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First report of *Xanthomonas citri Subsp. Citri* causing citrus canker on grape fruit (*Citrus paradisi)*, washington naval (*Citrus sinensis*), kaghzi limon (*Citrus aurantifolia* Swingle), lemon (*Citrus limon*) and pomelo (*Citrus maxima*) in Pakistan

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Citrus fruit production is largely affected by different bacterial and fungal pathogens. In Pakistan bacterial diseases like citrus bacterial canker (CBC) pose severe risk to citrus economy. Diagnoses of such diseases could be helpful to avoid the epidemics in nurseries or orchids. In 2011-12, citrus canker symptoms i.e., callus-like outgrowths on leaves and fruits of grape fruit (*Citrus paradisi),* Washington naval (*Citrus sinensis*), Kaghzi Limon (*Citrus aurantifolia swingle*), lemon (*Citrus Limon*) and pomelo (*Citrus maxima*) were noticed in Sargodha district of Punjab, Pakistan. Bacteria i.e., yellow mucoid, Xanthomonas-like isolates, were isolated from these lesions. Bacteria isolated from these lesions were cultured and total DNA was isolated. A diagnostic fragment of 581 bp based on rpf genes of *Xanthomonas citri pv. citri* was amplified, cloned and completely sequenced. BLAST and evolutionary analysis revealed that these isolates show 100% sequence similarity and group with *Xanthomonas citri subsp. citri* from Argentina (CP023285) and Reunion (CP018858), (CP018854). To our knowledge, this is the first formal report of *X. campestris* pv. *citri* pathotypes A on *Citrus paradise*, *Citrus sinensis*, *Citrus maxima*, *Citrus Limon* and *Citrus aurantifolia swingle* in Pakistan

Keywords: *Citrus paradise; Citrus sinensis*; *Citrus aurantifolia swingle*; *Citrus Limon*; *Citrus maxima; Xanthomonas citri* pv. *Citri;* rpf genes; pathogenicity test.

INTRODUCTION

Among fruit crops, Citrus (Family: *Rutaceae*) is major fruit crop worldwide both area- and production-wise (Hynniewta *et al.,* 2011). Lime, grapefruit, lemon and tangerines are preferred cultivars among cultivated species (Hynniewta *et al.,* 2011). Top citrus fruits producing countries are China, United States of America, India, Brazil and Spain (Liu *et al.,* 2012) while Pakistan is at 13th position. In Pakistan out of five provinces, Punjab contributes a major share in terms of production as well as area (Economic survey of Pakistan, 2012). Pests and diseases majorly affect citrus yield in Pakistan and per hectare yield is low as compared to countries with well-managed orchids (Economic survey of Pakistan, 2012). In tropical and subtropical areas like Pakistan, bacterial disease of citrus i.e., citrus bacterial canker (CBC), pose a severe risk to citrus economy (Bansal *et al.,* 2017; Gottwald *et al.,* 2002; Polek, 2007). CBC characterized by callus-like outgrowths on leaves and other parts of the plant like fruits and twigs, is caused by *Xanthomonas citri pv. citri* (Gabriel *et al.,* 2000). Due to widespread and damaging nature, severity of this disease, farmers have eradicated orchids in many countries (Bansal *et al.,* 2017; Gottwald *et al.,* 2002). *Xanthomonas. citri pv. citri* causes infection in multiple citrus species i.e., *Citrus aurantifolia*, *C. latifolia*, and *C. macrophylla*, and *C. paradisi* (Gottwald *et al.,* 2002; Ference *et al.,* 2017). Three types of CBC i.e., Asiatic type, Cancrosis B and Cancrosis C caused by *X. axonopodis* pv. *citri* **(***Xac***)**, *X. axonopodis* pv. *aurantifolii* and *X. axonopodis* pv. *aurantifolii* prevail in the world (Das, 2003; Gottwald and Graham, 2000). Asiatic type canker is very severe form of

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canker and is present in all citrus growing countries of the globe.

Accurate and timely detection of the *X. axonopodis pv. citri* is important to design resistant strategies against CBC. Detection of several pathogens is usually based on strain features, physiology and bacterial sensitivity (Schaad, 1988; Civerolo, 1984; Goto *et al.*, 1980), antibody based and DNA homology based identification, and different other techniques like restriction fragment length polymorphism (RFLP) and polymerase chain reaction (PCR) (Cubero and Graham, 2002; Miyoshi *et al.*, 1998). We have designed this study to detect the pathogen in canker lesion, so that we can take measures to prevent the further spread of pathogens.

MATERIALS AND METHODS

***Sample collection and DNA extractions*:** In 2011-12, infected leaf samples showing symptoms of callus-like outgrowths on grape fruit (*Citrus paradisi),* Washington naval (*Citrus sinensis*), Kaghzi Limon (*Citrus aurantifolia swingle*), lemon (*Citrus Limon*) and pomelo (*Citrus maxima*) were collected from district Sargodha, Punjab province of Pakistan. Asymptomatic samples were collected as negative control. Before extraction of DNA leaves and fruits were washed with distilled water then dried and cleaned with tissue paper. Infected portion of the sample was cut and put in an Eppendorf tube and immediately, placed on ice and properly labeled. DNA was also extracted on the same day of sample collection. Modified CTAB method (Jaufeerally-fakim and Dookun, 2000) was used to isolate total genetic material from the leaves and bacterial culture.

***Isolation and culturing of bacteria causing citrus bacterial canker*:** Surface sterilized leaf was placed on clean tissue paper in laminar flow cabinet and sprayed 70 % ethanol both side. Then leaf lesion was wiped with tissue paper and dried. Leaf lesion was placed in sterile petri plate. Sterile scalpel blade was used to halve and then quarter the excised lesion. Four pieces of lesion of same sample were placed on nutrient agar (NA) plate. NA agar plates were properly labeled with sample number and date. Plates were incubated in 28ºC for 7 days. For sub-culturing bacteria were streaked again to new NA agar plates. Sub-culturing was done 3 times to obtained pure culture bacteria. When pure pale yellow colonies were obtained, a single colony was picked and cultured into 5 ml liquid NA culture.

***PCR-based amplification of CBC pathogens*:** Amplification of diagnostic DNA fragment of pathogen from suspected samples was established using pathogen specific diagnostic primers Xac01 and Xac02 (da Silva *et al.,* 2002). PCR reaction conditions were optimized to amplify the DNA from *Xanthomonas axonopodis pv. citri* strain as described by (da Silva *et al.,* 2002). Amplification via PCR was confirmed by loading 5µl of PCR products in 0.8 % agarose gels in prepared in 0.5X TBE buffer and visualized in IRMECO gel documenting system.

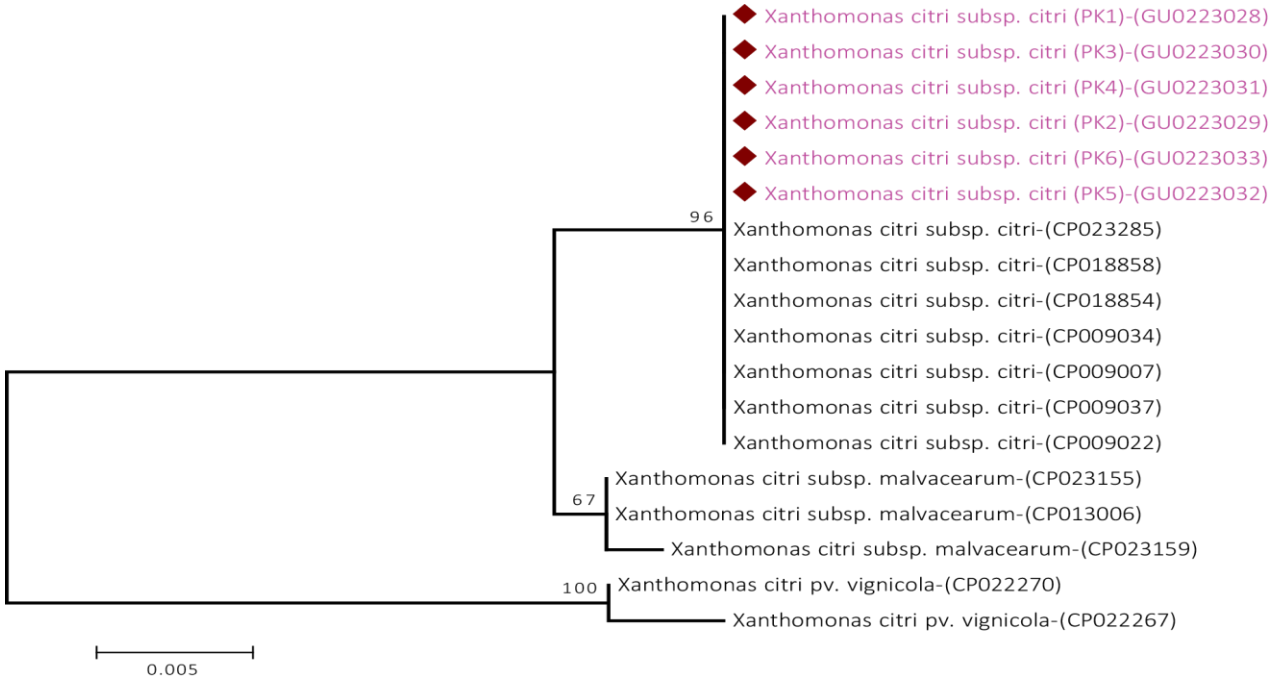

Figure 2. Phylogenetic dendrogram based upon selected sequences of *Xanthomonas citri* pv. *citri*. Sequences used for comparison were downloaded from GenBank. The database accession number in each case is given. The sequences of isolates from present study are indicated by pink color and square shape.

***Sequence and phylogenetic analysis*:** Vector i.e., pTZ57R/T (Fermentas) was used to clone PCR amplified fragment from each sample and completely sequenced (Sanger *et al.,* 1977). For phylogenetic analysis all sequences i.e., isolates from this study and sequences of other closely related strains of almost same size downloaded from databank. DNA STAR software (v8; Madison, WI, USA) was used to assemble and analyze these sequences. Evolutionary trees were generated to study phylogeny, first by the alignment of sequences using CLUSTAL W that was followed by Neighbor joint method to construct phylogenetic trees using MEGA7 program (Kumar *et al*., 2016).

RESULTS AND DISCUSSION

Due to low quantity and quality of citrus fruit, Pakistan receives fewer prices in international market as compared to countries with well-managed citrus orchids. Pakistan is far behind in average citrus yield per hectare in comparison with other citrus producing countries. Bacterial diseases like citrus greening and citrus canker, are responsible for 35-45% yield losses and sometimes in severe disease condition no yield at all. In 2011-12, a survey was conducted of different orchids of Sargodha and severe bacterial canker disease was observed at different citrus cultivars. Infected leaf samples of grape fruit (*Citrus paradisi)*, Washington naval (*Citrus sinensis*), Kaghzi Limon (*Citrus aurantifolia swingle*), lemon (*Citrus Limon*) pomelo (*Citrus maxima*) were collected from district Sargodha, Punjab province of Pakistan. Samples were collected from three infected plants of each cultivar from different orchid. Pathogen isolations from lesions were done using nutrient agar (NA) at 25-30 ^{0}C from all citrus cultivars, and yellow mucoid, Xanthomonas-like isolates were selected. These bacteria showed no morphological difference from pathogens already reported (Richard D *et al.,* 2017). These bacteria were cultured and total genetic material was separated from these bacteria and subjected to PCR amplification. The specific primers Xac01 and Xac02 (da Silva *et al.,* 2002) designed on *rpf* genes of *Xanthomonas citri*, were used to PCR amplify a fragment of 581 bps from bacterial isolates. Amplified fragment from each sample was cloned in pTZ57R/T vector (Fig. 1B, C) and completely sequenced (Sanger *et al.,* 1977). Sequence analysis showed that all bacterial pathogen isolates were *X. citri* pv. *citri* and 99-100% similar with each other. This high sequence homology found between the isolates is correlated with other published reports where similar results were found (Bui *et al.,* 2009; Carvalho *et al.,* 2005; Cubero *et al.,* 2002). As grafting is used to propagate economically important citrus cultivars like *C. paradise* and *C. sinensis* worldwide at a large area, there is less genetic diversity found in the *X. citri* population globally (Graham *et al.,* 2004). Due to genetic similarity of the host plant there is less selection pressure on the pathogen. All six sequences were submitted to GenBank with acc # GU0223028 to GU0223033. BLAST analysis revealed that amplified region showed 100% sequence homology with *X. citri* pv. *citri* (CP023285), (CP018858) and (CP018854) (Richard D *et al.,* 2017) reported from Argentina (South America) and Reunion (East of France). These results were quite surprising as none of these isolates show close sequence homology with isloates from near by regions like India and China. Since, it is a contagious pathogen, it is possible that it was introduced through trade between Pakistan and Americas. In phylogenetic analysis these sequences grouped together with the serotypes from South America, Reunion and USA (Fig. 2). Citrus is major fruit crop of Pakistan contributing towards GDP of the country substantially. Understanding pathogen causing citrus canker at molecular and sequence level is important step towards engineering resistance against *X. citri pv citri.*

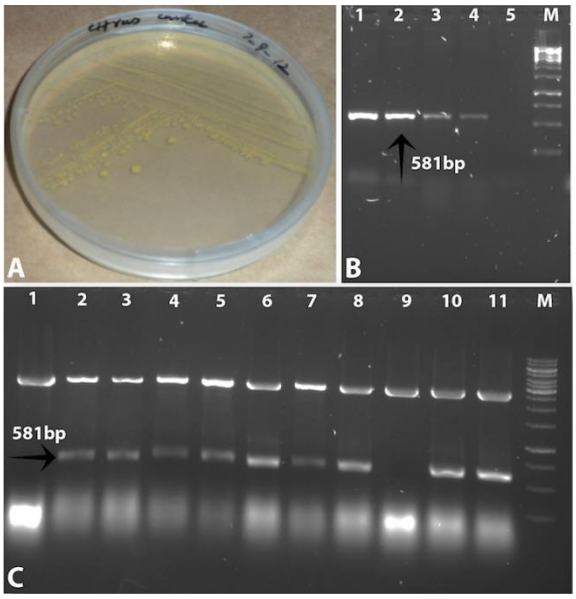

Figure 1. Bacterial isolation and cloning: A) Bacterial Isolation; A plate showing isolated yellow mucoid, Xanthomonas bacteria; B) PCR amplification of 581 bp fragment, based on *rpf* genes (da Silva et al., 2002), lane 1-4 PCR amplification while lane 5 is negative control; C) Cloning of amplified product into pTZ57R/T vector, lanes 2 to 8 and lane 10, 11 are clones. M is 1Kb DNA marker (Fermentas).

The objective of this study was to provide citrus research community with information about *Xanthomonas citri* pv. *citri* infecting different cultivars of citrus in Pakistan. This is first time we are reporting *X. citri* pv. *citri* on grape fruit (*Citrus paradisi)*, Washington naval (*Citrus sinensis*), Kaghzi Limon (*Citrus aurantifolia swingle*), lemon (*Citrus Limon*) pomelo (*Citrus maxima*) in Pakistan, though we were

suspecting it for a long time. Infectivity of *X. citri pv. citri* was also established as it is important to understand how plant pathogens function in natural conditions. Sequence information plays a vital role in the identification of pathogens and increases the capacity for diagnostics.

***Author's Contribution*:** JN, MAI, NN and AR performed all the experiments. MM, NA, MSN and AY were involved in design of the experiments and data analysis. MM, IL, MSN and AY wrote the paper while all the authors approved the manuscript.

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***Competing interests*:** The authors declare that they have no competing interests.

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