citrus orchards of Punjab. The pathogen *Xanthomonas citri* subsp. *citri* (*Xcc*) was isolated and purified on yeast dextrose calcium carbonate agar medium. Pathogenicity was confirmed by both attached and detached leaf assays. On water agar media, *Xcc* exhibited canker like symptoms on leaves of Crizo citrange, Duncan pumelo and Mexican lime whereas attached leaf assay was done on rough lemon plant and pathogenicity was confirmed after development of symptoms i.e. lesions surrounded by yellow halo. Hypersensitive response of bacteria was confirmed by application of bacterial inoculum on tobacco plant and *Xcc.* produced necrotic spots at the point of inoculums application. Isolated pathogen *X. citri* was characterized on biochemical and molecular basis. For molecular detection, DNA of isolated *Xcc.* was performed by SDS method and also through commercial Kit. Extracted DNA was subjected to polymerase chain reaction (PCR) where six set of primers based on 16S rRNA, pathogenicity and internal transcribed spacer were used for its characterization. Most of isolates showed positive results by all primers while few isolates from Sweet Orange gave no result by primers designed on pathogenicity region. Bacterial isolates produced amplification products by PCR were also subjected to RFLP by *EcoR1* and *Xbal* restriction enzymes. RFLP analysis depicted significant variation in two isolates. Phylogenetic analysis revealed that the Pakistani isolates are closely related to the isolates from Japan, India, Spain and distantly related to the isolates reported from Brazil and USA.

Key words: Biochemical test, PCR, RFLP and Phylogenetic analysis, *Xanthomonas citri* pv. *citri*.

Introduction

Citrus (*Citrus sinensis*) is an important fleshy, juicy and edible fruit plant of Rutaceae family, consists of eleven genera and twenty-seven species (Mabberley, 1997). In the world, total cultivation of citrus fruits is 124.246 million where China ranked 1[st]in citrus production worldwide; Brazil and United States are ranked at 2nd and 3rd respectively, (FAO, 2018). The world production of four major types of citrus fruit is predominated by sweet orange with a contribution of 70%, mandarins with 13% contribution, lemons and lime with 10% and grapefruits and others with remaining 6% (Rogers *et al*., 2013). Pakistan is ranking among the top ten producers of the world for citrus production (Pruvost *et al*., 1992a) with 2.4 million tons of production (GOP, 2018). Pakistan exports about 533 thousand tons of citrus (GOP, 2016). Citrus production is constrained by insect pests, nutritional imbalances, improper cultural practices and numerous diseases which restrict the citrus yield per unit area (Sattar & Hafiz, 1952). Numerous diseases e.g. gummosis, citrus canker, CTV, citrus greening and citrus decline etc. attack the citrus plants. But the worst enemy to the citrus plantations is citrus canker caused by microorganism *Xanthomonas axonopodis* subsp. citri (Awan *et al*., 1992). The disease causes huge loss to citrus fruit and the severity of disease depends upon the type of specie and the predominant climatic conditions (Graham *et al*., 2004). For the quality citrus production, as nutrients management is important including micro- and macro-nutrients which improve and maintain the quality of fruits through different post-harvest techniques at the same time it is also important to protect the fruits against different pests and diseases attack citrus plants including citrus canker, citrus decline, cirrus greening, gummosis and especially protect against the citrus canker which is the most distractive disease for the fruit quality of the citrus species in the major growing areas of Pakistan and other parts of the world.

Many viral, nematode, fungal and bacterial diseases are responsible for low quality and poor yield of citrus. Citrus nematodes, Alternaria brown spot, Citrus canker, *Citrus tristeza virus*, Citrus wither tip and citrus greening are major diseases that attack on citrus cultivars of

Pakistan. Citrus canker disease has unique importance among all citrus diseases; it seriously damages the fruit quality and health of plant. Citrus canker is present in the whole world where citrus is produced (Koizumi, 1985b). Among citrus diseases, citrus canker is the most feared disease which affects all types of citrus cultivars. Extensive damage occurs due to citrus canker and the severity of this infection varies with different citrus cultivars depending upon climatic conditions. In South-East Asian countries, India and Japan this disease is endemic. Citrus canker has spread to almost all countries that produce citrus except Europe (Graham *et al*., 2004). It is believed that citrus canker has introduced from South East Asia and now present in more than 30 countries worldwide. Generally, canker does not occur in an arid citrus growing area, and from some areas it has been eradicated. However, due to the widespread occurrence of the disease, Citrus canker is a continuous threat to citrus growers in canker free areas. On citrus canker, a lot of research is being carried out throughout the world which has been reviewed by (Civerolo, 1984; Rossetti, 1977).

Citrus canker is the most economically destructive plant disease disturbing the citrus all over the world. Causal organism of the citrus canker disease is *X. axonopodis* subsp. *citri* which is a bacterial pathogen. Citrus canker is affecting all citrus cultivars in Rutaceae family worldwide in tropical and subtropical climate having moist conditions. This disease affects all parts of the citrus above the ground and young plants are more susceptible to this disease (Graham *et al*., 1992). This disease is spread more rapidly in climatic conditions were high wind, rain, warm temperature occur at the same time new shoots and fruits emerge (Maloy & Murray, 2001).

Very first time citrus canker was founded in 1910 in the USA (Luthra & Sattar, 1942). After that citrus canker was also observed in all countries that produce citrus (Das, 2003). Symptoms of citrus canker disease appear as necrotic raised lesions on fruits, leaves and also on stem, and severe attack may cause fruit drop, defoliation and may lead to tree decline (Schubert *et al*., 2001). Grapefruit is more susceptible among other cultivars of citrus followed by the other cultivars (Gottwald *et al*., 2002) and the susceptible cultivars are difficult to grow for profitably in the presence of citrus canker (Graham, 2001). The Asiatic type of canker (canker A), *X. axonopodis* subsp. *citri*, caused by a group of strains originally found in Asia, is the most widespread and severe form of the disease. Cancrosis B, caused by a group of *X. axonopodis* subsp. *aurantifolii* strains originally found in South America is a disease of lemons, key lime, bitter orange, and pomelo. Cancrosis C also caused by strains within *X. axonopodis* subsp. *aurantifolii*, only infects key lime and bitter orange. A* strains, discovered in Oman, Saudi Arabia, Iran, and India, only infect key lime (Gottwald *et al*., 2002).

In Pakistan, the citrus industry is declining due to the lower quality and poor availability of fruits that lead to economic losses. Therefore, the present research was designed to study, the identification, biochemical and molecular characterization of *X. citri* strains that are re-emerging in this area.

Material and Methods

Sample collection and pathogen isolation: Samples were collected from different regions of Punjab. The prevalence of citrus canker in citrus orchard was observed by characteristic cankerous symptoms on leaves, twigs and fruits. Samples were collected in plastic envelops and brought in Bacteriology laboratory of Department of Plant Pathology, BZU, Multan. Samples were washed to remove contaminants and preserved in the refrigerator in clean plastic bags. Pathogen was isolated on Nutrient Agar (NA)(Bio Basic Inc. CA) media (Katkar *et al*., 2016). The diseased samples were cut into small pieces along with some healthy portion. The diseased bits of (0.5 to 1cm) were disinfected with 1.00% sodium hypochlorite (NaOCl) solution followed by washing twice in autoclaved distilled water, and then dried on sterilized blotter paper. The bits were then placed in sterilized petri plates containing NA media and kept in incubator at 27 ± 1°C temperatures for 48 hrs. The single purified colonies of that bacterium were streaked with the help of a disinfected wire loop to NA and YDC (Yeast Dextrose Calcium Carbonate) media (Bhure *et al*., 2019) in order to obtain purified bacterial culture (Islam *et al*., 2014). The bright yellow, circular and viscous colonies of the bacterium (*Xcc*) were developed on cultural media plates (Naqvi, 2019).

Quadrant streak method: Single colony of bacteria was isolated by quadrant streak method (Behlau *et al*., 2014). A small amount of bacterial culture was aseptically transferred to the edge of agar plate. Then with the help of sterilized loop spread it in a zig zag manner along one quadrant of the plate from the edge inward approximately 1.5 cm. Continue to streak all quadrants of plate in a similar zigzag manner and loop was sterilized and cooled at each interval. Plates were incubated and colonies were observed after 24 hours of incubation.

Isolation from diseased plants by ooze test: Infected samples were washed thoroughly with distilled water and then surface sterilized with 70% ethyl alcohol followed by 2 or 3 times washing with sterilized water. Samples were cut into small pieces of 2x2 mm discs. These discs were dipped in 5 to 7ml distilled water in the test tubes. The tubes were kept undisturbed for 20-30 minutes. The water turns turbid because of overflowing of bacteria from the discs and turbid bacterial ooze was utilized for bacterial isolation on nutrient agar medium by using streak plate method. The plates were incubated in upward down position in incubator at 27±2°C for 24 to 48 hours.

Growth of Bacteria on differential microbiological media: In order to examine the physiological characteristics of the pathogen, various media viz., nutrient agar, yeast dextrose calcium carbonate agar, peptone sucrose agar and new modified yeast sucrose calcium carbonate nutrient agar were used (Thimmegowda, 2006). A loop full of 48 hours old bacterial culture was serially diluted and from 1×10^5 dilution tube, 100µl of bacterial suspension was

uniformly spread with sterilized L shape spreader to attain well isolated bacterial colonies. The inoculated plates were kept at 28 ± 1°C in the incubator for 48 to 72 hrs. Observations for the growth pattern, colony color, colony shape and appearance were recorded (Table 1).

Pathogenicity test: Koch's postulates are the baseline for declaring any microorganism as pathogen and for *Xac* this pathogenicity test need to be performed. For pathogenicity test, sweet orange was used while for aggressiveness; panel of indicator hosts such as Duncan grapefruit; red blood and kinnow were inoculated with purified isolates of *Xac*. For pathogenicity test, all isolates of *Xac* were grown in liquid media or bacterial cells were scraped off from a freshly streaked agar plate and suspended in sterile distilled water for inoculation into citrus. The concentration was adjusted to 10^8 CFU mL^{-1} and leaves were inoculated through pinprick method and labeled with the respective isolate. Pathogenicity and aggressiveness of all isolates were determined based on already described disease rating scale (Ijaz *et al*., 1999).

$$\text{Number of lesions (\%)} = \frac{\text{Total lesions on leaves}}{\text{Total leaves}} \times 100$$

Bio-Chemical characterization of *X. citri* subsp. *citri*: Pathogenic bacteria that infect plants have diverse biochemical metabolic reactions which may be general or specific for each bacterial pathogen.

Gram staining: Smear of bacterial culture was prepared using a sterile tube, air dried and heat fixed in the shape of a thin film on a glass slide over the flame several times. Smear was submerged for 60 seconds with 0.5% aqueous crystal violet dye (BDH, UK) solution, washed under running tap water and then flushed for 40 seconds with Lugol's iodine solution (Sigma Aldrich, USA). After rinsed in water it was decolorize with 95% ethanol (Merck, Germany) The frost was again cleaned under tap water and coated with 10% Fuchsine Saffron solution (Bio Basic Inc. Canada) for 60 seconds. Smear on glass slide was finally rinsed under tap water and viewed under the compound microscope using immersion oil at a magnification of 100X (Otanga, 2013).

Potassium hydroxide (KOH) test: Potassium hydroxide (KOH) test verified gram staining results (Suslow *et al*., 1982). Solution of 3% KOH was prepared and its drop was put on a clean glass slide. A loop full of bacterial culture aseptically was taken from petri plate to make a viscous suspension in KOH, and mixed continuously for a minute in a circular motion and gently pulled the loop away from the suspension (Ryu, 1940).

Catalase Test: This test was used to determine the presence of catalase enzyme in test organism. 3% hydrogen per oxide solution was added on a clean glass slide. A single colony was picked and mixed in KOH gently to make a smear. Production of bubbles of free oxygen gas indicated the presence of catalase enzymes (Nookongbut *et al*., 2018).

Oxidase test: This test was performed to check the presence of enzyme cytochrome-c-oxidase in electron transport chain of bacteria. In this assay, 1% of Kovac'sreagent (Sigma Aldrich, USA) was poured in core of Whitman filter paper No.1. A loop full of 48 hours old bacterial culture was rubbed gently on a substrate reagent with the help of platinum loop. The purple color appeared within 15 to 60 sec showed that the pathogen was oxidase positive but if no color was developed or developed after 1 min, the pathogen was found to be oxidase negative (Steel, 1961).

Starch hydrolysis test: The media has been prepared by dissolving 15g of fine powdered nutrient agar in 500 ml distilled water. The 2g rice starch was separately added in 10 ml of distilled water. The starch was added in molten NA by continues stirring and progressive heating on hot plate to make a homogenous mixture. After sterilization media was poured in petri plates and bacterial culture was streaked aseptically. The plates were incubated for seven days at 28 ± 1°C. By scraping the bacterial culture, the plates were flooded with 3% Lugol's iodine solution (King & Phillips, 1978).

Hydrolysis of gelatin: This test was used to determine the ability of test bacteria to produce gelatinase enzyme. The media was prepared by adding 2g beef extract 2.5g peptone, 60g gelatin in 500ml distilled water. The media was poured in sterilized test tubes. A loop full of 24 hrs old *Xcc* culture was transferred to test tubes containing gelatin media and incubated at 27°C. The tubes were then placed at 4ºC, and results were determined after 72 hrs (Thimmegowda *et al*., 2008).

Bacteriocin production test: The isolates of *Xcc* were transferred to Petri dishes containing YPDA medium (0.6g peptone, 3g dextrose, 3g yeast extract, 15g agar, and 1000 ml distilled water). After growth at 30ºC for 24 hrs, the colonies were transferred to other Petri dishes containing YPDA medium with a dispenser content two filter paper discs measuring 4 mm in diameter, with each disc corresponding to one strain. After growth of the strains on the culture surface (30ºC, 24 hrs), the Petri dishes were placed in an inverted position under an exhaust hood. Chloroform (1ml) was added to the lid of each dish and incubated for 2 hrs for bacterial inactivation. For the determination of bacteriocin production, the dishes were overlaid with 5ml semisolid melting (45ºC) YPDA medium containing 1ml of a bacterial suspension previously cultured in liquid YPD medium. The Petri dishes were incubated for 24 hrs at 30ºC and the presence or absence of an inhibition halo around the bacteriocin producing colonies was recorded (Baltrus *et al*., 2014).

Lecithinase test: The egg yolk suspension was made from fresh egg**.** The egg was washed under tap water with detergent and then sterilized the egg surface with 70% ethanol for 10 minutes. The egg carefully passed over the flame on spirit lamp 2 to 3 times, and then egg

was broken aseptically and the yolk was separated in sterilized cylinder to prepare 40% v/v emulsion in distilled water. 1.5 ml of 40% v/v emulsion of egg yolk was transferred into molten cooled nutrient agar media before pouring into plates. Bacterial culture was spot inoculated into egg yolk media and incubated at 28 ± 1°C for 72 hrs (Naqvi *et al*., 2016).

MacConkey agar test: This test was performed to determine the enteric gram negative bacteria and to check the lactose fermentation of test organism. The media was prepared by dissolving 24g of MacConkey agar in 500ml distilled water. The pH 7.0 was maintained and media was autoclaved. After pouring, the bacterial culture was streaked on the media and incubated for 24 hours at 37°C (Gottig *et al*., 2009).

Tween-80 Hydrolysis Test: For tween-80 hydrolysis test, autoclave media (5g NaCl, 0.1g $CaCl_2.2H_2O$, 10g peptone, 15g of agar in 1000ml distilled water, pH 7.4) was prepared and 1% of Tween 80 was added to the molten media. After pouring media into the sterilized petri plates, the isolates of *Xac* were streaked and incubated at 27°Cfor seven days. Presence or absence of opaque milky precipitate around bacterial colonies is indication of positive or negative test respectively (Sierra, 1957).

Arginine dihydrolase test: An inoculum from a pure culture was transferred aseptically to a sterile tube of arginine dihydrolase broth. The inoculated tube was incubated at 35-37°C for 24 hours and the preliminary results were determined. The microbe must first use the glucose present to cause the pH to drop. This was indicated by a change from purple to yellow. Once the medium has been acidified, the enzyme arginine dihydrolase is activated. The culture was incubated an additional 24 hours at 35-37 C to allow the microbe to now use the arginine. The final results were then obtained by observing the tube after 48 hours.

Growth on Asparagine medium: All the isolates were allowed to grow on Asparagine medium (Asparagine as sole source of carbon and nitrogen) at 28°C for 48-72 hrs. This is used as a specific diagnostic test for Xanthomonas because they are not able to grow on it while others gram negative and yellow color bacteria like Enterobacteriaceae and many Pseudomonads can grow on it.

Hyper-sensitivity reaction: Hypersensitivity reaction examined the pathogenic nature of the bacterium on Nicotiana tobaccum plants by using the injection infiltration/ penetration technique to noticed the necrosis or tissue break down on leaves of tobacco (Hayward, 1993).

Molecular Detection of *Xanthomonas citri* subsp. *citri*

DNA extraction and PCR amplification: DNA was extracted from fresh bacterial culture by SDS DNA extraction method. A loop full of bacterial colony was transferred in Eppendorf tube and 800 µl of extraction buffer (Tris HCL, EDTA and NaCl) containing 2% v/v β-mercaptethanol and 2% w/v PVP, was added in each tube and mixed gently, 2µl of RNAse A and Protease K were also added. The tubes were incubated at 65°C in water bath for 20 minutes. Tubes were treated with. After cooling 225µl Potassium acetate was added and kept on ice for 1 hour. Centrifuged at room temperature with 12000rpm for 10 min and supernatant was taken in new centrifuge tube. Chloroform and isoamyl alcohol (24:1) was added to the supernatant and mixed. Centrifuged at 12000rpm for 10 min and supernatant was precipitated in to a pellet with equal volume of frozen isopropanol. Tubes were washed twice with cold 75% ethanol. Air dried overnight and 100µl TE buffer was added in each tube. Detection of *Xcc* was carried out by using different primers from different target genes (Table 2).

Ingredients for PCR included 10X PCR buffer 5µL, DNTPs 0.5 µL, MgCl2 1.7µL, Primer 2µL (Forward and reverse), Taq DNA polymerase 0.3 µL, dH2O 14.5 µL and DNA template 1 µL (Table 3).

Specific primer designed for PCR reaction: Due to strain variability the already designed DLH primer was found unable to detect *Xcc*. That's why new primer DLH AF1/DLH AR2 was designed in current study. Specific primers of *Xcc* were designed by Mega X7software. The primers DLH AF1-TTGGTGTCGTCGCTTGTAT and DLH AR2- TGTTGCCTGCGGGCGTCAA were designed to target *pthA* gene of bacterium having complete sequences with Accession Numbers retrieved from NCBI. Designed primers were analyzed by BLAST search to confirm the specificity for detection. The expected amplification products were 222 bp.

Restriction fragment length polymorphism (RFLP): The DNA extracted from all bacterial isolates produced amplification products by PCR through universal primer 27F/ 1492R were used for RFLP analysis. For RFLP 1µl of each enzyme *EcoR1* and *Xbal* (Fermentas, Thermoscientific, USA) was used to digest 5 µl PCR product in a reaction mixture of 2.5µl enzyme buffer, 16.5µl sterilized water followed by incubation in water bath at 37°C for 1-2 hour. After incubation, 2 to 3 drops of bromophenol dye were added to stop the further reaction and each treated sample was visualized under the UV light after gel electrophoresis.

Sequencing and Phylogenetic analysis: PCR products were purified for Sanger sequencing. The obtained sequences were aligned with Mega X7 software and subjected to BLAST analysis on NCBI site (www.ncbi.nlm.nih.gov) and compared with the isolates from different regions submitted in GenBank (Altschul *et al*., 1997). Configuration was-done by using MUSCLE expertise and maximum probability of phylogenetic trees of *X. citri* subsp. *citri* was done using Mega X7 software (Tamura *et al*., 2011).

Table 1. Composition of different media for isolation of bacteria.

Media	Peptone gm/L	Beef extract gm/L	NaCl gm/L	Yeast extract gm/L	Calcium carbonate gm/L	Sucrose gm/L	Glucose gm/L	K_2HPO_4 gm/L	$MgSO_4$ gm/L	Agar gm/L	pH
NA	5	3	8	---	---	---	---	---	---	15	7.4
YDC	---	---	1	10	20	---	20	---	---	15	7.4
NSA	5	3	8	---	---	20	---	---	---	15	7.2
YSCNA	5	3	5	10	20	20	1	0.5	0.25	15	7.4

Table 2. List of Primers used for Detection of *Xanthomonas* Spp.

Sr. No.	Target gene	Primer name	Sequence	References
1.	Pathogenicity	J-pth 1/J-pth 2	5-CTTCAACTCAAACGCCGGAC-3 5-CATCGCGCGCTGTTCGGGAG-3	(Cubero & Graham, 2002)
2.	Pathogenicity	DLH 1/DLH 2	5-TTGGTGTCGTCGCTTGTAT-3 5-CACGGGTGCAAAAAATCT-3	(Hartung, Daniel, & Pruvost, 1993)
3.	16S rDNA	FD1/RP2	5-AGAGTTTGATCCTGGCTCAG-3 5-ACGGCTACCTTGTTACGACTT-3	(Weisburg, Barns, Pelletier, & Lane, 1991)
4.	16S rDNA	P16SF1/P16SR2	5-GCCAGCAGCCGCGGTAATAC-3 5-GCGCTCGTTGCGGGACTTA-3	(Adriko *et al.*, 2012)
5.	*FyuA*	X-fyuaF/X-fyuaR	5-GCCGGTGGACTACGATTGGAATTA-3 5-GTCGCGGCGCCACTTCA-3	(Adriko *et al.*, 2014)
6.	Internal transcribed spacer	X-ITS F3/X-ITS R2	5-GGCGGGGACTTCGAGTCCCTAA-3 5-CTGCAGGATACTGCCGAAGCA-3	(Adriko *et al.*, 2014)

Table 3. PCR profiles with each primer.

Primer	Initial denaturation	Final denaturation	Annealing	Extension	Cycles	Final extension
J-Pth	94°C	95°C/30 Sec	54°C	72°C/30 Sec	30	72°C/10 Min
DLH	94°C	95°C/30 Sec	50°C	72°C/30 Sec	30	72°C/10 Min
FD1/RP2	94°C	94°C/30 Sec	57°C	72°C/2 Min	35	72°C/10 Min
P16S	94°C	94°C/30 Sec	58°C	72°C/45 Sec	35	72°C/10 Min

Results

Isolation and Purification of *Xanthomonas citri* subsp. *citri*: The unique opaque yellow colonies of *Xac* exude out from leaves placed on nutrient agar plate's after48 hours of incubation at 30ºC temperature. Yellow raised colonies are due to Xanthin produced by members of genus *Xanthomonas*. Along with visual observation, the colony color of all *Xac* isolates varied from yellow to light yellow. The size and shape of colonies was small to medium, convex and mucoid (Table 4).

Pathogenicity tests

Detached leaf assay: The results showed that *Xcc* inoculum, when inoculated on detached leaves of different citrus cultivars i.e., Mexican Lime, Dunkin Pumelo and *Carrizo citrange,* produced raised brown lesions surrounding by yellow halo on adaxial surface and water soaked lesion on abaxial surface after 7 to 10 days of inoculation. These symptoms were identical to Asiatic citrus canker hence the pathogenicity of *Xcc* isolates was confirmed.

Attached leaf assay: Pathogenicity test was carried out by using pin prick and inoculum spray method**.** The results showed that the prepared inoculum of isolate *Xcc* 1 and *Xcc* 2 produced typical canker symptoms on host plants of *Carrizo citrange* and Dunkin pumelo. After 14 days of inoculation, symptoms began as spots and gradually turned into pustules with a raised margin around the halo spot. Brown callus were also found around the inoculation site on the lower side of the leaves after 14 days of inoculation at 28ºC. Mexican lime and *Carrizo citrange* were inoculated by pin prick method while spray inoculation was done on Dunkin pumelo and Rough lemon. The results showed that pin prick method was more successful than spray inoculation method (Table 5).

Starch hydrolysis test: A clear golden zone was observed at the surroundings of bacterial growth after 7 days of incubation at 27ºC when media containing starch was discolored with Lugol's iodine. *Xcc* hydrolyzed the starch and indicated the zone. Positive results were observed by all isolates of *X. citri*subsp.*citri.*

Biochemical characterization

Gram staining: The gram negative bacteria retain the color of saffranin and appear as pink to red whereas, the gram positive bacteria retained the color of crystal violet and also seen under 100X objective with oil emersion. Bacterium was like very minute rods which were stained pink red color as shown in (Fig. 1A).

KOH test: All isolates of *Xcc* were able to form thread like string when treated with 3% Potassium hydroxide solution which indicates that the bacterium was gram negative (Fig. 1B).

Catalase test: Bubbles were formed by bacteria by producing the catalase enzyme which indicates that *Xcc* protect itself from toxic products of oxygen metabolism. Bubbles formation indicates that bacteria were gram negative (Fig. 1C).

Oxidase test: All bacterial isolates gave negative result in response of oxidase test confirming the absence of cytochrome-c-oxidase enzyme (Fig. 1D).

Starch hydrolysis test: A clear golden zone was observed at the surroundings of bacterial growth after 7 days of incubation at 27ºC when media containing starch was discolored with Lugol's iodine. *Xcc* hydrolyzed the starch and indicated the zone. Positive results were observed by all isolates of *X. citri.* subsp. *citri* (Fig. 1E).

Gelatin hydrolysis test: Nineteen isolates were able to hydrolyze the gelatin medium and converted the gel into liquid. One isolate showed negative response as that was too old culture and hence gave negative result. The control tube remained negative i.e. semi solid gel (Fig. 1F).

Table 4. Growth pattern of *Xcc* on different nutritious media.

Media	Colony morphology			Growth
	Color	Shape	Appearance	
NA	Bright yellow to yellow	Circular	Flattened	Fast growing
YDC	Light yellow to bright yellow	Convex	Mucoid and slightly raised	Medium growing
NSA	Light yellow and shiny	Irregular	Flattened and mucoid	Medium growing
YSCNA	Yellow to bright yellow	Circular / irregular	Glistening and flattened	Fast growing

Table 5. Pathogenicity and aggressiveness of *Xcc* isolate on attached and detached leaf assay.

Isolates	Host plant	Pathogenicity test		Virulence
		In vitro	*In vivo*	
Xcc 1	Mexican lime	++	++	Highly susceptible
Xcc 2	Dunkin pumelo	++	++	Highly susceptible
Xcc 3	*Carrizo citrange*	+	+	Susceptible
Xcc 4	Rough lemon	-	-	Resistant
Xcc 5	Mandarins	-	-	Highly resistant

If No of lesions > 5: Pathogenic (+), > 10: Highly pathogenic (++), < 5: Non Pathogenic (-).

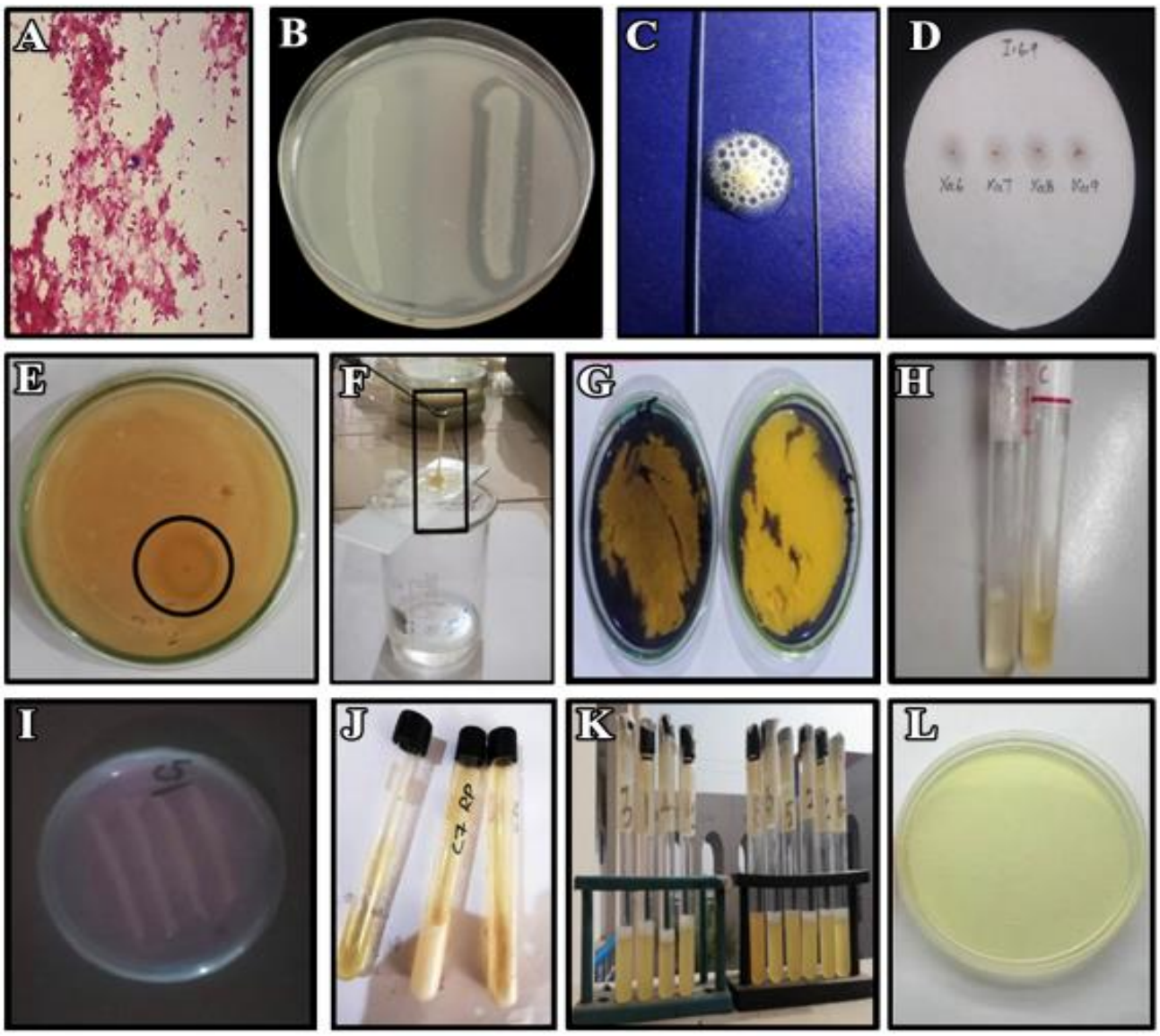

Fig. 1. Biochemical tests performed for the detection of various activities of bacterium (A) Gram staining of Xcc (B) Lecithinase Test (C) Catalase Test (D) Oxidase Test (E) Bacteriocin Production (F) KOH test (G) Starch hydrolysis (H) Gelatin Hydrolysis (I) MacConkey Agar (J) Tween 80 hydrolysis (K) Arginine dihydrolase (L) Growth on Asparagine media.

Bacteriocin production test: *Xcc* isolates as producer and Asiatic canker strains as indicators indicated the best production observed after 48 hours of incubation. The isolates of *Xcc* were also screened for capacity to inhibit the growth of Asiatic canker isolates. *X. citri* subsp. *citri* strain C9 was inhibited by Asiatic citrus canker isolates. The inhibition zones were ranged from 3 to 6 mm from edge of *Xcc* colonies (Fig. 1G).

Lecithinase test: Diffused zones of white and opaque were observed around the bacterial colonies in culture plates. 17 out of twenty Xcc isolates showed positive response and three isolates were unable to produce diffused zones and hence indicated negative result (Fig. 1H).

MacConkey agar test: *Xcc* isolates when streaked on MacConkey Agar indicated the growth of colonies by producing the lactose hence confirming that isolates have gram negative response. Only two isolates were unable to grow on MacConkey agar (Fig. 1I).

Tween 80 hydrolysis test: All twenty isolates of *Xanthomonas citri*subsp.*citri* were observed able to hydrolyze the tween 80, showing the existence of dense milky white region around the bacterial colonies after incubation at 27ºC for 7 days. Hence, confirming the characteristic property of genus *Xanthomonas* (Fig. 1J).

Arginine dihydrolase test: The results showed that the stab method-inoculated different isolates of *X. citri*subsp.*citri* were unable to utilized arginine, no red color appeared after incubation and found the Arginine dihydrolase test to be negative (Fig. 1K).

Growth on Asparagine medium: Growth on Asparagine medium test was done to indicate the growth of *Xanthomonas* on Asparagine medium, but they were not able to grow on it while others gram negative bacteria can grow on it (Table 6, Fig. 1L; Fig. 1A-L).

Table 6. Responses of the isolated bacteria in different biochemical test media.

Biochemical tests	Reaction	Appearance
Gram reaction	**-ve**	Small, rod, pink color colony
KOH test	+ve	Thread like slime
Catalase test	+ve	Bubble formation
Kovacs' Oxidase	**-ve**	No color
Starch hydrolysis	+ve	Clear zone in Iodine Stained medium
Gelatine hydrolysis	+ve	Liquefy the gelatin medium
BPT*	+ve	Inhibition zone around the colony
Lecithinase Test:	+ve	Diffused opaque zone around the colony
MacConkey Agar Test:	+ve	No pink color around the colony
Tween 80 hydrolysis	+ve	Milky White Precipitate
Arginine dihydrolase	**-ve**	Slants remain same
Growth on asparagine medium	**-ve**	Slants remain same
Hyper-sensitive Test	+ve	Necrotic spots on the inoculated leaves

*Bacteriocin production test

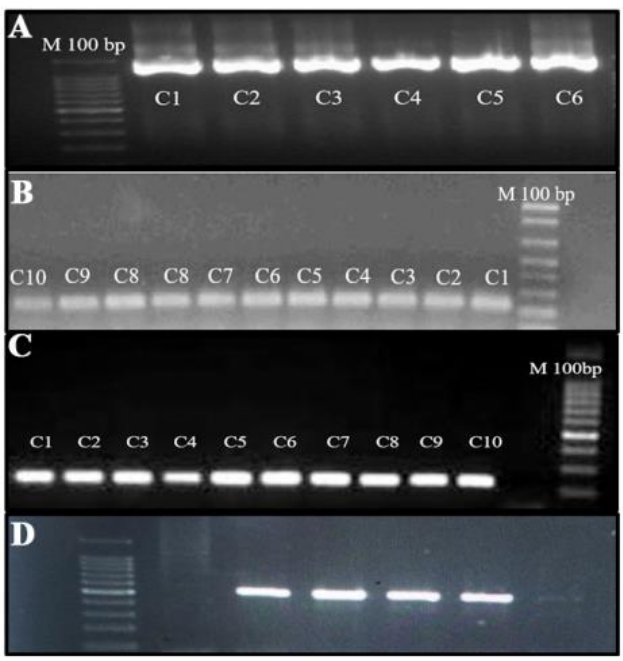

Fig. 2. 1.5% TBE agrose gel electrophoresis showing (A) Amplicon size of 1492 bp with primer 27F/1492R (B) Fragment of 222bp newly designed specific primer of DLH (C) Amplification of 197bp fragment with specific primer of J-pth (D) 596bp fragment with primers P16S. Lane M 100bp DNA ladder.

PCR based detection of *Xcc*.: Different primers were used for the detection of *Xcc.* All the collected samples were first tested with universal primers and then with specific primers. All the isolates were tested with universal primers i.e., Fd1/rp2 and 17F/1492R showed amplicon size 1500bp and 1492bp respectively. The isolates which showed positive result for universal primers were further tested by specific primers. Different specific primers based on different gene region were tested, but the primer DLH based on pth-A gene did not amplified the required band for any isolate so a new primer was designed based on pathogenicity region of the bacteria. Expected band of DLH was amplified with 222bp fragment were obtained with this new primer for almost all isolates of citrus canker from different citrus cultivar and then PCR products were visualized on 1.5% agrose gel electrophoresis (Table 7, Fig. 2A-D).

Restriction fragment length polymorphism (RFLP): All the bacterial isolates used in this study produced amplification products using different primers. PCR products were restricted with two enzymes separately. Both the enzyme EcoR1 and Xbal restricted the bacterial strains, out of five strains the enzyme EcoR1 restricted the four strains at the same site except the one strain C3while the enzyme Xbal restricted the strain C3. The amplified and digested fragments showed that bacterial isolate C3 is might be a different strain then all other strains (Fig. 3A-B).

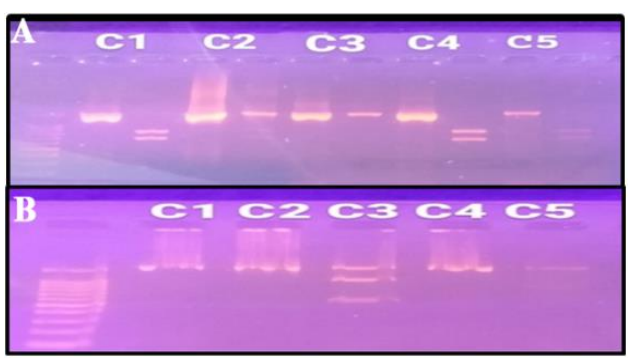

Fig. 3. RFLP profiles of different bacterial strains of Xcc obtained after digestion with the restriction enzyme (A) Eco R1. Lanes 1, 3, 5, 7, 9 are amplified PCR product, lanes 2, 4, 6, 8, 10 are restricted PCR products (B) Restriction enzyme Xbal. Lanes 1-5 are restricted PCR product.

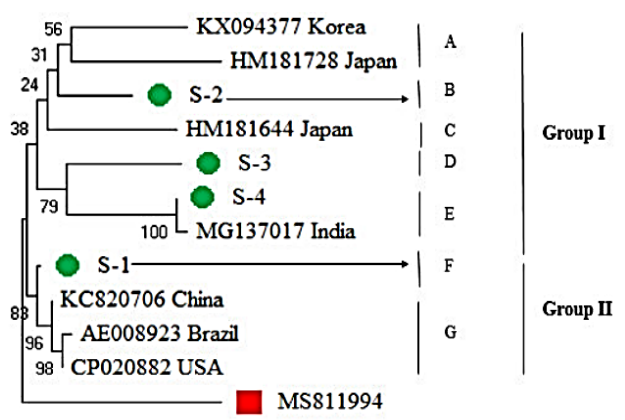

Fig. 4. Phylogenetic tree of X. citri subsp. citri based on 16S rDNA region.

Phylogenetic analysis: MEGA X software was-used for the construction of Phylogenetic analysis, isolates collected from Pakistan were analyzed along with a sequences of other isolates deposited in NCBI. The tree was constructed keeping 1000 bootstrap value. The maximum likelihood tree depicted that the *Xcc* isolate detected from Pakistan closely related to the isolate reported from Japan (HM181728), India (MG137017), Spain (KC820706) and distantly related to the isolate reported from Brazil (AE007923) and USA (CP020882). Generally, the distribution of the phylogenetic tree shows developed and two major groups I and II (Fig. 4). Group I is further divided into subgroup A, B, C, D and E. Group II is also further divided in to subgroup F and G. The new identified isolates from Pakistan falls in subgroups B, D, E and F along with isolates of China, Japan, Korea and India, Grape Fruit (*Citrus paradisi*) showed a 100% Sequence identity with isolate of India (Mg137017) and Brazil (AE008923). Lemon (*Citrus limon*) showed 99% identity with isolate of USA (CP020882) and 98% sequence identity with an isolate of China (KC820706). All isolates of Pakistan showed high sequence identity with other isolates of *Xcc. Agrobacterium tumefaciens* was used as outgroup (MS811994) (Fig. 4).

Table 7. Detection of *Xanthomonas citri* subsp. *citri* through different primers.

Sr. No.	Location	Host	No. of samples	+ve for J-pth	+ve for DLH	+ve for X-ITS	+ve for X-Fyua	+ve for Fd1/rP2	+ve for P16S
1.	Multan	Grapefruit	2	1	0	0	2	2	2
		Lemon	8	2	0	3	5	8	8
		Kinnow	3	0	2	2	3	3	3
		Sweet orange	1	0	0	0	1	1	1
2.	Khanewal	Lemon	5	1	0	1	4	5	4
		Kinnow	3	2	0	1	1	3	3
		Grapefruit	2	0	1	0	1	2	1
3.	Layyah	Grapefruit	1	1	0	1	1	1	0
		Lemon	1	0	0	1	1	1	1
		Lime	3	1	1	2	3	3	2
		Sweet orange	2	0	0	1	2	2	2
		Kinnow	2	0	1	1	2	2	1
4.	Rahimyar Khan	Kinnow	5	2	1	4	4	5	5
		Lemon	2	1	2	1	1	2	2
		Sweet orange	3	0	0	2	2	3	3
5.	Sargodha	Kinnow	4	1	2	3	3	4	3
		Sweet orange	2	0	1	2	2	2	2
		Grapefruit	5	2	3	4	4	5	4
		Lemon	3	1	2	1	2	3	3
		Lime	2	0	0	0	2	2	1
6.	Toba Tac Singh	Kinnow	3	2	0	2	1	3	3
		Lemon	2	1	1	1	2	2	2
7.	Sahiwal	Grapefruit	3	2	0	2	3	3	3
		Kinnow	4	2	3	3	2	4	4
		Lemon	2	1	1	1	2	2	2
		Lime	2	1	2	1	2	1	2
		Feutrell early	1	1	1	1	1	0	1
		Sweet orange	2	2	2	1	2	0	2

Discussion

Canker is one of the most economically devastating bacterial disease of citrus plant caused by *X. citri* subsp. *citri* occurs in large areas of the world's citrus growing countries of the world including Pakistan (Civerolo, 1984; Das, 2003). Now this disease is emerging day by day which may cause massive losses to the citrus industry in the coming years. Under favorable circumstances, it is difficult to manage disease in traditional mode once the disease is established. Thus, an appropriate management strategy is required to control the disease. The *Xcc* was identified by conducting studies on its morphological, biochemical and cultural features as per standard microbiological procedure. Twenty isolates were studied on the basis of their biochemical characteristics and were detected by different primers. Characteristics symptoms of bacterial citrus canker were observed during the collection of diseased samples as thin, circular, watery, translucent spot on the lower and upper surface of the leaf. The spots are grayish, spongy and hard corky surface with the progression of the disease. Similar symptoms were recorded by different researchers in various citrus cultivars while working on citrus canker disease (Das, 2003; Tesha, 2018; Naqvi *et al*., 2022; Ali *et al*., 2023).

It is obvious that nutrient agar is a general purpose medium for the isolation of the any bacterial species. The isolation of bacterium *Xcc* formed yellow mucoid

colonies on NA and YDC agar media after incubation of 24-48 hours at 28±2ºC. The colonies were similar with the previous results studies of (Arshadi *et al*., 2013; Katkar*et al*., 2016). Typical yellow single colonies were purified by sub-culturing a single colony of each isolate, giving convex round and mucoid colonies on YDC agar medium (Izadiyan & Taghavi, 2020). Our results also support the findings of Abhang (2015) who used NA media for culturing of seven different isolates of *Xcc* (Isokar *et al*., 2020). Schaad *et al.,* (2001) also previously reported the phenotypic characteristics of *Xcc* and current study supports their results.

Pathogenecity test was carried out for confirmation of pathogen and found different pathogenic behavior on panel of host. Pathogenicity tests were done on detached leaves of *Mexican lime*, *Duncin Pumelo* and *crizzo citrange*, and on Lemon plants for the confirmation of pathogenicity. It was observed that Mexican lime leaves were more susceptible to *Xcc* as compared to *Duncin pumelo* and *crizzo citrange*. Yellow Hallo was noticed on rough lemon plants after 10-15 days of inoculation and our results confirmed the earlier studies on pathogenicity tests of *Xcc* by (Francis *et al*., 2010), and they revealed that the lesions produced on detached and attached leaves with *Xcc* resembled those of canker formed by natural stomatal infections in the field. Our results were also coincide with previous results of (Lin *et al*., 2008) and (Al-Saleh *et al*., 2014) who performed the pathogenicity of purified *Xac* strains on detached grapefruit leaves of Duncan (Al-Saleh *et al*., 2014). Albeit, this study is baseline for formulating appropriate management strategy in controlling citrus canker disease which has already become a threat to the citrus production in Pakistan.

For the determination of hypersensitive response (*hrp*) by *Xcc*, the bacterial suspension was applied on tobacco leaves as an inoculum. *Xcc* showed the *hrp* response by forming necrotic spots around the point where inoculum was applied and results of present study are same as reported earlier by (Razaghi *et al*., 2012)as they inoculated *Xanthomonas citri* subsp. *malvacearum* on non-host plants i.e., tobacco, they observed the yellow necrotic spots after some days of inoculation.

The bacterium was identified and characterized on the basis of biochemical tests viz., gram staining, KOH test, Catalase test, Kovacs' Oxidase, Starch hydrolysis, Gelatine hydrolysis, Lecithinase Test, MacConkey Agar Test, Tween 80 hydrolysis, Arginine dihydrolase, Growth on asparagine medium as mentioned by (Jabeen *et al*., 2016). The gram staining results showed that all twenty isolates of *Xcc* were found as gram negative and current study supported the results of (Arshiya *et al*., 2014) and they concluded that *Xanthomonas axonopodis* is bacteria which give gram negative response and turns color to pink. Catalase test was also observed to be positive when 3% hydrogen per oxide solution was added in the bacterial culture, formation of bubbles of free oxygen gas indicated the presence of catalase enzymes in the bacteria. Our results confirmed the work of (Mubeen *et al*., 2015).

As a result of KOH test, all isolates made a threaded loop confirming the positive result, this result was similar to (Kharde *et al*., 2018), they reported that all the isolates formed thread like slime after addition of KOH in bacterial culture. In starch hydrolysis test the bacteria were found to hydrolyze the starch in the media and isolated *Xcc* made a visible distinguishable zone which was identical to the results of (Joko *et al*., 2000)who reported the hydrolyzation of starch at two days incubations. Cultured bacterium responded a positive result i.e., formation of milky white dense zone at the surroundings of *Xcc* colonies. Isolated pathogen condensed the gelatin medium and hence positive for gelatin liquefaction test as done previously by (Lin *et al*., 2005).

Lecithinase test was also observed to be positive which showed an obscure halo around the bacterial colonies when grown on egg yolk agar medium, hence confirmed the activity of bacterial lecithinase to break down the egg yolk lecithin to insoluble diglycerides (Esselmann & Liu, 1961). Same results were observed by (Naqvi *et al*., 2016) when the opaque diffused white zones around the *Xcc* colonies appeared. When *Xcc* isolates were streaked on MaCconkey agar, the colonies were grown by the production of lactose enzyme which is a typical character of gram negative bacteria. (Fu & Tseng, 1990) previously done this test on the strains of *X. campestris* and they disclosed that bacteria were giving rise to red colonies, which indicates the utilization of lactose. Our results coincide with the findings of (Muneer *et al*., 2007) who described that *Xcc* was a gram negative bacterium for gram reaction, also negative for lecithinase and oxidase test but tween-80, starch hydrolysis, acid production and anaerobic nature on solid agar based media varied amongst the different strains of the bacterium. Similar studies were also carried out by (Reddy & Ou, 1976) who showed that no significant biochemical groups were found in a collection of forty-two different strains which were gathered from ten Asian countries. Hence, we understand that the absurdity found in wording can be due to the reason of cultural infectivity or dissimilarities in experimental methods due to the human error. All isolates of *Xcc* were found unable to produce an enzyme cytochrome-C, and gave negative response but positive in the case of other *Xanthomonas* species. Bacteriocin production test conducted to check the cross protection ability of *Xcc,* some of the isolates were kept producers while other isolated were subjected to indicators. The typical isolates of Asiatic bacterial canker suppressed the growth of isolates which were kept as indicators. The results confirmed the work of (Ali *et al*., 2017; Islam *et al*., 2014; Mubeen *et al*., 2015) who used several biochemical tests to identify and characterize different strains of citrus canker causing bacteria. In the current research, after confirmation; the bacterium was further studied for its cultural and physiological characteristics and it was grown on four different solid agar based medium to determine its growth habits (Ali *et al*., 2023). The new modified yeast sucrose calcium carbonate nutrient agar medium (devised by us) showed luxurious growth of pathogen and showed maximum recovery of the bacterial colonies because it was rich in sucrose and glucose which has been discovered to be the best carbon sources afterward galactose, fructose, mannose and mannitole as described by (Thimmegowda *et al*., 2008) while starch, xylose and lactose were considered as the inferior carbon sources (Jabeen *et al*., 2016).

Our results coincide with (Watanabe, 1963) who observed that sucrose ($C_{12}H_{22}O_{11}$) was the most beneficial carbon source for the excellent pathogen growth followed by mennose, galactose, glucoses, and maltose. Similarly, our results are in accordance with (Sun *et al*., 2004) and (Jabeen *et al*., 2016) who found glucose and sucrose as the best carbon sources for the lush growth of the pathogen. Besides this, (Watanabe, 1963) found succinic acid as another good carbon source for the luxuriant growth of pathogen while considering the nitrogen sources, l-glutamic acid was one of the most favorable one followed by l-cysteine. We suggest that while preparing the artificial medium for the bacterial growth, maximum weightage should be given to sucrose rather than the glucose because surely it will provide more carbon sources to the bacterium as compared to the glucose. It is evident by the chemical formula of the both compounds which showed sucrose to possess double carbon atom i.e., twelve carbon atoms ($C_{12}H_{22}O_{11}$) while glucose have only six carbon atoms ($C_6H_{12}O_6$) (Naqvi *et al*., 2022).

In the current research the bacterium produced light yellow to bright yellow, circular to irregular, slightly raised to flattened and slimy to glistening bacterial colonies with the average size ranging from 1.0 to 5.2mm as described by (Schaad *et al*., 2001) that the yellow color and mucoid colonies is the characteristic of Xanthomonads because of the existence of extracellular polysaccharides (EPS) in sugar containing media. Our results are parallel with (Jabeen *et al*., 2016) who described that diseased leaf samples of canker conferred bright yellow, circular, mucoid and smooth colonies of the bacteria when plated on yeast dextrose calcium carbonate agar medium. Similar studies were performed by (Jansson *et al*., 1975) who proved peptone sucrose agar medium to be the best for the physiological experiments of the tested bacterium.

For molecular detection of isolated *Xcc isolates*, DNA was extracted by SDS-PAGE method and by using (Solarbio universal genomic DNA extraction kit from Beijing Solarbio Science & Technology Co, Ltd). The Extracted DNA was subjected to chain reaction for detection. Different primers based on 16S rDNA, Pathogenicity, *Fyua* and ITS genes, were used during this study. Three set of primers were designed on 16S rDNA region i.e., 27F/1492R designed by (Lane, 1991) and used to detect *Xcc*by (Ibrahim *et al*., 2019), all twenty isolates gave same band size by this primer as reported already. fd1/rp2 was also designed on 16S rDNA region by (Weisburg *et al*., 1991) and tested for *Xcc* species detection by (Mikiciński *et al*., 2020). Another primer P16S based on same region was also used in our study and we got amplification size of 596 base pairs, earlier in 2016, this primer was used to test *Xanthomonas* in multiplex PCR reaction mixture by (Adriko *et al*., 2016). X-ITS primer based on ITS region of *Xanthomonadae* family was used for DNA amplification and few of isolates gave positive results. This primer was designed by (Adriko *et al*., 2014) and used to detect *Xanthomonas* species by (Zarei *et al*., 2019).

Primers J-pth1/J-pth2 was tested and designed on pathogenicity gene *pthA* of *Xcc* (Cubero & Graham, 2002) and gave the amplification of 197 base pairs and current study supports already reported results. In last, DLH primer was used in our study for genomic amplification on basis of pathogenicity gene, (Hartung *et al*., 1993) and reported the amplicon size of 222 base pairs. In this study, bacterial strains that were isolated from different citrus cultivar were detected by RFLP analysis, which showed that the one of the isolated strain of bacteria was different from the other strains. Similar research was done by (Ah-You *et al*., 2007) who suggested that strains should be reclassified within the *X. citri* specie.

Xcc isolates detected from Pakistan are closely related to the isolates reported from Japan, India, and Spain and distantly related to the isolates reported from Brazil and USA. Generally, the phylogenetic tree is divided into two major groups I and II. The new identified isolates from Pakistan falls in subgroups B, D, E and F along with isolates of China, Japan, Korea and India.

Conclusion

Morphological and biochemical characterization is the baseline to perceive pathogen population, its epidemiology and biological phenomenon, which enhances aggressiveness of pathogen. Therefore, the pathogen *X. citri* subsp. *Citri* responsible for canker disease was isolated from different areas and different citrus cultivars, the identifiedand finally characterized on biochemical and molecular basis. Molecular detection by different primers confirmed the identity of the bacterium *Xcc.*

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