The Journal of Animal & Plant Sciences, 29(1): 2019, Page: 117-124
ISSN: 1018-7081

MORPHOLOGICAL, BIOCHEMICAL AND GENETIC CHARACTERIZATION OF CITRUS CANKER PATHOGEN (*XANTHOMONASAXONOPODIS*) FROM CITRUS CULTIVARS OF PUNJAB, PAKISTAN

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ABSTRACT

The objective of the present study was to identify citrus canker pathogen (*Xanthomonas axonopodis*) and disease severity among different citrus cultivars collected from citrus growing area (CRI) of Punjab, Pakistan. Morphological, biochemical and molecular markers characterized the pathogen. Total 95 samples were collected from grapefruit (*C. paradis*), kumquats (*C. japonica*) and lemon and limes (*C. limon*) and subjected to biochemical analysis by API strip. Three different primer sets targeting 16SrDNA and internally transcribed spacer region (ITS) were used to check genetic variability by PCR. The infectious pathogen colonies were found to be small, yellow, mucoid and gram negative by gram staining. The oxidase, urease tests were negative and casesin hydrolysis, citrate utilization, methyl red test, catalase tests were positive for the pathogen via API strips. All the collected samples were positive for the disease by PCR and the disease severity was also measured by percentage mean. The highest disease severity (82%) was observed in grapefruits followed by lemon and limes (70%) while the lowest (30%) was in kumquats. The present results revealed that citrus cultivars have variability towards the susceptibility of citrus canker and phylogenetic analysis revealed that A type citrus canker is prevalent in selected region of Pakistan.

Keywords: citrus canker, biochemical tests**,** *Xanthomonas axonopodis,* Biochemical assay, PCR.

INTRODUCTION

Citrus is an important fruit crop with annual worldwide production of 2.4 million metric tons in 2015-16. In Pakistan, citrus fruits are grown on an area of 206,569 hectares with annual production 2.36 million tons in 2015-16 and more than 98% of citrus fruit is produced only in Punjab (Pakistan Bureau of Statistics, 2016).

The yield and quality of citrus fruits faces many production constraints including diseases caused by fungal, viral, bacterial and nematode pathogens. Citrus nematode, citrus gummosis, citrus canker, citrus greening and *citrus tristeza* virus are commonly prevalent in Pakistan. Among these diseases, citrus canker affects the plant growth and fruit quality in all citrus growing areas of the world (Pruvost *et al.,* 2002). The infectious agent of the disease is *Xanthomonas axonopodis* (Sahi *et al.,* 2007). From Pakistan it was first reported in Punjab and after which it becomes epidemic throughout the country. All the cultivars are susceptible for citrus canker such as grapefruit, sweet oranges, lemons, limes, rootstocks and their hybrids (Gottwald *et al.,*2002).

Citrus canker has been grouped into three types A, B, C based on host rang and geographical distribution (Graham *et al,* 2004). The Asiatic form A caused by *Xac* is most virulent form throughout the world including Pakistan (Canteros *et al.,* 2017). The citrus canker B (false canker) caused by *X. citri*pv. *Auranti folii* was reported in Argentina, Uruguay and Paraguay on lemon (*Citrus limon*). The cancrosis C also caused by *X. citri*pv. *aurantifolii* has been found only in São Paulo state in Brazil. The two known hosts of C type canker are mexican lime and sour orange (Schubert *et al.,* 2001).

The symptoms caused by citrus canker are pustules to necrotic lesions consisting of erumpent corky tissues surrounded by oily or water-soaked margins and yellow halo. The disease severity causes defoliation, dieback, premature fruit drop and blemished fruit. Lesions on the fruit are most economically important damage, which makes the fruit not acceptable for fresh market, and have very little price. The integrated control measures of the disease are the production of disease free stock, copper sprays and decrease in pathogen spreading caused by leaf miner or wind (Das, 2003; Schubert and Sun, 2003 ; Graham *et al.*, 2004).

Early rapid and specific detection of canker-associated bacterium is important for plant disease management (Mansfield *et al.,* 2012). Without diagnosis of the disease, it cannot be control at specific time (Mccartney *et al.,* 2003). Conventional assays to detect and identify bacterial pathogen involved isolating the pathogen on selective media and its identification through biochemical analysis (Gottwald *et al.,* 2001). But molecular based methods can overcome many shortcomings in conventional methods. PCR based molecular markers are generally more specific and much reliable (Grote *et al.,* 2002).

Keeping in view the reduction in market value of citrus due to citrus canker in Pakistan, the present study was aimed to provide knowledge about the prevalence of CBC in Pakistan citrus varieties. By the use of the biochemical assay and genetic diversity by PCR, 95 citrus samples were screened for *Xac* isolates. Genetic diversity by PCR assay targeting different genomic regions of the pathogen was first studied in Pakistan.

MATERIALS AND METHODS

Collection of samples: For the detection and assessment of incidence of CBC ninety-five different citrus samples were collected from "Citrus Research Institute (CRI), Sargodha, Pakistan" in summer (June, July, August) 2016. The symptoms were yellow to dark brown lesions as shown in Fig 1. The leaves were collected in labeled polythene bags and stored at 4°C prior to use.

Isolation of pathogen and preparation of Glycerol stocks: The canker lesion along with 2mm of the peel around was excised from infectious citrus leaves and left for 10 minutes in sterile water for the release of the bacteria. The extract of the tweezed bacteria was streaked on the selective media GYP (Galactose yeast peptone: peptone 0.5g, yeast extract 0.5g, agar-agar 1.5g, glucose 1g, distill water 100ml) media for 2 days on 28°C. Suspected single colonies of *X. axonopodis* were further purified and stored in 50% glycerol stocks at -80°C.

Morphological Characterization: The morphological characteristics such as colony appearance, shape, margins and pigmentation were used to identify *Xac* pathogen associated with citrus canker. The bacterium shape was identified by the standard gram's staining technique. The bacterium shape, size, arrangement and staining reaction was observed under 100X microscope.

Biochemical tests: The pathogen was identified biochemically by performing twenty different biochemical tests using API-E 20 strip. The strips were used by following the manual described by the bio Merieuxs API kit.

DNA Extraction: Two different methods were optimized for the DNA extraction. First method was used for the *Xanthomonas* cells grown *in vitro*, while the method II was used for the extraction of total DNA from the canker lesions on leaves from all citrus varieties. Each method is described below:

In first method total purified genomic bacterial DNA was isolated by phenol chloroform method from *in vitro* cultured bacteria Sambrook *et al.* (1989). In second method citrus leaves with canker lesions were cut and washed with 70% ethanol. The total genomic DNA was extracted by following CTAB (hexa decyl trimethyl ammonium bromide) method by Murray& Thomson (1980) with some modification (Naz *et al.,* 2015).

DNA quantification: The DNA quantification was done by measuring the optical density (OD) of DNA by UV/VIS Spectrophotometer (Optizen NANO Q).

PCR amplification: Samples were analyzed by conventional PCR Primus-96 Thermal cycler by three different primer sets that amplified 16S rDNA and ITS region of the citrus canker pathogen (Table 2). The Dream Taq green PCR master mix (2X) Cat#1018 was used which comprises of 2X Dream Taq Green buffer, 0.4 mM each dNTPs, 4 mM $MgCl_2$. The final concentration of primer and template from cultured cells and plant material was 0.4uM and 15ng respectively. The PCR thermo cycling conditions were an initial denaturation steps at 94°C for 3 min followed by the 36 cycles of denaturation at 94°C for 30s, annealing at 50°C for 30s and elongation at 72°C for 45s, followed by extension at 72°C for 10 minutes. The amplified product was subjected to electrophoresis through 1% agarose gel and analyzed in gel documentation system (Wealtec, Dolphin)

Cloning and Sequence analysis: The amplified PCR products were purified using GF- 1 Gel recovery kit (K# GF-GF-100), cloned into pTZ57R/T vector (Invitrogen) and transformed into *E.coli* competent cells. After cloning the plasmid was purified by GF- 1Plasmid purification kit (K#GF-PL-100). Recombinant plasmid was confirmed by colony PCR and restriction digestion. The plasmid was sequenced by ABI Prism DNA sequencer (Perkin-Elmer, USA). The sequences were aligned and compared with reference sequences available on GenBank database by BLASTn (https:// blast. ncbi. nlm. nih.gov/Blast.cgi.).

Phylogenetic analysis: The Phylogenetic analysis was done using the neighbor joining method in MEGA7 (Kumar *et al.,* 2016). The reference sequence from GenBank and plasmid sequence was used for evolutionary analysis and *Candidatus Liberibacter asiaticus* sequence from GenBank (Accession No. KY038851.1) was used as out-group. The tree with bootstrap values was created from 500 replicates taken to represent the evolutionary history and branches reproduced in less than 50% bootstrap replicates were collapsed. The evolutionary distances were calculated by p-distance method and the analysis involved 7 nucleotide sequences. All positions containing gaps and missing data were eliminated. A total of 376 positions were included in the final dataset.

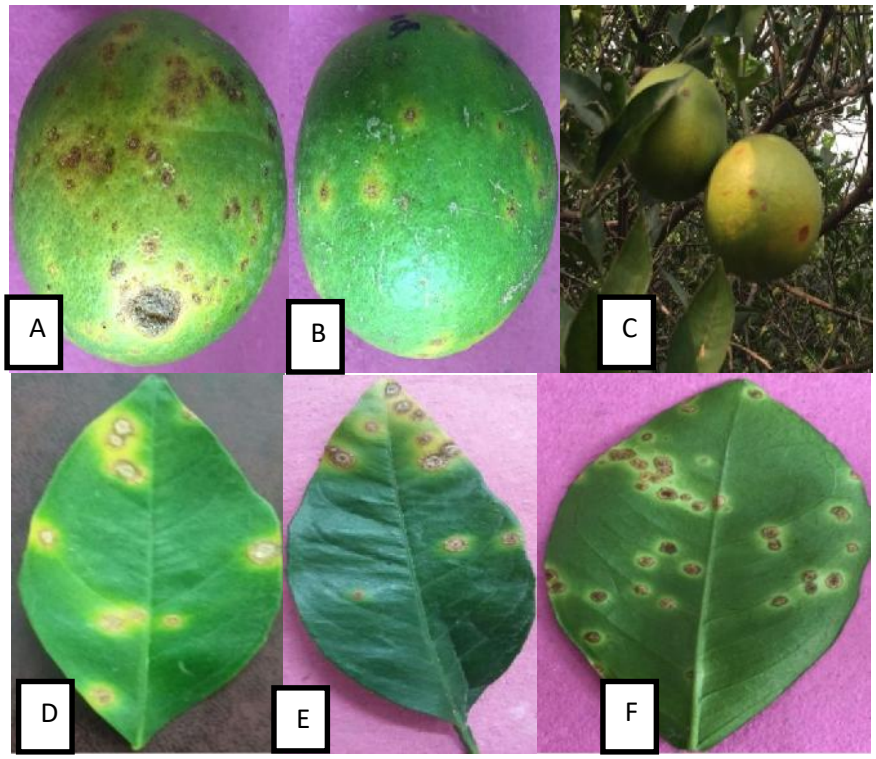

Fig. 1. Fruit symptoms of CBC on sweet orange younger lesions on early fruit (A), older lesions (B), older lesions on later fruit(C), Whole leaf symptoms of CBC on leaf (D) younger lesions, older lesions on top (E) and bottom of sweet orange leaf (F).

Table 1. **Oligonucleotide primers pair used for Polymerase chain reaction (PCR).**

Primers	Sequences	Product size (bp)	Amplified regions
HF	5'-CACGGTGCAAAAAATCT-3'	222	
HR	5'-TGGTGTCGTCGCTTGTAT-3'		16S rDNA
XCF	5'-AGGCCGGTATGCGAAAGTCCCATCA-3'	424	ITS
XCR	5'-CAAGTTGCCTCGGAGCTATC-3'		
XACF	5'-CGCAT CCCCACCACCACCACGAC-3'	500	ITS
XACR	5'-AACCGCTCAATGCCATCCACTTCA -3'		

RESULTS AND DISCUSSION

The identification of *Xac* was carried out from citrus different cultivars by morphological, biochemical and molecular markers.

Morphological Characterization: The leaves lesions placed on GYP agar plate showed the bacterial colonies after 24 hours at 28°C identified the bacterium as *Xac*. The colony color of the isolates was variable from light yellow to dark yellow (Fig 2A). The colonies of *Xac* bacterial isolates were small, round, mucoid and yellow due to accumulation of xanthomandian. The results were in line with previous study of Das (2003) who reported that seven isolates of the *Xac* isolates were gram negative and yellow in color on selective medium. The typical yellow coloration of the colonies was due to the pigmentation of xanthomonadin, which is produced by different *Xanthomonas*. The colonies were mucoid due to the production of extracellular polysaccharide slime by the addition of glucose in the culture medium (Singh and Thind, 2014). The size, shape and arrangement of colonies were found to be small to medium, convex and single (Fig 2B). The characteristics of the bacterium are in table 2. Gram staining test from 5 isolates was found as gram negative and rod shaped bacteria. All morphological properties were consistent with those previously described for pathotype A by Sun *et al.* (2004).

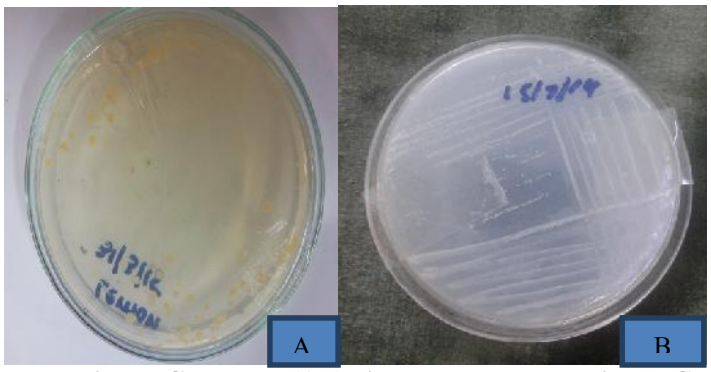

Fig. 2.Yellow to dark yellow colonies on GYP plate (AB), light yellow *Xac*colonies on GYP agar plate (C).

Table 2. Morphological Characteristics of *Xanthomonas axonopodis.*

Isolates	Pigmentation	Configuration	Shape	Texture	Margins	Opacity
Xac01	Yellow	Small, single	Rod	Mucoid	Smooth	Translucent
Xac02	Yellow	Small, single	Rod	Mucoid	Smooth	Semi-Translucent
Xac03	Yellow	Small, single	Rod	Mucoid	Smooth	Translucent
Xac04	Yellow	Small, single	Rod	Mucoid	Smooth	Semi Translucent
Xac05	Yellow	Small, single	Rod	Mucoid	Smooth	Translucent

Biochemical Characterization: The biochemical properties were studied by API-E20 strip, which involves different biochemical tests. In biochemical tests the bacterial isolates were positive for the citrate utilization, H_2S production, urea hydrolysis, deaminase, acetoin production and hydrolysis of gelatin. But they were negative for the fermentation of sorbitol, mannitol and glucose. The different biochemical tests are listed in Table 3: Beta galactosidase, arginine hydrolase, lysine decarboxylase, ornithine decarboxylase test were all negative for all strains. Xac1, Xac2, Xac3, Xac4 and Xac5 all strains showed the positive results towards citrate utilization and H_2S production. Xac 04 was positive for urea hydrolysis while all other strains were negative for the respective test. Juhasz *et al.* (2013) also reported that *Xac* isolates were positive for catalase, KOH and H_2S production; hydrolysis of starch, gelatin liquefaction and fermentation of different sugars such as sucrose. Deaminase utilization test for all the strains was positive with brownish red coloration. In present study the isolates fermented different carbohydrates such as rhaminose, sucrose, arabinose and oxidase, but they were negative for the fermentation of sorbitol, mannitol and glucose Glucose mannitol sorbitol and inositol fermentation tests were negative by Xac1, Xac2, Xac3, Xac4 and Xac5. Xac1, Xac02, Xac03, Xac04 and Xac05 were positive for rhaminose and sucrose utilization, while melibiose and amygdalin fermentation was negative. These findings are in line with Mohammadi, *et al.* (2001) who suggested that eighteen different isolates of *Xac* from Marathwada region of India showed utilization of arabinose, rhaminose and sucrose.

Table3. Biochemical test of *Xanthomonas axonopodis* pv*citri* used for differentiating various trains.

Tests	Biochemical test	Results Coloration	Xac1	Xac2	Xac3	Xac4	Xac5
ONPG	Beta-galactosidase	Colorless	-	-	-	-	-
ADH	Arginine dihydrolase	Yellow	-	-	-	-	-
LDC	Lysine decarboxylase	Yellow	-	-	-	-	-
ODC	Ornithine decarboxylase	Yellow	-	-	-	-	-
CIT	Citrate utilization	Blue	+	+	+	+	+
H_2S	H_2S production	Black deposit	+	+	+	+	+
URE	Urea hydrolysis	Yellow	-	-	-	+	-
TDA	Deaminase	Brown red	+	+	+	+	+
IND	Indole production	Red	-	-	-	-	-
VP	Acetoin production	Pink	+	+	+	+	+
GEL	Gelatinase	No diffusion of black	+	+	+	+	+
GLU	Glucose fermentation	Blue/blue-green	-	-	-	-	-
MAN	Mannitol fermentation	Blue/blue-green	-	-	-	-	-

INO	Inositol fermentaion	blue/blue-green	-	-	-	-	-
SOR	Sorbitol Fermentation	blue/blue-green	-	-	-	-	-
RHA	Rhaminose Fermentation	Yellow	+	+	+	+	+
SAC	Sucrose fermentation	Yellow	+	+	+	+	+
MEL	Melibiose fermentation	Blue	-	-	-	-	-
AMY	Amygdalin fermentation	Blue	-	-	-	-	-
ARA	Arabinose Fermentation	Yellow	+	+	+	+	+
OX	Oxidase	Violet	+	+	+	+	+

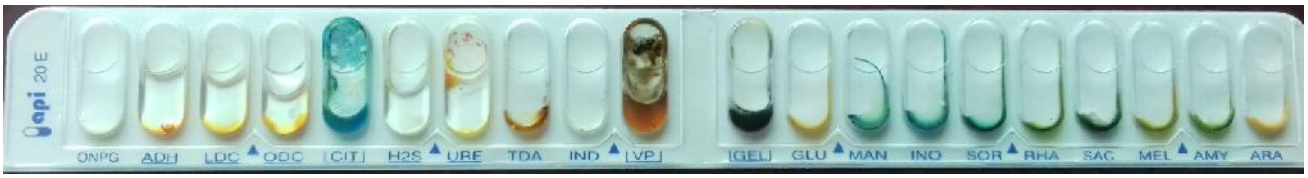

Fig. 3. Twenty different biochemical tests by API E-20 strip.

Detection of *Xac* by PCR: The PCR was used for the identification of the CBC causing pathogen by using three different primer sets amplifying different regions of the citrus canker pathogen. An amplified fragment of 222bp from 16S rDNA, 424bp from XCF/XCR and 500bp from XACF/XACR was observed from all collected citrus cultivars. Results of PCR amplification have been depicted in Table 4.Specific PCR products were detected from all primer sets. The18-mer oligonucleotide HR/HF primer pair tested for *Xac* was amplified by 78% *Xanthomonas* species in grapefruit group of citrus. The highest degree of disease 95% was identified by shamber and lowest 60% by pomelo in grapefruit. The predicted amplified product was obtained from 30% isolates of *Xac* from different varieties of citrus belonging to kumquats group and 70% from lemon and lime group members. The 16S rRNA sequences based primers (HF/HR) amplified specific region in all citrus cultivars, these results were corroborate with those reported by Cubero and Graham (2002).

Zafarullah *et al.* (2016) reported that the 16S rDNA gene was considered unsuitable due to the high levels of sequence similarity in this region and revealed very limited diversity. Therefore ITS based primers have high discriminating power, so specific primers based on ITS region amplified a specific band in all strains. Two sets of primer based on ITS region XCF/XCR and XACF/XACR were used for the rapid identification of all strains. The XCF/XCR primer amplified a PCR product of *Xac* by 80% varieties of citrus belonging to grapefruit group. The 40% isolates from kumqats, 70% from lemon and lime were identified by this primer pair and no amplified product was observed from healthy plants. The other set of primer XACF/XACR amplified PCR product of 500bp from 82% grapefruits, 40% from kumquats and 77% from isolates belonging to lemon and lime group(Fig 4). In these results all the citrus cultivars amplified by XACF/XACR primer pair as Park *et al.* (2006) also mentioned that this primer set amplified DNA fragment from *Xac* in different citrus cultivars.

In next step the rate of disease susceptibility among different citrus groups was calculated by percentage mean. The highest percentage of disease susceptibility was observed in grapefruit while kumqats showed the lowest percentage of disease (Table 4).To check the specificity of the primer other negative controls such as healthy plant and non-template control showed negative result for the pathogen. These findings were similar with the previous reported findings by Burhan *et al.*(2007) that intensity of disease severity was partially similar in different cultivars such as maximum (56.62%) was found in Mars Early, immediately followed by Olinda Valencia. Singh *et al.* (2014) also find out that grapefruit have the highest degree of disease susceptibility among different citrus groups due to high level of gene expression which code for extra cellular polysaccharide (EPS) in *Xac* pathogen to enhance the canker development.

Table 4. Different varieties of citrus used to evaluate different set of primers, based on 16S (HF/HR), and internally transcribed spacer region (XACF/XACR, XCF/XCR) for PCR identification of *Xac.*

Groups	Varieties	Total. No. of samples	HF/HR (%age mean of identification)	XCF/XCR (%age mean of identification)	XACF/XACR (% age mean of identification)
Grapefruit	Shamber (SH)	5	94	95	100
	Star ruby(Kakalíková *et al.*)	5	60	60	70
	Red blush(RB)	5	75	80	80
	Pink Ruby(PR)	5	80	80	80
	Ray Ruby(RR)	5	60	60	58
	Ruby blood(Rb)	5	85	90	90
	Pomelo(P)	5	55	60	70
age of Susceptibility toxic			78	80	82
Kumqat	Nagami(N)	5	15	20	17
	Marumi(MA)	5	55	60	55
	Meiwa(ME)	5	30	40	40
% age of Susceptibility toxic			30	40	40
Lemons & lime	Volkameriana	5	50	60	60
	Eureka	5	60	60	60
	Mesero	5	35	40	35
	Lisbon	5	98	100	100
	Local mitha	5	78	80	80
	Pehawasrimittha	5	78	80	78
	Tahiti lime	5	80	80	75
	Kagzi lime	5	80	80	80
	Lakeland	5	98	100	98
% age of Susceptibility *to Xac*					

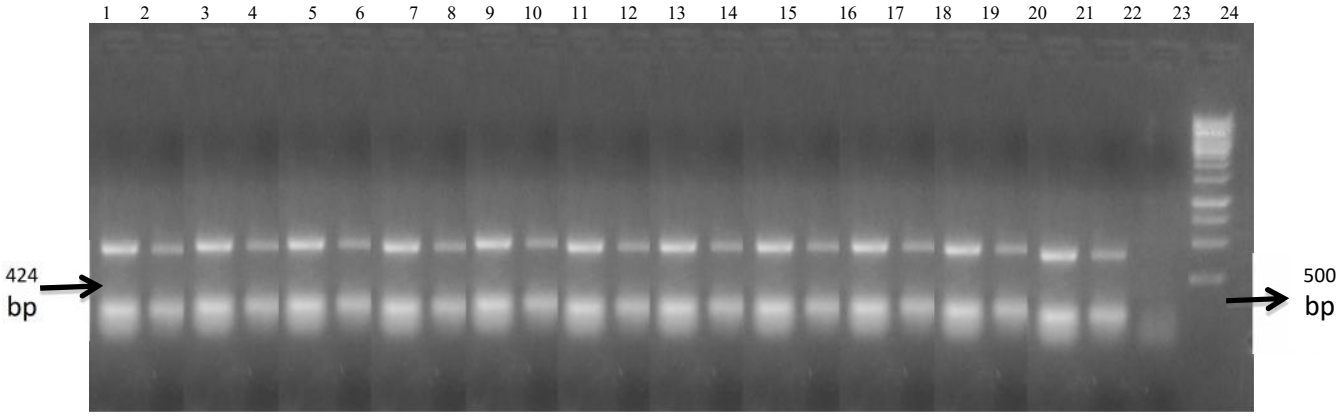

Fig. 4. Specific PCR amplification of *X. axonopodis pv. citri* from different varieties of citrus with primer set XCF and XCR. Lane 1-22 listed in Table 1, lane 23: healthy plant (negative control), lane (1 kb DNA ladder Marker Enzynomic). Arrow showing 500bp band in the ladder.

Phylogenetic Tree: A phylogenetic tree constructed by neighbor joining method clustered all *Xanthomonas axonopodis* taxa into a monophyletic group. The group was divided into two subgroups. First group was consisting of the isolates with Accession No. CP011827.2 AF442739.1, 20163533C_XCF, and other group containedDQ991181.1, KF926678.1 and AF442744.1 (Fig. 5). The sequence of ITS region of *X. axonopodis isolates* from shamber (shown as 3C_XCF) showed 100% homology to reference sequence of *X. axonopodis pv. citri* isolate from China (Accesion No. CP011827.2). *Xac*was 99%similar to the sequence of the region of the isolate of USA (AF442739.1). The other group showed less similarity with the sequence of the ITS region of the pathogen. The *Candidatus Liberibacter asiaticus* (Accession No.KY038851.1) appeared as a separate group in evolutionary analysis which has no similarity with the other groups. Overall results confirmed that

infectious pathogen A type is prevalent in Pakistan. The sequencing and phylogenetic analysis showed 100% homology to pathotype A strain from USA and China which confirmed the prevalence A type CBC in selected regions of citrus germplasm of Pakistan

Citrus canker is a devastating disease, which is affecting the economy of Pakistan very badly. In present study we have detected the pathogen by morphological, biochemical and molecular characterization. Pathogen was found to be affecting almost all the citrus varieties to different extent. Therefore there is a need to follow up the correct cultivation of citrus cultivars to decrease disease incidence in nurseries and to prevent the spreading pathogen (*Xac*) to new emerging groves. This study would be helpful for future planning of characterization and control of this disease.

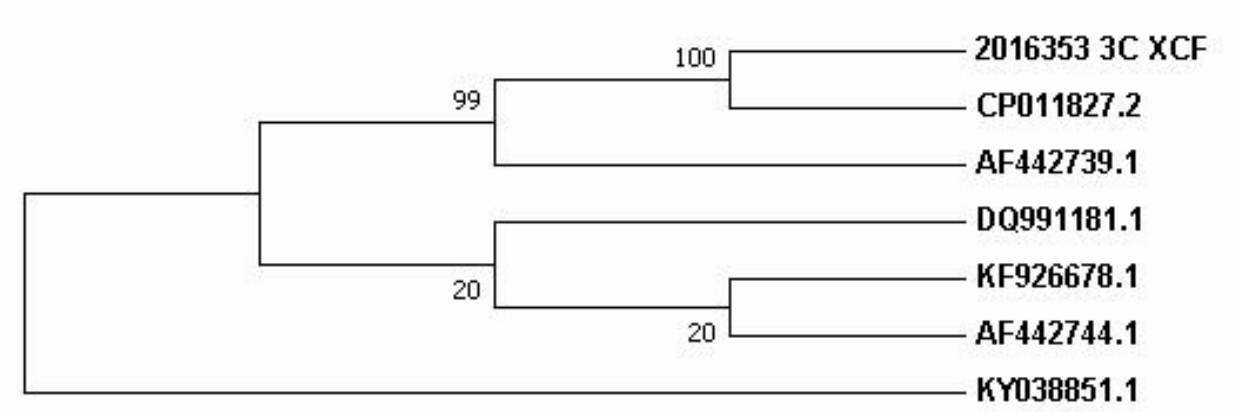

Fig.5: Phylogenetic analysis of *Xac* based on nucleotide sequences by using MEGA7 software. The percentages of replicate trees in bootstrap values (500 replicates) are given at the branch node.

REFERENCES

Burhan, M., S.T. Sahi, and S. Ahmad (2007). Screening of citrus cultivars for source of resistance against citrus canker under field conditions. Pakistan J. Bot. 39: 1867- 1871.

Canteros, B., A. Gochez, and R. Moschini (2017). Management of Citrus Canker in Argentina, a Success Story. J. Plant Pathology 33 (5): 441.

Cubero, J. and J.H. Graham (2002). Genetic relationship among world-wide strains of *Xanthomonas* causing canker in citrus species and design of new primers for their identification by PCR. Appl. Environ. Microbiol. 68: 1257-1264.

Das, A. (2003). Citrus canker. A review. J. Appl. Hortic. 5(1): 52-60.

Gottwald, T.R., G. Hughes, J.H. Graham, X. Sun, and T. Riley (2001). The citrus canker epidemic in Florida: The scientific basis of regulatory eradication policy for an invasive species. Phytopathology. 91(1): 30-34

Graham, J.H., T.R.Gottwald, J. Cubero, and D.S. Achor (2004). *Xanthomonas axonopodis* pv. citri: factors affecting successful eradication of citrus canker. Mol. Plant. Patho. 5(1): 1-5.

Grote, D., A. Olmos, J.J. Kofoet, E. Tuset, E. Bertolini, and M. Cambra (2002). Specific and sensitive detection of *Phytophthora nicotianae* by simple and nested-PCR. Eur. J. Plant. Pathol. 108(3): 197–207.

Juhasz, C.C., A. Leduc, C. Boyer, F. Guérin, C. Vernière, O. Pruvost, I. Wonni, and L. Ouédraogo (2013). First report of *Xanthomonas citri* pv. citri causing Asiatic citrus canker in Burkina Faso. J. Plant Dis. 97(12):1653.

Kumar, S., G. Stecher, and K. Tamura (2016). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol. Bio.evol. 33 (7): 1870-1874.

Mansfield, J., S. Genin, S. Magori, V. Citovsky, and M. Sriariyanum (2012). Top 10 plant pathogenic bacteria in molecular plant pathology. Molecular plant pathology. 13(6): 614-629.

McCartney, H.A., S.J. Foster, B.A. Fraaije and E. Ward (2003). Molecular diagnostics for fungal plant pathogens. Pest Manag Sci. 59: 129–42.

Mohammadi, M., M.R. Mirzaee, and H. Rahimian (2001). Physiological and biochemical characteristics of Iranian strains of *Xanthomonas axonopodis* pv. citri, the causal agent of citrus bacterial canker disease. J. of Phytopathology. 149: 65-75.

Murray, M.G. and W.F. Thompson (1980). Rapid isolation of high molecular-weight plant DNA. Nucl. Acids Res. 8: 4321.

Naz S., H. Kausar, F. Saleem, and A. Zafarullah (2015). Characterization of abiotic stress genes from different species of *Eucalyptus*. Pakistan J. Bot. 47(4): 1217-1223.

Pakistan Bureau of Statistics (2016).http:/ /www.pbs.gov.pk.

Park, D.S., J. W. Hyun, Y. J. Park, J. S. Kim, H. W. Kang, J. H. Hahn, and S. J. Go (2006). Sensitive and specific detection of *Xanthomonas axonopodis pv. citri* by PCR using pathovar specific primers based on hrpW gene sequences. Microbio.161(2): 145-149.

Pruvost, O., B. Boher, C. Brocherieux, M. Nicole, and F. Chiroleu (2002). Survival of *X. axonopodis* pv. *citri* in leaf lesions under tropical environmental conditions and simulated splash dispersal of inoculum. Phytopathology. 92(4): 336-346.

Sahi, S.T., M.U. Ghazanfar , M. Afzal, A. Rashed and A. Habib (2007). Incidence of citrus canker disease caused by *Xanthomonas campestris* pv. *citri* (Hasse) dows on Kinnow (*Citrus reticulata*) and its chemotherapy. Pakistan J. Bot. 39(4): 1319.

Sambrook, J., E.F. Fritsch, and T. Maniatis (1989). Molecular Cloning: A Labolatory Manual, second ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NewYork.

Schaad, N.W., E. Postnikova, G. Lacy, A. Sechler, I. Agarkova. P.E. Stormberg., V.K. Stormberg, and A.K. Vidaver (2006). Emended classification of xanthomonad pathogens on citrus. Appl. Microbiol and biotech. 29(8): 690–695

Schubert T.S, S.A. Rizvi, X. Sun, T.R. Gottwald, J.H. Graham, and W.N. Dixon (2001). Meeting the challenge of eradicating citrus canker in Florida. J. Plant Dis. 85(4):340-56.

Schubert, T.S. and X. Sun (2003). Bacterial citrus canker. Plant Pathol. J. Circular No. 377. Fl. Dep. Agric. and Cons. Services. Division. Plant industry. 1-6.

Singh D and S.K. Thind (2014). Prevalence, isolation and standardization of growth media for *Xanthomonas axonopodis* pv. *citri* causing citrus canker. Plant Dis.29(2), 188-192.

Sun, X., R. E Stall, J.B. Jones, J. Cubero, T.R. Gottwald, J.H. Graham, W.N. Dixon, T.S. Schubert, P.H. Chaloux, and V.K. Stromberg (2004). Detection and characterization of a new strain of citrus canker bacteria from Key/Mexican lime and alemow in South Florida. PlantDis. 88 (11): 1179-1188.

Zafarullah, A., S. Naz, and F. Saleem (2016). Detection and molecular characterization of *Candidatus liberibacter* spp. causing Huanglongbing (HLB) in indigenous citrus cultivars in Pakistan. Pakistan J. Bot. 48(5):2071-2076.