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Distribution and pathotype identification of *Xanthomonas citri* subsp. *citri* recovered from south-western region of Saudi Arabia

Mohammed A. Al-Saleh, Arya Widyawan, Amgad A. Saleh and Yasser E. Ibrahim*

Plant Protection Department, College of Food and Agriculture Sciences, King Saud University, P.O. Box 2460, Riyadh 11451, Kingdom of Saudi Arabia.

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We investigated the distribution of *Xanthomonas citri* subsp. *citri* (*Xcc*) pathotypes in south-western region of Saudi Arabia. A total of 76 *Xanthomonas* like strains were isolated from different citrus species showing bacterial canker symptoms from citrus commercial farms, backyard orchards and nurseries. These strains were subjected to biochemical, molecular and pathogenicity tests on leaves of grapefruit (*Citrus paradisi*). Based on symptoms induced on leaves of grapefruit, these strains were divided into two groups. There were 21 strains that induced typical erumpent canker lesions with water-soaked margin, belonging to *Xcc* type A. On the other hand, strains that produced flat necrotic lesions with water-soaked margin on grapefruit leaves belonged to *Xcc* A* type (55 strains). The physiological and biochemical tests, ImmuneStrip ® assays and 16S rDNA analysis confirmed the identity of the two *Xcc* pathotypes: A and A*. Our results indicate that, the two pathogenic variants co-exist in south-western region of Saudi Arabia and this could further favour the generation of new genetic variants through recombination and horizontal genetic exchange. The generation of new aggressive pathotypes may threaten the citrus plantation in Saudi Arabia.

Key words: Disease diagnosis, pathogenicity, citrus bacterial canker.

INTRODUCTION

Citrus bacterial canker (CBC) caused by *Xanthomonas citri* subsp. *citri* (*Xcc)* (Schaad et al., 2006), is an important disease in many citrus production areas world-wide including Saudi Arabia (Gottwald et al., 2002; Ibrahim and Bayaa, 1989). The pathotypes of the casual agent are primarily separated by their geographical origin, host range and certain genotypic characteristics (Brunings and Gabriel, 2003). The common well-known pathotypes are A, B, and C. The citrus canker B (cancrosis B or false canker) was reported in Argentina, Uruguay and Paraguay on lemon (*Citrus limon*) (Schubert et al., 2001). The C type of citrus canker (cancrosis C) is also caused by *X. citri* pv. *aurantifolii*. It has been found only in São Paulo state in Brazil and it has only two known hosts; Mexican lime and sour orange. The symptoms induced by the three forms of canker are similar and induce erumpent and corky lesions surrounded by oily or water-soaked margins on leaf, fruit, and stem tissues (Civerolo, 1984). The Asiatic form or pathotype A of *Xcc* is widespread throughout the world and affects the widest range of citrus hosts (Schubert et al., 2001; Juhasz et al., 2013; Hoarau et al., 2013). However, several groups within pathotype A with restricted host range have been identified. Group A* was reported for the first time by Vernière et al. (1998) in Southwest Asia. This group was isolated from Mexican lime trees in several countries in Southwest Asia; including Saudi Arabia, Oman and Iran. The pathogen produces typical

*Corresponding author. E-mail: yasereid@ksu.edu.sa. Tel: 00966-114678428. Fax: 00966-114678423.

erumpent bacterial canker lesion on Mexican lime but not on grapefruit (Vernière et al., 1998). Recent studies reported the presence of group A* in Thailand, Cambodia, Ethiopia and Burkina Faso (Bui et al., 2007; 2008; Derso et al., 2009). In 2012, typical CBC symptoms were observed on leaves, stems and fruits of Mexican lime trees in south western region of Saudi Arabia where CBC had not been previously detected (personal observation). Interestingly, other CBC susceptible citrus trees near the infected ones (within 3 m of a diseased tree) did not show any CBC symptoms. Initial laboratory pathogenicity tests on grapefruit leaves revealed that there were two different types of symptoms produced by *Xcc* strains. The purpose of this study was to determine which strains of *Xcc* are present in south-western region of the Saudi Arabia, specifically the citrus major producing areas e.g. Al-Bahah, Aseer and Jazan.

MATERIALS AND METHODS

Surveyed areas

Ten locations belonging to three citrus-growing regional areas as mentioned before were surveyed for citrus bacterial canker strains distribution on commercial farms, backyard orchards and nurseries. The disease incidence of citrus canker for each block was calculated by expressing the number of diseased trees as a percentage of the total number of trees inspected. The severity of canker in each 5-tree block was rated visually on the following scale: 0 = no symptoms, 1 = isolated leaf lesions, 2 = lesions restricted to one side of the canopy, 3 = lesions distributed over the entire canopy and 4 = greater occurrence of leaf lesions than in 3 (Agostini et al., 1985).

Isolation of bacterial strains

Seventy six citrus samples with bacterial canker-like symptoms were collected from seventeen orchards located in various regions. Samples of diseased leaves, stems and fruits were collected from each orchard planted with either one variety or mixed varieties from four to five trees per row. Both sampled trees and rows were selected at random. Samples were sealed in plastic bags and forwarded to the quarantine laboratory at the King Saud University, College of Food and Agriculture Sciences, Plant Protection Department. Samples were washed under running tap water for 10 min. Infected areas were soaked in 1% sodium hypochloride solution for 30 s and rinsed in sterile distilled water three times. One lesion, and 2 mm of the peel around it, was cut into small pieces with a sterilized scalpel, comminute in Phosphate Buffer Saline (PBS, pH 7.2) and left for 10-20 min at room temperature. 100 µl of the PBS extract were streaked onto plates of nutrient agar media supplemented with 1% glucose (NGA) (Lelliot and Stead, 1987) and incubated at 28°C for 48-72 h. Bacterial colonies with yellow pigmentation were picked up and transferred to new NGA plates for purification and further tests.

Pathogenicity tests

Inoculum preparation

Isolates of *Xcc* were grown on NGA plates and incubated at 28°C for 24 h. Bacterial cells were suspended in sterile distilled water and the bacterial suspension was adjusted to 10^8 CFU ml^{-1} (OD_{660}= 0.06).

Detached leaves

Pathogenicity of purified *Xcc* strains was evaluated on detached grapefruit leaves cv. Duncan. Surface of young leaves was disinfected with 70% ethanol, washed with sterile water and placed on the surface of 1% water agar with their abaxial surfaces facing upwards (Vernière et al., 1991). Ten wounds per leaf were performed with a needle and droplets (10 µl) of bacterial suspensions of 1×10^8 CFU $ml^{-}1$ were placed on each wound. Leaves were incubated for 1-2 weeks at 28°C until symptoms appearance. Negative controls had leaves treated with sterile water.

Attached leaves

Grapefruit cv. Duncan fully expanded leaves were infiltrated by pressing the opening of a syringe without a needle gently against the abaxial leaf surface supported by one finger with 1×10^8 CFU $ml^{-}1$ of *Xcc* strains (Vernière et al., 1991). Plants were maintained in the greenhouse at 28-30°C.

Physiological and biochemical characterization

A total of 76 strains were identified according to tests described by Fahy and Persley (1983), Lelliott and Stead (1987) and Vernière et al. (1991).

ImmuneStrip ® assay

ImmuneStrip assay was conducted according to the instruction in the manual of *Xcc* immuneStrip ® test proposed by Agdia, Inc. (Catalog No. STX92200). Bacterial suspensions were diluted with the immunoStrip buffer. The strips were dipped into the immunoStrip buffer for 5 min until both the control line and the test line appeared. Positive results gave both control and test lines, while negative results gave only the former one.

Amplification and sequencing of 16S rDNA gene

DNA extraction

Total DNAs of *Xanthomonas* strains were extracted using the method described by Llop et al. (1999). The DNA was stored at -20°C until further use for PCR.

Sequence analysis

Amplification of the 16S rDNA was done by using primers 27F and 1492R (Lane, 1991). The PCR products were sent to the Advanced Genetic Technologies Centre, University of Kentucky, Lexington, USA to be cleaned and sequenced. DNA sequences were cleaned and alignment using BioEdit Software and the cleaned sequences were searched against other 16S rDNA sequences deposited in the NCBI GenBank database.

Statistical analysis

In cases where disease incidence data were not normally distributed, analysis of variance and separation on means was performed

Table 1. Strain designation, host plant, location and pathotype identification of *Xanthomonas citri* subsp. *citri* strains recovered from south-western of Saudi Arabia.

Number of isolate	Strain designation	Host plant	Region	Pathotype identification	Accession number
12	Aseer 1-12	*C. sinensis*	Aseer	A	JQ890091*
15	Aseer 13-27	*C. aurantifolia*	Aseer	A&A*	JQ890092
20	Bah 1-20	*C. aurantifolia*	Al-Bahah	A&A*	JQ890093
5	Jaz 1-5	*C. limon*	Jazan	A	JQ890094
24	Jaz 6-29	*C. aurantifolia*	Jazan	A*	JQ890095

*No nucleotide polymorphisms was detected among the 16S rDNA region of Saudi *Xcc* strains.

Table 2. Distribution, incidence and severity percentage of canker disease caused by *Xanthomonas citri* subsp *citri* on citrus leaves in south-western region of Saudi Arabia.

Region	locations	Variety	incidence (%)[1]	Severity (%)[2]	Citrus trees age (year)
Al-Bahah	Al-Bahah	Mexican lime	77abc	1.99b	10
	Al-Makhwah	Mexican lime	74c	1.83b	8
	Baljurshi	Mexican lime	74c	1.77b	8
Asser	Abha	Mexican lime	83ab	2.90a	6
	Mahail	Sweet orange	78abc	2.01ab	12
	Balqarn	Mexican lime	83ab	2.65ab	10
Jazan	Abu Arish	Mexican lime	84a	2.98a	10
	Jizan	Lemon	76abc	1.85b	8
	Sabya	Mexican lime	83ab	2.91a	8
	Samitah	Mexican lime	80b	2.35ab	10

[1] Average of 15 trees with canker symptoms; [2]Mean of five canker ratings (1 rating per 5 tree block) where 0 = no disease and 4 = sever (see text). Means within a colum followed by the same letters are not statistically different at $P \leq 0.05$ (Tukey test).

on arcsine transformed data, but percent data were reported. Disease severity data were analyzed according to the Kruskale Wallis nonparametric test statistics. Analysis of variance was performed and means were separated according to the Tukey test. The experiments were repeated twice.

RESULTS

Orchard survey and bacterial isolation

A total of seventy six *Xcc* strains was isolated from infected leaf, stem and fruit samples collected from symptommatic Mexican lime (59 strains), sweet orange (12 strains) and lemon (5 strains) plants from different locations (Table 1). Bacterial citrus canker was detected in all citrus growing regions surveyed in Saudi Arabia (Table 2). Typical canker symptoms were observed not only on leaves but also on fruits and twigs of citrus trees (Figure 1). Also, water soaked regions around the raised corky lesions on the fruits were observed. No chlorosis was observed around the corky lesions on twigs. The overall disease incidence on leaves was 79.2% with at least one lesion per leaf. Disease severity expressed as percent infected leaf area was 32.2%. The highest disease incidence was recorded in Jazan on Mexican lime followed by Asser, Balqarn and Sabya, respectively. Canker severity on Mexican lime recorded in Abu Arish (2.98%) was significantly higher than in Baljurshi (Table 2). Based on the field host range and pathogenicity tests, it appeared that the *Xcc* variant that occurred in all locations of Al-Bahah, one location in Aseer and two locations in Jazan regions had similar host ranges to that of the atypical Asiatic (*Xcc*-A*) form.

Pathogenicity tests

Based on pathogenicity tests on grapefruit leaves, all strains were divided into two groups. In detached leaves, bacterial strains gave two different kinds of symptoms. Strains of group one gave erumpent callus-like lesions which surrounded by thin water soaked margins (Figure 2a), while the second group caused no symptoms post inoculation (Figure 2b). In case of attached leaves, the first group strains was pathogenic and showed water soaking regions following hyperplasia and hypertrophy and necrosis (*Xcc*-A) (Figure 3a), while the second group (*Xcc*-A) gave only flat necrotic appearance on the infiltrated areas (Figure 3b). Hypertrophy and hyperplasia were observed 6 days after inoculation. The flat necrotic lesions were clear

Figure 1. Canker symptoms caused by *Xanthomonas citri* subsp. *citri Xcc*-A* strains on Mexican lime (a) and *Xcc*-A strains on Sweet orange (b) which bacteria were consistently isolated.

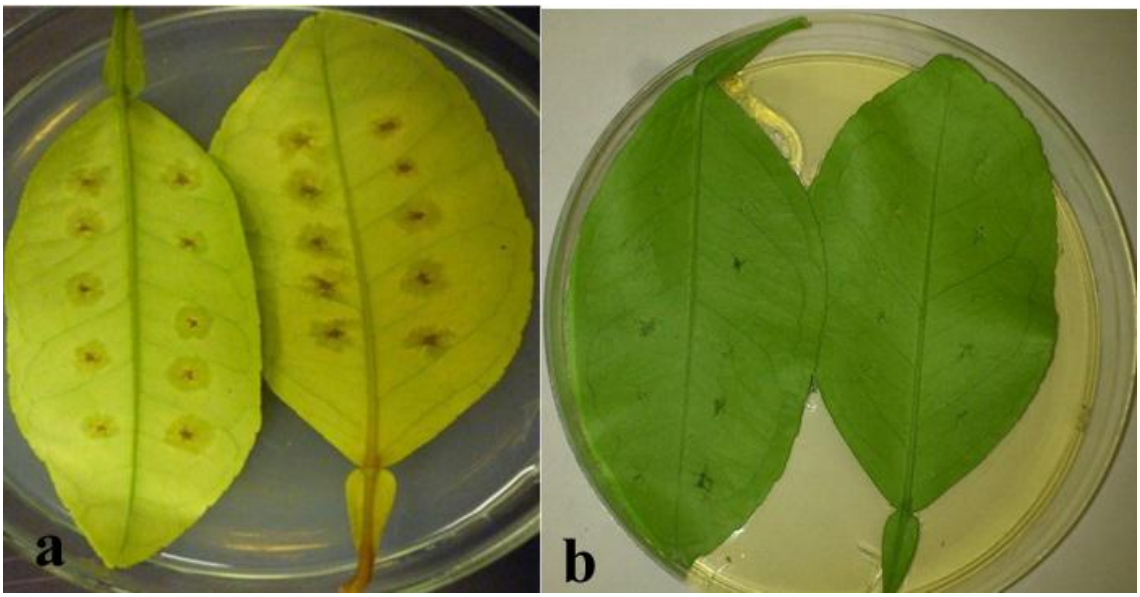

Figure 2. Response on Duncan grapefruit in a detached leaf assay after one week post inoculation with (a): *Xcc*-A strains, (b): *Xcc*-A* strains. All *Xcc*-A strains produced erumpent callus-like tissue with water-soaked. In contrast, the *Xcc*-A* strains caused no symptoms after inoculation.

and the hyperplasia developed well and resulted in necrotic regions. No symptoms were observed on control plants inoculated with sterile water in both inoculation methods. All *Xcc* strains were re-isolated from inoculated leaves and re-identified by phenotypic characters.

Physiological and biochemical characterization

Data in Table 3 show that all *Xcc* strains were Gram-negative; rod shaped, motile, aerobic, non-fluorescent on King's medium B, but grew with characteristic mucoidal colonies on media containing glucose. They were able to hydrolyze casein, gelatine, starch, Tween 20 and 80. In addition, all isolates had the ability to grow on media supplemented with 1, 2, and 3% NaCl. All the strains were oxidase negative, arginine-dihydrolase positive and did not macerated potato discs. Moreover, the strains utilised sucrose, cellubiose, L-rhamnose and L-arabinose. All strains grew at 36 and 4^{o}C but not at 40^{o}C. The identity of bacterial strains was also confirmed with the immunoStrips designed for detection of *Xcc*. Collectively, the biochemi-

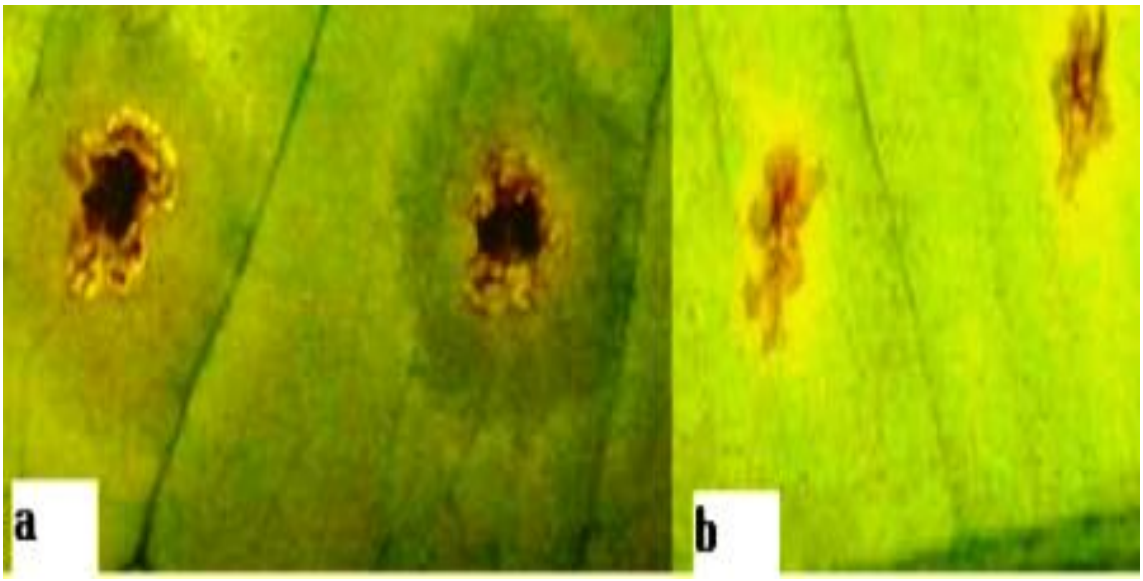

Figure 3. Symptomatology of *Xanthomonas citri* subsp. *citri* strains (A and A*) on attached grapefruit leaves cv. Duncan. Typical callus-like lesions with water soaked margins developed by *Xcc*-A while, b) flat less water soaked margin belonged to *Xcc*-A*.

Table 3. Biochemical and physiological characteristics of Saudi Arabia strains of *Xanthomonas citri* subsp *citri,* the causal agent of citrus canker disease.

Characteristic	Reaction
Gram reaction	-
Oxidative/fermentative tests	O
Arginine dihydrolase	-
Fluorescent pigmentation	-
Growth at 36°C	+
Growth at 40°C	–
H_2S generation from cysteine	+
Indole test	–
Levan production	+
Litmus milk test	Alkaline
MR-VP	–
NaCl tolerance: 1, 2 & 3% (w/v)	+
Nitrate reductase	–
Potato rot	+
Hydrolysis of: casein, gelatin, starch,Tween 20 and 80	+
Utilization of	
L-arabinose	+
L-rhamnose	+
Cellubiose	+
Sucrose	+

+ Positive reaction; -negative reaction.

cal identification of the strains confirmed their identity as *Xcc*.

Identification with 16S rDNA gene sequencing

The bacterial strains were also confirmed at the molecular level by sequencing 16S rDNA gene. The 16S rDNA analysis could not discriminate *Xcc* Saudi Arabian strains. Comparison between the partial sequences of 16S rDNA of Saudi strains and the other sequences of 16S rDNA deposited in GenBank showed that isolates were *Xcc.*

DISCUSSION

In our study, we aimed to determine the distribution of *Xcc* strains in the main citrus-growing areas of Saudi Arabia. Our survey showed the presence of *Xcc*-A and/or A* strains in the visited orchards with different extent of infection. During the survey, citrus canker was found on Mexican lime, sweet orange and lemon varieties. In all Al-Bahah locations, two locations in Jazan and one location in Aseer, other citrus varieties did not show any citrus canker symptom and that, hence, indicated the host specificity of the causal agent. The fact that, citrus canker disease was observed in commercial farms, backyard orchards and nurseries might indicate that the pathogen was probably introduced with the plant material used in citrus plantations. In addition, all the surveyed locations for canker disease used the flood irrigation system. The use of this system and contaminated seedlings might have disseminated citrus canker in these areas. The group A* was reported for first time with the same symptoms on Mexican lime by Vernière et al. (1998) in Southwest Asia and in Thailand and Cambodia (Bui et al., 2007; 2008) and Ethiopia (Derso et al., 2009), while, the group A^w strains produced brownish, flat and necrotic lesion on grapefruit (Sun et al., 2004).

Vernière et al. (1998) used several biochemical tests to identify and differentiate different pathotypes of citrus canker bacteria. The tests included hydrolysis of gelatine and casein, in addition to the growth on 3% NaCl. They reported that citrus canker pathotype A gave positive results on the three previous tests, while pathotype B had negative results for these tests. Patothype C gave a positive result only on hydrolysis of casein. In the present study, all bacterial isolates recovered from Saudi Arabia had the ability to hydrolyze both casein and gelatin and grew on media supplemented with 3% NaCl. We concluded that these tests were not able to discriminate between the Saudi Arabia strains. Vernière et al (1998) also reported that phenotypic tests based on carbon source utilization usually do not discriminate *Xcc*-A* strains from *Xcc*-A. In order to differentiate between groups of pathotype such as A, A^w and A* another test is needed. Generally, bacterial strains in this study were identified as *Xcc* according to Fahy and Persley (1983); Schaad (1988) and immunoStrip test. To confirm the identity of different Saudi strains causing citrus canker disease, the 16S rRNA sequences showed that both strains were *Xcc* with a similarity of 99%. Moreover, the analysis of 16S rDNA sequences did not give any discrimination between Saudi strains. Our results corroborate with the previous research that showed 16S rDNA sequences of different pathotypes of *Xanthomonas* causing citrus canker disease (A, B and C) were *Xcc* with a similarity of 99% (Lee et al., 2008).

Fox et al. (1992) reported that the 16S rDNA gene was considered unsuitable for discriminating and identifying closely related strains due to the high levels of sequence similarity in this region.

The results of our study based on physiological, biochemical, genetic analyses and pathogenicity tests showed that two strains (*Xcc*-A and *Xcc*-A*) were associated with bacterial citrus canker in Saudi Arabia. Identification of both pathotypes in citrus nurseries in Saudi Arabia suggests that there is a potential large scale distribution of these strains within the citrus orchards in this region. As diseased citrus nursery plants are a major source of primary inoculum, sanitation of citrus nurseries against citrus canker in Saudi Arabia is a prerequisite for improving disease management.

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