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PATHOGENIC VARIABILITY AMONG DIFFERENT ISOALTES OF *XANTHOMONAS AXONOPODIS* PV. CITRI

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ABSTRACT

Citrus production is subjected to the attack of a number of production threats. Citrus canker, caused by *Xanthomonas axonopodis* pv *citri (Xac)* is one of them. In the present study five different isolates of the bacterium (*Xac*) including *Xac1, Xac2, Xac3, Xac4*, and *Xac5* isolated from grapefruit (*Citrus paradesi*), lime (*Citrus limon*), rough lemon (*Citrus jambhiri*), red blood malta (*Citrus sinensis*) and kinnow (*Citrus reticulata*), respectively were used. These isolates were tested to find the pathogenic variability on three test hosts including grapefruit, lime and rough lemon. The results of these isolates on pathogenicity, symptoms development and host susceptibility indicated that the isolate *Xac*3 showed greater virulence on all test hosts, followed by the isolate *Xac*4, while the response of other three isolates was intermediate. Among the test hosts, grapefruit gave the highest degree of susceptibility to all the strains, while lime and lemon were ranked second and third, respectively.The results of the study suggest variation in the pathogenic nature of these isolates.

Keywords: Citrus canker, isolate, variability, *Xanthomonas axonopodis* pv *citri*.

INTRODUCTION

Citrus (*Citrus* spp.) is an important fruit crop of the world. In Pakistan the total area under citrus cultivation is 194 (000 ha) with an annual production of 2147.3 (000 tons), with Punjab as the leading citrus producer in the country (GoP, 2011-12). Nutritionally balanced citrus fruit exists in number of varieties which makes it unique in consumption among all the fruit crops and is also an important source of foreign exchange. Like any other crop citrus also faces many production constraints including insect pests, various diseases, nutritional imbalances and improper cultural practices which limit its yield per unit area (Hafiz and Sattar, 1952). Citrus canker is an important bacterial disease that affects the citrus production both quantitatively and qualitatively. It attacks nursery and citrus

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plants with the symptoms on leaves, twigs, stem and fruits, while the lesions on fruit reduce its market value (Stall and Seymore, 1983).

Citrus canker exits in number of strains infecting different citrus cultivars throughout the world. Civerolo (1985) reported five different pathogenic types of this disease depending upon the kind of citrus hosts, symptoms and the isolation of the bacteria on different nutrient media. These forms include canker A or Asiatic canker, canker B or false canker, canker C or Mexican lime cankeriosis, canker D or bacteriosis and canker E or the nursery form of citrus canker. Among these types of citrus canker, Asiatic form of this disease is the most serious threat to citrus worldwide due to a wide host range and virulence (Stall and Seymore, 1983). In their studies Gottwald *et al.* (1991) and Marta *et al.* (2010) described that the bacterium of citrus canker reproduces in the diseased lesions present on different tissues of citrus plant. The availability of free moisture helps this bacterium to ooze out and spread to the adjoining trees. They have also observed that this disease is severe on the side of the trees which are exposed to wind and rain. Similarly, in their finding they reported that the long distance spread of the disease results due to heavy winds, storms and with the movement of infected plant material. Rao and Hingorani (1963) have reported that the bacterium (*Xac*) can survive up to six months in the infected leaves but the cankers on other tissues like twigs and branches serve as a source of longer survival upto 76 months. The temperature range from 20-30°C is considered as the most suitable for the disease development (Reddy, 1984). The role of Asian leaf miner (*Phyllocnistis citrella* Stainton), in the dissemination of citrus canker is being considered very important, however, it has not been reported as disease vector yet. In their observation Sinha *et al*. (1972) and Cook (1988) reported that the feeding of leaf miner results in the formation of galleries on citrus tissues below the epidermis. Once these galleries come in contact with bacteria, considerable amount of inoculum is produced. According to Das (2003) a considerable amount of money is being spent to overcome this menace throughout the world as citrus canker results in trade related problems. Keeping in view the importance of citrus canker, the present study was conducted to find out the pathogenic variability among the strains of *Xanthomonas axonopodis* pv *citri*.

MATERIALS AND METHODS

The diseased samples of citrus canker including infected leaves, twigs and fruits were collected from different nurseries and orchards in citrus growing areas. The diseased samples were cut into small pieces along with some healthy portion, which were cleaned and surface sterilized with 1% chlorox and 70% ethanol for 2-3 minutes, respectively, later rinsed with sterilized water and dried on blotter paper. These pieces were placed on Nutrient Agar (NA) medium for the preliminary isolation of bacterium. The yellowish mass exuded from the diseased lesions was purified on the Yeast Dextrose Calcium Carbonate Agar (YDCA) medium. The isolates were tested for the hypersensitive reaction on tobacco plants as reported by Klement *et al.* (1964). For the assessment of pathogenicity of these isolates, the cultures from each of these samples were taken and

multiplied on NA medium. Inoculum suspension was prepared by adjusting the concentration of isolates suspension at 10^8cfu/ml. Pathogenicity tests were conducted on detached leaves as reported by Koizumi (1971). Five leaves of each of the test host i.e. grapefruit, lime and rough lemon, kept in three replicates were inoculated with the bacterial suspension of each isolate using the pin prick method as described by Akhtar *et al.* (1995). The control leaves were inoculated with sterilized distilled water. The inoculated leaves were incubated at 25°C under 12 hours of light. The observations for the water soaking of leaves and lesion development were made after 4, 7 and 10 days of inoculation. Data on the number of leaves infected after inoculation and number of lesions per leaf were collected and analyzed statistically with the help of Staistix 8.1. Version.

Table1. The details of the isolates with their respective hosts.

Isolates	Host	
	Common name	Botanical name
*Xac*1	Rough lemon	*Citrus jambhiri*
*Xac*2	Red blood malta	*Citrus sinensis*
*Xac*3	Grapefruit	*Citrus paradesi*
*Xac*4	Lime	*Citrus limon*
*Xac*5	Kinnow	*Citrus reticulata*

RESULTS AND DISCUSSION

Isolation of pathogen (*Xanthomonas axonopodis* pv c*itri)*

The culturing of the disease specimen produced yellowish growth of the bacterium on nutrient agar medium which was further purified on YDCA. The cultural characteristics like colony growth, color, shape and texture of the bacterial isolates observed were in accordance with Akhtar *et al.* (1996) and Islam *et al. (*2013).

Hypersensitive reaction (HR) on tobacco plants

Table 2. shows the hypersensitive response of the all five isolates on tobacco plants, among these five isolates *Xac*1 and *Xac*5 gave a positive HR after 48 hours of inoculation while *Xac*3 and *Xac*4 gave a positive HR after 24 hours and *Xac*2 isolated from red blood malta produced hypersensitive reaction after 72 h of inoculation. The inoculated leaf tissue showed water soaking and necrosis. These results are in accordance to those reported by Akhtar *et al.* (1996). All the positive HR isolates were used for further studies.

Pathogenic variability in citrus canker isolates

The results of the inoculation of these isolates on detached leaves are shown in Table 3, which indicate that the isolate *Xac*3 produced more extensive and clear infections with greater water soaking, followed by necrosis around the wound-inoculated surface of the detached leaves of all three test hosts, however its

reaction was severe on grapefruit as compared to the other two test hosts and on rough lemon it was the least. This response of grapefruit was in accordance with the studies of Stall *et al.* (1995) who have reported the susceptibility of grapefruit to different strains of citrus canker. The *Xac3* was followed by the isolate *Xac*4 from lime on the basis of symptoms development on the detached leaves, the isolate *Xac*2 ranked third among all five test isolates in its pathogenic behavior. However, it was more severe on grapefruit, followed by lime. *Xac*5 and *Xac*1 were rated fourth and fifth on the basis of infection and lesion development, which took more time than the rest of the isolates tested and produced less water soaking and necrosis around the lesion as compared to the strains *Xac*3, *Xac*4 and *Xac*2. Schubert *et al.* (2001) reported that the pathogenicity is of great importance in the recognition and diagnosis of the strains. According to the studies conducted by Marta *et al.* (2010) the detached leaf assay is of vital importance in the screening of citrus germplasm against citrus canker and to study the variation in the lesion development under a quantified inoculum levels.

Table 2. Hypersensitive (reaction on tobacco plants) and Gram Reaction of *Xanthomonas axonopodis* pv. *citri* isolates.

Isolate	Time (hours)			Gram reaction	KOH test
	24h	48h	72h		
*Xac*1	No reaction	+	+	G -ve	+
*Xac*2	No reaction	No reaction	+	G -ve	+
*Xac*3	+	+	+	G -ve	+
*Xac*4	+	+	+	G -ve	+
*Xac*5	No reaction	+	+	G -ve	+

Table 3. Leaves infected by isolates using pin prick method of inoculation.

Isolates	Test hosts		
	Grapefruit	Lime	Rough lemon
*Xac*1	0.1533E	0.1067D	0.0600E
*Xac*2	0.2833D	0.1767C	0.1067D
*Xac*3	0.6833A	0.5533A	0.4000A
*Xac*4	0.4833B	0.4000B	0.2000B
*Xac*5	0.3767C	0.3533B	0.1533C
Control	0.0000F	0.0000E	0.0000F

Alpha 0.05

Table 4 shows the results of the experiment conducted on the development of the number of lesions produced by these isolates on the detached leaves. The results showed that the isolate *Xac*3 produced extensive and clear lesions with more water soaking and necrosis this was followed by the isolate *Xac*4. The lesion development of the other three isolates was comparatively low with less water soaking and more time. Koizumi (1979) reported that the formation of lesion on citrus tissues and the reproduction of bacteria depend on the type of resistance in the host. However, Stall *et al*. (1980) observed that the number of bacterial cells per lesion is not always associated with the type of resistance

in citrus plants while the availability of free moisture on the host tissue is essential for successful infection. Das (2003) reported that these pathovars of citrus canker are different on the basis of their geographical distribution and their pathogenic behavior on citrus cultivars. The effect of these five strains in producing the pathogenic reaction was higher on grapefruit as compared to lime and rough lemon. Rough lemon is generally used as a rootstock in our conditions because of its adoptability and the ability to withstand the adverse conditions. The results of the study suggest that all these isolates belong to the Asiatic form on the basis of symptom development on the test plants and their isolation on nutrient media. The studies conducted by other workers including Civerolo (1985) and Goto *et al.* (1980) show that higher susceptibility of lime was possibly due to the genetic makeup of the lime and the environmental conditions that prevail in the field and the response of these isolates to all these conditions. The variability among these isolate is very much clear which may be due to the wide host range and the production of certain compounds in the citrus plants after the infection of the bacteria as reported by Rasoulina *et al.* (2013) and Silva *et al.* (2013). In their observations, Khan and Hingorani (1970) and Al-Saleh *et al.* (2014) found the difference in the behavior of *Xac* strains against the test cultivars and suggested the presence of genetic specificity between the strains and citrus plants/cultivars. Civerolo (1984) has found a number of other plants in the family rutaceae other than *Citrus* and *Poncirus* as the host and reported that primary basis of strains classification is the differential pathogenicity of these isolates on various citrus hosts. Similarly, Golmohammadi *et al*. (2007) and Mavrodieva *et al*. (2004) used the conventional polymerase chain reaction (PCR) for comparing the isolates of citrus canker and observed it as an important method of screening these isolates.

Table 4. The mean number of lesions/ leaf produced by the citrus canker isolates.

Isolates	Test hosts		
	Lime	Rough lemon	Grapefruit
*Xac*1	0.4267 D	0.2533 C	0.5733 C
*Xac*2	0.6000 B	0.2667 C	0.7333 B
*Xac*3	0.7333 A	0.5733 A	0.8667 A
*Xac*4	0.4800 C	0.3467 B	0.5600 C
*Xac*5	0.3467 E	0.2000 D	0.4667 D
Control	0.0000 F	0.0000 E	0.0000 E

Alpha 0.05

Based on the pathogenic data of these isolates and the response of the test hosts, significant variation exists among these isolates. Goto (1985) and Das (2003) have observed the production of extracellular polysaccharides (EPS) in the culture media and in the citrus tissues. The bacterial cells in diseased lesions are enclosed in EPS and disperse by rain splash. These EPS molecules protect the pathogen from desiccation which enhances the survival of the bacterial colonies. Hussain *et al.* (2010) reported that the severity of the citrus canker pathogen varies among different species of citrus and depends upon the existing environmental conditions. The results of the present studies suggest pathogenic

variability in these isolates and a varying response of each citrus variety to these strains. According to Civerolo (1984) this variation might be due to the fact that the CBCD strains are inherently variable and variability is a major characteristic of this group. Farimah *et al.* (2013) have also described the genetic variation in the citrus canker bacterium. Gottwald *et al*.(1993) and Schubert *et al*. (2001) reported that strains of *Xac* can be identified and characterized from the other pathovars with the help of such pathogenicity test on a set of susceptible and resistant citrus hosts using detached-leaves or leaf-disks. Such pathogenicity tests play an important role in the diagnosis of citrus canker diseases. Citrus canker is a major threat to world citrus production, because of its wide pathogenicity spectrum, cultivation of citrus canker susceptible varieties and the emergence of the new strains. Thus such studies are of great importance to find out the variation among the isolates of the citrus canker.

CONCLUSION

The present studies suggest variability in the pathogenic behaviour of citrus canker isolates which were isolated from the citrus plantations in different citrus growing areas. The isolate *Xac*3 was the most virulent among five isolates while grapefruit showed maximum susceptibility as a test host. The response of the isolates on three test hosts, their easy isolation on nutrient media and the susceptibility of grapefruit suggests that these isolates belong to canker A group of this disease.

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