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Assessment and molecular characterization of citrus canker causing pathotypes

Sumyia Iqbal*, Shazia Iftikhar, Anam Rasool*

Department of Environmental Sciences, Fatima Jinnah Women University, Rawalpindi. Pakistan

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Abstract

Citrus is one of the most popular and significant commercial fruit of Pakistan. Outbreak of Citrus Canker diseased in different varieties causes enormous economic loss. The present research work was conducted to identify and characterize citrus canker causing Pathotypes in citrus varieties from the orchards of Khanpur, district Haripur and Islamabad (National Agriculture Research Council). The survey was conducted in orchards of Khanpur and Islamabad during March, 2015 to find out the incidence, prevalence, severity and disease index as well as for sampling from selected orchards. Varieties encountered during the survey included Grapefruit, Malta (musambi), Red Blood, Ruby Blood and Lemon. Causal organisms were isolated and grown on nutrient agar (NA) and Potato dextrose agar (PDA) for morphological studies. Firstly, Pathogens were identified on the basis of morphological characters.The incidence of Khanpur and National Agriculture Research Council, Islamabad orchards observed were about 10% and 95% respectively. Complete description of macro and microscopic characters was prepared. The purified cultures of bacterial pathogen was then identified and characterized. The identified microbial pathogens include *Xanthomonas citri*. DNA was successfully extracted and amplified by using TaKaRa Ex taq™ version kit with primers 9F and 15-10R, to confirm the identification by gene sequence. Identified Strains were stored for further study. It is recommended on basis of present study to carry out further experimentation on pathogenicity of these pathotypesforfinding out the citrus varieties resistance against this disease. There is also need to control this pathogen using different biological/chemical agents and genetic engineering techniques.

* **Corresponding Author:** AnamRasool ✉ anam.rasool32@gmail.com

Introduction

Citrus, the edible fruit belonging to family *Rutaceae* have been considered as the best source of vitamin C, folic acids and dietary fibers. It is rich in antioxidant thus used for treating skin, lungs and kidney cancers, birth defects and cardiac diseases. Thus it contributes to a stable and healthy life (Baghurst, 2003).All fruits that are grown in Pakistan 29.55%of the total agricultural orchards under fruit cultivation have been reported to be covered by citrus fruits (Ahmad and Mustafa, 2006). In Pakistan researches have suggested the cultivation of citrus increase from 68.50 to 194.07 thousand acres from the year 1959 to 2009 (Qureshi *et al.,* 2014), with production of 1.98 million tons annually but the relative ratio between the cultivating area and the production of citrus is lower than the other countries and hence the contribution of Pakistan in world citrus production is only 2%.

Although citrus orchards are kept in great esteem yet its present status is threatened by a number of problems, in which citrus canker caused by *Xanthomonas* bacterium is considered to be the first cause in the large queue of bacterial diseases of citrus trees (Das, 2003). The disease, caused by the bacterium *Xanthomonas citri*, befalls in large areas of the world's citrus-growing countries including Pakistan. All citrus varieties are subtly to vastly liable to the disease. This disease causes widespread damage to the fruit; defoliation, dieback and fruit drop occurs and infected fruits are less appreciated or entirely unmarketable. The severity of the contamination varies with different species and variations and the prevalent climatic conditions (Gottwald *et al*., 2002).

This disease is characterized by eruptive lesions on leaves, stems and fruits; deceases the fruit worth and production (Lakshmi *et al*., 2014). It is also significant due to its socio-economic influence as *Xanthomonas* is considered a quarantine organism (Provost *et al.,* 2002), listed in Annex IIAI of Council Directive 2000/29/EU. Annexes III; IV AI and VB of this Directive list requirement for the introduction into the EU of citrus plants, including fruits, which could be a passageway for the entry of this pathogen. Its occurrence holds back the export of fruit to EU because citrus export of EU is forbidden if *Xanthomonas* is confirmed. (Roberts *et al*., 2005). Subsequently, it is essential that government, private sector and all the investors join hands and work together for the improvement of difficulties faced by the horticulture sector of the country.

Hence there is the necessity for the molecular understanding of this pathogen. This research is done to detect casual bacteria pathotype (*Xanthomonas citri*) of Asian countries in infected leaf samples which indicated symptoms of citrus canker. The findings of these studies will be useful for the recognition of commercial fruits with suspicious symptoms. It will also assist the pathologist to work on citrus canker management, as well as epidemiologists, to save the substantial export loss that the country is facing from this disease.

The suspected presence of citrus canker in Pakistan has been documented in several publications, but the present study is a step forward in utilization of molecular markers which will identify the main casual pathotype. The finding of this study will help the future researcher to identify citrus canker resistance in transgenic lines by study the pathotype of the citrus canker in Pakistan. This will also helpful in aiding the future researchers eager for control of disease in an ecofriendly manner.

Material and methods

Sampling

For the assessment of citrus canker disease, regular survey was conducted in Khanpur and Islamabad (NARC). For survey and samples size Derso *et al*., (2006) specific methodology was followed while the questionnaire included orchard's name, grower's name, variety's name, and harvesting time were developed. Samples were taken to Environmental laboratory of Fatima Jinnah Women University for further characterization of citrus canker.

Procedure

The samples comprised of numerous leaves, but analyses were only performed on lesions observed. Each lesion was cut with sterile scalpel sterilized with 0.85% saline water, examined for bacterial ooze within two to five minutes. Sterile disposable loop was used to collect small volume of exudate, and then agar plate was inoculated. Inoculation was done on Nutrient Agar (NA) using sterile loop at 25-28˚C for 3-6 days. *Xanthomonas* like colonies were chosen and purified on NA for further analyses (Department of Agriculture, Australian Government 2014).

Characterization

Physiological studies were accomplished by determining macroscopic characteristics of each isolate. Bacteria were characterized on the basis of cultural aspect of colony like appearance, color, shape and texture (Singh & Thind, 2014). Growth rate of bacterial single colony streaking on Nutrient Agar (NA) and Potato Dextrose Agar (PDA) was observed after 24 hours at 28°C.For Molecular characterization, single colonies were being picked up from agar medium, resuspended in 20µl TE buffer (10 mM Tris-HCl pH 8.0. 1 mM EDTA) and boiled at 95 °C for 5±15 min. The suspension was centrifuged for a short interval of time and 1µl of the supernatant was used for the polymerase chain reaction (PCR) (Katsivela *et al.*,1999).

Amplification

The amplification of 16S rRNA genes from chromosomal DNA(Deoxyribonucleic Acid) was done by PCR using universal forward and reverse primers: 9F (5'-GAGTTTGATCCTGGCTCAG-3') and 1510R (5'GGCTACCTTGTTACGA-3'). The PCR was done using a Gene Amp 9600 thermo cycler and circumstances explained earlier (Ahmed *et al.*, 2007). Quality was checked by gel electrophoresis on a 1% (w/v) agarose gel. PCR products were purified and sequenced by Macrogen (Korea). The Sequence data of the closely published strains, used for building up phylogenetic tree, were retrieved from the DDBJ (DNA Data Bank of Japan) database by BLAST searches. The editing and alignment was accomplished using CLUSTAL X. The phylogenetic tree was built up using the unambiguous data of nucleotides (nts). It was constructed using the neighbor-joining method (Saitou &Nei 1987).

Results

Prevalence, incidence, severity and disease index was observed and also studied in reference to the environmentally driven factor for the surveyed citrus orchards of Khanpur and NARC Islamabad, in plantation season mainly March and July. The average rainfall value of citrus plantation seasons of Khanpur and NARC were compared with the mean severity shown in fig. 1.

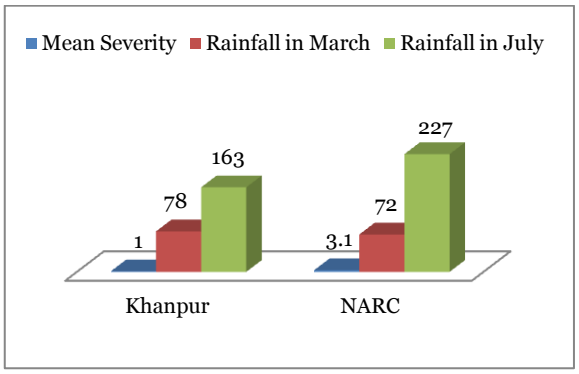

Fig. 1. Relationship between disease severity and rainfall of Kanpur and NARC.

The average values of temperature or rainfall were also compared with the mean incidence, tree age with reference to locations shown in fig. 2.

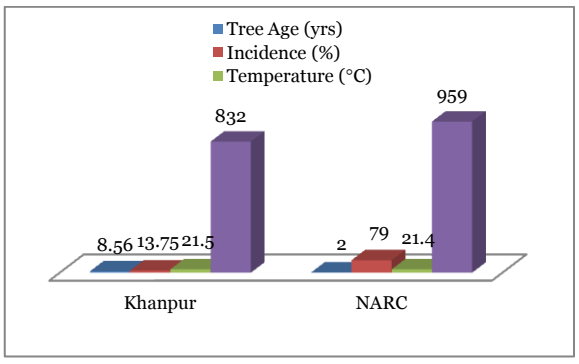

Fig.2. Comparison of percent incidence of diseased citrus, temperature, tree age and rainfall values in relation with surveyed sites.

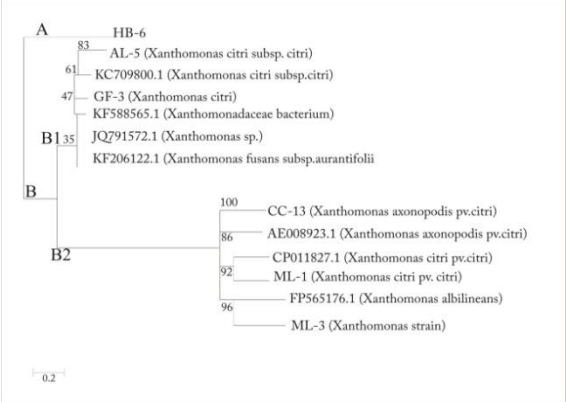

Fig. 3. A Neighbor-joining phylogenetic tree obtained, Using BioEdit software gaps were removed during alignment for construction of the rooted tree. At the branching points bootstrap values were given which expressed as percentages of 1000 replications.

The bacterium isolated from infected samples on nutrient medium plates showed cultural characters with yellow to stack yellow colored colonies with mucoid to glistening surface (Table 1). Bacterial colonies had convex elevation with circular shape and entire margins with appearance of colonies within 3-5days. Bacterial colony had different Growth rate and color when streaking on two different media; Nutrient Agar (NA) and Potato Dextrose Agar (PDA) and observed after 24 hours at 27°C. Growth on Nutrient Medium was faster and stack yellow color colonies observed. On the other hand the growth on Potato Dextrose Medium was slow and the colonies were pale yellow.

Table 1. Selected Samples were morphologically identified as *Xanthomonas citri subsp. citri*, confirmed after sequencing results.

S. No	Code	Source	Host	Color		Media Growth				
				On NA	On PDA	Growth time	Shape	Elevation	Surface	Margin
1	GF3	Leaves	*Citrus. paradisi*	Stack Yellow	Pale Yellow	2 days	Circular	Convex	Mucoid and glistening	Entire
2	ML1	Leaves	*Citrus. aurantiifolia*	Stack Yellow	Pale Yellow	3 days	Circular	Convex	Mucoid and glistening	Entire
3	ML3	Leaves	*Citrus. aurantiifolia*	Stack Yellow	Pale Yellow	3 days	Circular	Convex	Mucoid and glistening	Entire
4	AL4	Leaves	*Citrus. limon*	Stack Yellow	Pale Yellow	2 days	Circular	Convex	Mucoid and glistening	Entire
5	HB6	Leaves	*Citrus.sinensis*	Stack Yellow	Pale Yellow	2 days	Circular	Convex	Mucoid and glistening	Entire
6	DD10	Leaves	*Citrus.sinensis*	Stack Yellow	Pale Yellow	3 days	Circular	Convex	Mucoid and glistening	Entire
7	CC13	Leaves	*Citrus.sinensis*	Stack Yellow	Pale Yellow	3 days	Circular	Convex	Mucoid and glistening	Entire

Discussion

Pakistani fruit receives low price as compared to other well managing orchard countries though it is popular due to its taste in both local as well as in international markets. Conversely citrus canker is becoming an alarming threat to fruits and its export producing economy occurs in large areas of the world's citrus growing countries, including Pakistan (Das, 2003) as well as for the neighboring countries. The citrus cultivars previously known to be resistant to this disease have now become susceptible. Once this disease becomes endemic, it is very difficult to manage with commercial methods.

In the present study, survey was carried out in Khanpur and NARC and assessed for occurrence of

disease. Disease prevalence and incidence were evaluated for each location. During the survey NARC showed maximum, while in Khanpur the prevalence was reported low which might be attributed to absence of favorable environmental factors for development of this disease (fig. 1-2). Increased rainfall and decreased temperature were significant with the increase in citrus canker in Islamabad area mainly in NARC, because the distribution of the bacterium is mainly by splash dispersal at low temperature. The temperature along with different environmental factors (relative humidity, wind speed, rainfall, sunshine and clouds) had great influence on the citrus canker disease development (Vernière *et al*., 2003). Evidence provided by Khan *et al.* (1992) concluded that a low temperature range (8–11°C) in the months of January and February during the year played a significant role in the development of citrus canker disease.

Bock *et al.* (2005) also observed that the most important ways of dispersal of citrus canker were rain splash and wind. Investigations done few decades back show that rainstorms of short duration and high intensity were apparently associated with disease increase (Bertoni & Mills. 1987). Khan and Abid (2007) reported that out of the six variables, assessment, minimum temperature and wind speed influenced citrus canker disease development most significantly. Wind-driven rain splash played a dynamic role in the dispersal of bacterium (Gottwald *et al.*, 1997). Results of the study conducted are consistent with Gottwald *et al.*, (2002) who reported that rapid spread of disease across regions was favoured by rain, thus Islamabad (NARC) weather mainly the wind-driven rain splashes providing the most optimum conditions for the pathogen development.

Another possible explanation for absence of the disease on Khanpur cultivars could be that the disease occurred on young citrus plants. The average trees age of Khanpur orchards was 8.56 years, while in NARC the average age of all the trees ranged from 1-2 years (fig. 2). Ngugi *et al.*, (2002) also explained that younger plants are most vulnerable to canker in his research on prevalence, incidence and severity of sorghum diseases in Western Kenya.

During the survey, different citrus varieties encounter mainly Red Blood, Malt a (musambi), Mexican lemon, Harvard Blood, Daisy, Cara Cara, Graph Fruit, American lemon and Ruby blood in which few of them showed flat, brownish and necrotic lesions on mature as well as young leaves mainly varieties come across in NARC showed 100% prevalence. In number of studies pathotype A showed flat, brownish and necrotic lesion on grapefruit (Sun *et al.*, 2004). Another strain A* with the similar symptoms on Mexican lime was reported by Vernière *et al.,* (1998) in Southwest Asia, Cambodia and in Thailand (Bui *et al.*, 2008) and Ethiopia as well (Dersoet *al.*, 2009).

Morphological characteristics are the most useful aspects with which isolated pathogens are identified and discriminated. In the present study Isolates pathogens were identified morphologically. The optimum temperature observed for *Xanthomonas* growth ranged from 25°C to 28°C after 3-6 days. In recent studies the growth temperature for *Xanthomonas citri* bacterial inoculums was also observed 28°C after 72 hours even on different growth media (Singh & Thind. 2014) while some studies concluded that bacterium grow after incubation of 3-4 days at 25-28°C (Prokic´ *et al.*, 2012).

The most distinctive feature of *Xanthomonas* strain observed in this study was the development of separated convex, stack yellow, small colonies, Mucoid and glistening appearance of bacterium on Nutrient Agar (NA), while pale yellow colonies were observed in the present study. Similar findings were made on the Nutrient Agar (NA) for development of well separated bacterial colonies by Gracelin *et al.,* (2011).

Current studies comprising of morphological studies combined with DNA sequencing analysis identify and describe *Xanthomonascitri* from citrus leaves. DNA

was successfully isolated and amplified by polymerase chain reactions (PCR). PCR technique was proved very effective in amplification of 16S rRNA region of bacteria for the purpose of sequencing and ultimate detection of species. The 16S rRNA genes have become the standard for the determination of phylogenetic relationships, the assessment of diversity in the environment, and the detection and quantification of specific populations. Gene encoding the small ribosomal subunit (16S rRNA gene in bacteria) was selected because this gene contains both conserved and variable region (Fatima *et al*., 2012). DNA sequencing analysis verified the morphological identification of *Xanthomonas citri*. The 16S rRNA sequencing marker (27F and 1492R) were used as commanding tool in identification and determination of canker causing pathotype. Phylogenetic studies showed genetic variability among *Xanthomonas* species (fig. 3).

Morphological and molecular characterization confirmed that all the identified belonged from pathotype A because of its distinct characters.16S rRNA gene amplification was the initial step which will further help in taxonomic characterization (Fatima *et al*., 2012). This work can be further processed towards expression analysis of different proteins in order to distinguish pathogenic and non-pathogenic isolates.

Conclusion

Survey was done in Khanpur and Islamabad (NARC) Pathogen isolations were done using Nutrient agar media and stack yellow mucoid, *Xanthomonas*-like isolates were selected. The 16S rRNA of the seven isolates were amplified with primer set respectively, cloned and sequenced. BLAST analysis showed the highest sequence identity with those of *Xanthomonas citri* (Xcc-A). These results confirmed that the pathogen inducing typical CC symptoms on leaves of Islamabad citrus trees was Xcc-A.

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