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Etiological and Some Epidemiological Features of Bacterial Citrus Canker in Egypt

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ABSTRACT

Symptoms consistent with citrus canker were observed in many citrus groves located in different egyptian citriculture regions. the presence of the disease was confirmed using different diagnostic methods. attempts to pathogen isolation from symptomatic citrus samples enabled toobtain15 bacterial isolates with characteristics consistent with *xanthomonas citri* subsp. *citri*. the results of physiological and biochemical tests supported the membership probability of these isolates to *x. citri*subsp. *citri*. pathogenic response of these isolates was established by artificially inoculation of detached and attached leaves of grapefruit and valencia sweet orange. all isolates caused canker symptoms on both citrus species. subjection of the isolates to omnilog id system indicated that, the isolates strongly belong to *xanthomonas citri* subsp. *citri* with similarity around 90 %. the identity of the isolates was confirmed by pcr amplification using primers 2-3. the pcr amplification successfully amplified a 222 bp dna fragment from all isolates except one. considering the results of the performed tests, these bacterial isolates confirmed to be *x.citri* subsp. *citri* and accordingly, the symptoms that appeared on the affected trees represented citrus canker disease .in another trend, some epidemiological features of the disease were studied. the obtained isolates significantly varied in their aggressiveness. the disease was occurred in 31.7 %of surveyed groves which were distributed in all surveyed localities. disease incidence (number of symptomatic trees) within diseased groves recorded 8.49%. the total mean of disease severity on the diseased trees within localities reached 35.75%.

Keywords: citrus canker, *Xanthomonascitri*subsp. *citri*, grapefruit, epidemiology, disease incidence and severity.

INTRODUCTION

Citrus canker disease is a major threat for the citriculture worldwide. It causes severe economic impact to the citrus grower in tropical and subtropical citrus-growing areas and also limits the exports of citrus fruits and propagation materials owing to the quarantine status of the pathogen in some countries. The disease reduces the yield, disfigures the fruits, debilitates the trees and its presence in a nursery can cause serious to total loss (Gottwald *et al*., 2002b and Das, 2003).

The disease is caused by certain strains of xanthomonads but the most widespread and aggressive canker is induced by *Xanthomonas citri* subsp. *citri* (Schaad *et al*., 2006 and Bull *et al*., 2010) which is responsible for the so-termed Asiatic citrus canker or type A canker.

Citrus canker occurs on seedlings and on young and adult trees of susceptible hosts and the symptoms are formed on the leaves, shoots, twigs and fruits (CABI, 2006). The disease symptoms evolve under favorable conditions as initial water soaking appear about 7 to 10 days after infection followed by hyperplasia and necrosis at the site of infection. As the disease progresses and the conditions are highly conducive to infection, it causes defoliation, shoot dieback, and fruit drop (Brunings and Gabriel, 2003). *Xanthomonas citri* subsp. *citri* was found in southern Asia, Japan, North America, South America, Middle East and Africa (CABI/EPPO, 2006).

X. citri subsp. *citri* affects numerous species, cultivars, and hybrids of citrus and citrus relatives and has been found in all types of citrus, including grapefruit, lemons, oranges, sour oranges, tangerines, and limes (GISD, 2015).

Diagnosis of citrus canker pathogen can be achieved by checking the cultural and physiological characteristics, serological tests, molecular methods, bioassay of leaf discs or detached leaves and pathogenicity test (EPPO, 2009). Verniere *et al*, (1993) used the Biolog substrate utilization system to identify and assess metabolic variation among 148 strains of *X. citri* subsp. *citri* that originated from 24 countries and associated with various forms of citrus bacterial canker disease. They used the metabolic profiles of these strains to attempt strain identification.

The DNA-based methods proved to be powerful techniques and traditionally have been mostly used to improvement the identification and detection of plant pathogens due to their accuracy, specificity and sensitivity (Hartung *et al*., 1993, 1996; Cubero& Graham, 2002; Mavrodieva *et al*., 2004; Coletta-Filho *et al*., 2006; Golmohammadi *et al*., 2007).Although, several primer pairs are available for molecular diagnosis of *X. citri* subsp. *citri*, primers 2 and 3 that targeted a Bam HI restriction fragment length polymorphic DNA fragment specific to *X. citri* subsp. *citri* (Hartung *et al*., 1993) are the most frequently used because of their highly specificity and sensitivity.

Disease severity is depending on the susceptibility of the host plant species and cultivars (Goto, 1992) and definitely on strain aggressiveness. The pathogenic variation among strains of bacteria associated with citrus canker has been reported (Gottwald *et al*., 1993, Schubert *et al*., 2001 Das, 2002 and Madhuri *et al*, 2016).

The majority of epidemiological studies on citrus canker have concentrated on local disease increase and spread of *X. citri* subsp. *citri* within citrus nurseries and commercial plantations. Disease spread between geographical regions is most likely to occur via diseased plant material (Civerolo 1984 and Schubert *et al*., 2001). The spread of citrus canker through groves has been previously reported (Danos *et al*., 1981 and Gambley *et al*., 2009). Measuring the disease incidence and severity in different groves, provides an estimate of

when the disease first established in the location and the rates of spread of the disease and then, evaluate the seriousness of the disease (Danos *et al*., 1981 and Gambley *et al*., 2009).

The present work aimed to confirm the presence of citrus canker disease in Egypt and outlined the etiological features of the disease pathogen. It also detailed the most important features related to the epidemiology of the disease in the major citriculture regions in Egypt.

MATERIALS AND METHODS

Sampling and pathogen isolation

Symptomatic leaves, twigs, and fruits of sweet orange [Valencia and navel orange (*Citrussinensis*)] and grapefruit (*Citrus paradasi*)trees showing typical symptoms of citrus canker were collected from the major citrus growing areas in Egypt.The corky-like raised lesions, surrounded by a yellow halo were removed from each sample type and washed inrunning tap water for 4-5 minutes, sprayed with 70% ethyl alcohol and left to dry under sterile condition then cut into small pieces with sharp sterile scalpel. A portion (500 mg) of these cuttings was soaked in 10 mlof sterile saline solution (distilled water with 0.85% NaCl, pH 7.0) for 30 min. Another portion (50 mg) was pulverized in 2.0 ml of saline solution and left for 20 min. A loopfuls of the suspensions resulted from the both methods were streaked onto NGA medium (nutrient agar supplemented with 0.1% glucose),YPGA [yeast peptone glucose agar (0.5% yeast extract, 0.5% peptone, 1% glucose and 1.5% agar)]and Wakimoto's potato semisynthetic medium [25% potato broth, 1.5% sucrose, 0.5% peptone, 0.08% $Na_2HPO_4.12H_2O$, 0.05% $Ca(NO_3)_2 \cdot 7\ H_2O$, 2% Agar and distilled water, 1 litre; pH 7.2(Wakimoto 1967)].After incubation at 28 ºC for 4-6 days, plates were examined for the presence of round, convex, smooth-edged, mucoid and creamy yellow colonies. Colonies suspected to be *X. citri* subsp. *citri* were picked and streaked onto yeast dextrose calcium carbonate (YDC) medium(Schaad *et al*., 2001) and the isolates showed copious growth, very mucoid and richer yellow on this medium were selected and stored either in sterile distilled water at room temperature or on NA slants at 4 ºC and in 50% glycerol at -80ºC for further studies.

Pathogen identification

Morphological, physiological and biochemical characteristics

Colonies suspected of being *Xanlhomonas* were initially identified based on colony morphology on isolation media and presence ofmucoid growth on YDC medium (Schaad *et al*., 2001).

The suspected isolates were checked for Gram reaction using the KOH solubility test, motility, cell shape and spore forming. They were further examined for physiological and biochemical features as described by Schaad *et al*. (2001) and Fahy and Hayward (1983).They were tested for oxidative or fermentative metabolism of glucose, production of fluorescent pigment on King's medium B (KB), nitrate reductase activity, oxidase and catalase reaction, hydrolysis of starch, gelatin, casein and Tween 80, H_2S production from cysteine, arginine dihydrolase, utilization of sucrose, cellubiose, L-rhamnose and L-arabinose, growth at 36 and 40 ºC, Growth on 2 % NaCI, Tolerance to 0.02% triphenyl tetrazolium chloride, action on litmus milk and hypersensitive reaction on tobacco leaves.

Automated identification by OmniLog ID System

The automated identification system, OmniLog ID 1.0 (Biolog, Inc., Hayward, CA, USA) microplates was used to perform bacterial identifications according to their metabolic profiles (Verniere *et al*., 1993). The procedures were carried out according to the manufacturer's instructions.

Pathogenicity test

Detached-leaf assay

Pathogenicity testing of the suspected bacterial isolates were performed on detached leaves of grapefruit (*Citrus paradasi* cv. Ruby Red) and Valencia orange (*Citrus sinensis* cv. Olinda) to confirm their pathogenicity. The test was performed according to Davis *et al*. (2015) and (NDP 2016). The inoculum was prepared by growing each bacterial isolate on YPGA medium at 28°C for 3 days. Bacteria were suspended in 0.85% NaCl and spectrophotometrically adjusted to a concentration of 1×10^8cfu/ml.Soft and not yet fully expanded leaves of the tested plants were collected then washed for 10 min with running tap water, soaked for 2 min in a fresh solution of 1% sodium hypochlorite, rinsed three times in sterile distilled water and left to air dry. The prepared leaves were placed underside uppermost on a water agar plates (one leaf per plate). Each isolate was inoculated onto 5 leaves by placing droplets of 10 µl each from bacterial suspension at 3-5 separated sites on each leaf surface and pricking tissue through the droplets using sterile needles. The negative control leaves were prepared by the same manner except the sterile distilled water were used instead the bacterial suspension. After inoculation, the plates were warped by cling warp and incubated at 28 °C in an incubator equipped with white lights and checked daily. The resulted responses were recorded from day 4 after inoculation.

On citrus plants

Leaves, twigs, mature stems and fruits on young trees (3 to 4 years old) of potted (40cm pots filed by 1/1 sand clay soil) Valencia sweet orange (Cv. Olinda) and grapefruit (Cv. Ruby Red) were inoculated by bacterial suspension of each isolate at inoculum concentration of 10^8cfu/ml. In the case of leaf inoculation, young leaves of each type were gently rubbed by clean cheesecloth that was dipped in fine powder of Carborundum then sprayed with bacterial suspension until the leaves had moistened. In the case of twigs and mature stems inoculation, droplets of bacterial suspension were placed on the twigs and stems then gently pricked at the place of the droplets using a sterile needle. In the case of fruits inoculation, droplets of bacterial suspension were placed on attached immature fruits and gently pricked at the place of the droplets using a sterile needle. Negative controls were inoculated in the same way using sterile

distilled water instead the bacterial suspension. The inoculated plants were kept in greenhouse. The mean of temperature degrees in the duration of the experiments fluctuated from 24 at night and 32 at day. The resulted responses were recorded from 1 week after inoculation.

Identification by PCR.

DNA extraction

The total DNA was extracted from bacterial cells using the protocol described by Ausubel *et al*. (1997) with little modification. Prior to extraction, the bacterial isolates were grown on NA medium for 48 h at 28°C then subcultured in 2 ml of YP medium (0.3% yeast extract and 0.5% polypeptone) and shake incubated (120 rpm at 28°C) for 24 h. The bacterial cultures were centrifuged at 5000 rpm for 5 min, washed twice with a 1 M NaCl solution and resuspended in 1000 µl of extraction buffer (0.2 M Tris-HCl pH 8.0, 10 mM EDTA, pH 8.0, 0.5 M NaCl, 2% SDS and 250 µg/ml proteinase K). After incubation at 55°C for 1 h, 100 µl of 5 M NaCl was added to the suspension and mixed thoroughly then 80 µl of CTAB-NaCl solution (10% CTAB in 0.7 M NaCl) was added. The mixture was gently mixed and incubated at 65°C for 10 min. DNA was extracted using chloroform isoamyl alcohol (24:1) and phenol chloroform isoamyl alcohol (25:24:1). After centrifugation at 12000 rpm for 10 min, the supernatant was transferred into a new tube and the DNA was precipitated by adding 1 vol. of cold isopropanol and washed with 70% ethanol. The pelleted DNA was air dried and resuspended in 100 µl of TE buffer (pH 8.0) and the suspension was used as a DNA template.

PCR analysis

PCR was performed for all tested isolates using the primer pairs 2-3 [2 (5'-CACGGGTGCAAAAAATCT-3') and 3 (5'-TGGTGTCGTCGCTTGTAT-3')] designed by Hartung *et al*. (1993) that specifically targeted a *Bam*HI restriction fragment length polymorphic DNA fragment (222 bp amplicon size) specific to *X. citri*subsp. *citri*.

Following the protocol of Hartung *et al*. (1993), the PCR mixture components was prepared in a sterile vial and consisted of PCR buffer (50 mMTris-HCl, pH 9;20 mMNaCl; 1% Triton X-100; 0.1% gelatin; 3 mM MgCl2), 1 µMof each primer, 0.2 mMdNTP (deoxynucleotide triphosphate) and 1.25 U Taq DNA polymerase. A volume of 5 µl from the extracted DNA template was added to 45 µl of the PCR mixture to give a total of 50 µl per reaction. The PCR mixture components without DNA had been used as negative control.

PCR products were size-separated using 2% (w / v) agarose gel in 1× Tris-acetate-EDTA (TAE) buffer (40 mmol / litreTris-acetate; 1 mmol / litre EDTA; pH 8.0) run for 2 h at 110 V. The gel was stained with ethidium bromide and visualized under ultraviolet light and photographed.

Isolates aggressiveness

Experimental conditions

The aggressiveness of the identified isolates was evaluated after artificial inoculation into leaves of grapefruit (Cv. Ruby Red) and Valencia orange (Cv. Olinda) using two inoculation methods (Pricking and Carborundum methods, as described below). The reactions were evaluated 30 days after inoculation depending on the phenotype of the canker lesions in inoculation site and number of lesions / cm^2 of inoculated leaf regarding to inoculation method. Inoculum of each bacterial isolates were prepared as mentioned above (in detached leaf assay).Five plants of each citrus type were used and sex leaves from each plant were inoculated by each strain. The assays were repeated three times and the results average of all tests were reported. The inoculated plants were grown in a greenhouse in which the average of temperature was 32°C during the day and 25°C night. The details of inoculation methods and rating of aggressiveness are as follows:

Pricking method

As described by Shiotani *et al* (2000), a 3 µl from the bacterial suspension of each isolate was placed on six spots on each leaf then immediately pricked through the suspension with an insect pin (0.5-mm diameter). After pricking, the inoculum droplets were wiped off with sterile cotton. Differences in aggressiveness among the isolates were evaluated in regard to the expansion in resulted lesion by measuring the diameters of the lesions using digital Vernier caliper.

Carborundum method

In this method, the aggressiveness of the tested isolates was assessed following the method of Graham and Gottwald (1990) with some modification. The bacterial suspension of each isolates was mixed with fine powder of Carborundum to serve as wounding agent then the suspension was mechanically rubbed onto the lower surface of the tested leaves. Differences in aggressiveness among the isolates were evaluated by counting the number of lesions in four placements per each inoculated leaf in which, the area of each placement equals4 $cm^2$and then used to calculate the mean number of lesions/cm^2.The area used in counting was determined by the means of square shaped (2x2 cm) metallic template that was placed on 4 different patches from the leaf then counting the lesions inside it. Furthermore, the number equivalent to 1/10 of the total counted lesions in each square was randomly selected and the diameter of each lesion was measured by digital Vernier caliper.

Disease occurrence within citrus groves

Commercial citrus groves that located in the major citrus cultivation areas in Egypt were surveyed for the occurrence of citrus canker within citrus groves during the summer and autumnof 2015.The surveyed groves located in Wadi El-mollak, Al-Salhia(Al Sharqia governorate),Noubaria; Markaz Bader and Wadi El-natroun (AL-Beheira governorate).Presence of any symptomatic tree(regardless to the citrus type) in any grove was recorded as a diseased grove regardless to the number of diseased trees. Disease occurrence was expressed as a proportion of groves contains diseased tree (or trees) per total number of surveyed groves as a whole.

Disease incidence within trees in diseased citrus groves

Disease incidence was evaluated in the same locations and period as in disease occurrence but the assessment was conducted only in groves of sweet orange due to the paucity of other citrus types and the irregular frequency in plantation areas. Citrus trees in diseased groves representing each surveyed locality were visually investigated for the presence of citrus canker symptoms on the trees. Disease incidence was expressed as a proportion of trees expressing disease symptoms per total number of surveyed trees in the grove as a whole.

Disease severity in diseased citrus groves

A total of 20 symptomatic sweet orange trees representing each recorded (in disease incidence assessment) diseased grove were randomly selected and visually investigated in objective to assessment of disease severity. Using the rating scale described by Agostini *et al*. (1985), the severity level of each tree was rated as following: 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. The values of assessed tree were averaged and recorded as the disease severity rate of each grove as whole.

Statistical analysis

All experiments were conducted in randomized complete block design and the mean values were compared by the least significant difference (LSD) testing at p = 0.05. Means were compared using Duncan's multiple Range test at p = 0.05. All statistical analyses were performed using the statistical computer software SPSS (version 11).

RESULTS

Isolation

Isolation from symptomatic citrus tissue consistently yielded xanthomonad-like colonies from almost samples on all used isolation media. Typical colonies that resembled *Xanthomonas citri* subsp. *citri* appeared after 4 to 6 days at 28°C as light whitish yellow, mucoid, round, and smooth and had a "sticky" texture when touched with the loop (Fig 1A). By streaking the suspected colonies on YDC medium, only 15 isolates showed the characteristics growth (copious growth, very mucoid and richer yellow) in this medium (Fig 1B). Based on this last result, 15 isolates were selected, designated (XCC1to XCC15) and the subsequent experiments were accomplished on these isolates only.

Identification

Microscopical, physiological and biochemical characteristics

All the selected isolates manifested uniform microscopical, physiological and biochemical characteristics which concord with *Xanthomonas citri* subsp. *citri* Table (1). They were Gram negative and non-spore forming with straight rods cells. All isolates caused hypersensitivity response in tobacco leaves whereas, none of them produced fluorescent pigment on King's B medium. They reacted negatively with oxidase and nitrate reductase activity tests whereas thy reacted positively with catalase and arginine-dihydrolase tests.

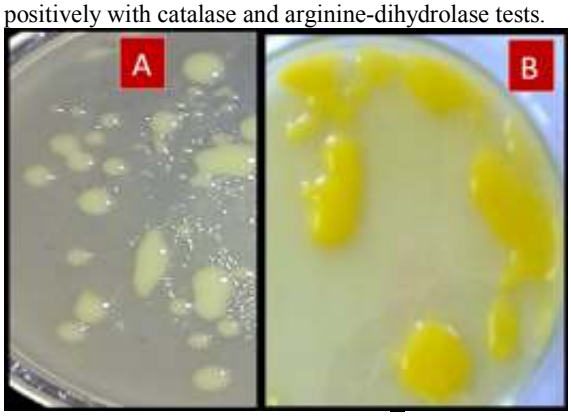

Fig. 1. The colony morphology of *X. citri* subsp. *citri* on NGA medium(A) and YDC medium (B) incubated at 28°C for 6 days. The colonies on NGA medium are light whitish yellow, mucoid, round, and smooth while on YDC medium are more copious growth, very mucoid and richer yellow.

Table 1. Microscopical, physiological and biochemical characteristics of fifteen *Xanthomonas citri* subsp. *citri* isolates obtained from symptomatic citrus plant materials.

Characteristic	Reaction
Growth on YDC medium	Mucoid
Cell shape	straight rod
Gram staining	+
Spore forming	-
HR in tobacco	+
Fluorescent pigment on KB medium	-
Oxidase test	-
Nitrate reductase activity	-
Catalase activity	+
Arginine-dihydrolase	+
Hydrolysis of:	
Casein	+
Gelatin	+
Potato starch	+
Tween 80	+
Glucose metabolism:	
oxidatively	+
Fermentatively	-
Action on litmus milk	Alkaline
H_2S from L-cysteine	+
Utilization of:	
Sucrose	+
Cellubiose	+
L-rhamnose	+
L-arabinose	+
Growth at	
36 °C	+
40°C	-
2 % (w/v) NaCl	-
0.02% triphenyl tetrazolium chloride	-

+ = Positive reaction - = Negative reaction

They were able to hydrolyze casein, gelatin, starch and Tween 80.Thy oxidatively utilized glucose and never fermentatively. They reacted alkaline on Litmus milk and generatedH_2S from cysteine.They were able to utilize sucrose, cellubiose, L-rhamnose and L-

arabinose as a carbon sources. Moreover, all isolates were able to grew at 36 but not at 40°C.Thygrow in the presence of 2% NaCl whereas their growth was inhibited in medium containing 0.02% triphenyl tetrazolium chloride.

Automated identification by OmniLog ID System

The pattern of 95 carbon source utilization of the 15 isolates was compared using Biolog GN microplate system. All of the tested isolates were confirmed to be *Xanthomonas citri* subsp. *citri* (*Xanthomonas axonopodis* pv. *citri*) with similarity percentage ranged from 74 to 92 % (Table 2).

Pathogenicity test

Detached-leaf assay

Generally, all isolates elicited typical symptoms of citrus canker on Valencia sweet orange (Fig. 2) and grapefruit (Fig. 3) leaves. The initial symptoms beginning to appear after 5-7 days from the inoculation date as tiny, slightly raised blister-like lesions (Fig.2 A). As the lesions aged (15 days later), they turned to light tan, then tan to brown (Fig. 2 B). at 30 days, the centers of the lesions become raised and spongy or corky (Fig. 2C). Eventually, the centers of the lesions become crater-like. None of control leaves showed similar symptoms.

Table 2. Biologidentity confirmation for fifteen isolates of *Xanthomonas citri* subsp. *citri*, isolated from symptomatic citrustissues.

Isolate	Identity	Similarity
XCC1	*Xanthomonas axonopodis* pv. *citri*	88
XCC2	*Xanthomonas axonopodis* pv. *citri*	91
XCC 3	*Xanthomonas axonopodis* pv. *citri*	90
XCC4	*Xanthomonas axonopodi s*pv. *citri*	74
XCC5	*Xanthomonas axonopodis* pv. *citri*	92
XCC6	*Xanthomonas axonopodi s*pv. *citri*	89
XCC7	*Xanthomonas axonopodis* pv. *citri*	92
XCC8	*Xanthomonas axonopodis* pv. *citri*	91
XCC9	*Xanthomonas axonopodis* pv. *citri*	88
XCC 10	*Xanthomonas axonopodis* pv. *citri*	88
XCC 11	*Xanthomonas axonopodis* pv. *citri*	91
XCC 12	*Xanthomonas axonopodis* pv. *citri*	87
XCC 13	*Xanthomonas axonopodis* pv. *citri*	90
XCC 14	*Xanthomonas axonopodis* pv. *citri*	89
XCC 15	*Xanthomonas axonopodis* pv. *citri*	89

Fig. 2. Progression of citrus canker symptoms on artificially inoculated Valencia sweet orange leaves in detached-leaf assay. A:7 days after inoculation date, B: 15 days after inoculation date, C: 30 days after inoculation date.

Fig. 3. Initial symptoms of citrus canker on artificially inoculated grapefruit leaf using pricking method in detached-leaf assay.

On citrus trees

On leaves of inoculated trees, the initial symptoms beginning to appear after 8-10 days from the inoculation date and the lesions were dispersed and abundant (owing to inoculation method) with a water-soaked margin surrounded by a chlorotic halo (Fig. 4A, B&C). Furthermore, the symptoms were relatively more expressive (purest and obvious)than in the detached-leaf assay test.

On twigs, lesions appeared initially as oily looking roughly circular spot. The lesions

become raised and blister-like, growing into white spongy pustules. These pustules then darkened and thickened into a brown corky canker with rough touch (Fig. 5).The young stems were girdling by the coalesced pustules (Fig. 6). On mature stems, pustules coalesced and split the epidermis along the stem length(Fig. 7).

On fruits, symptoms are similar to those on leaves and twig but the lesions tended to have more elevated margins and a sunken center (Fig. 8).

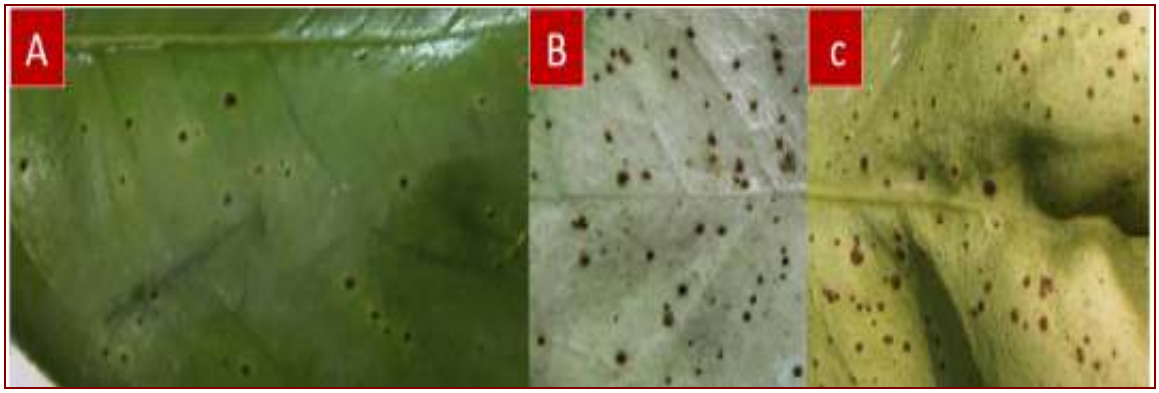

Fig. 4. Progression of citrus canker symptoms on artificially inoculated Valencia sweet orange attached leaves. A:10 days after inoculation date, B: 20 days after inoculation date, C: 30 days after inoculation date.

Fig. 5. Appearance of citrus canker symptoms (Raised white spongy pustules in the beginning then darkened and thickened into a brown corky canker with rough touch) on artificially inoculated grapefruit twigs.

Fig. 6. Artificially inoculated young stems of Valencia sweet orange girdling by the coalesced pustules of citrus canker.

Fig. 7. Symptoms of citrus canker on artificially inoculated mature stems of grapefruit (pustules split the epidermis along the stem length)

Fig. 8. Appearance of citrus canker symptoms on artificially inoculated grapefruit (left) and Valencia sweet orange (right) fruits (lesions with elevated margins and a sunken center).

Identification by PCR

The PCR amplification using the species-specific 2-3 primer pairs successfully amplified a 222 bp DNA fragment from 14 of the tested isolates. No amplification was observed with isolate designated XCC4 with these primers (Fig. 9).

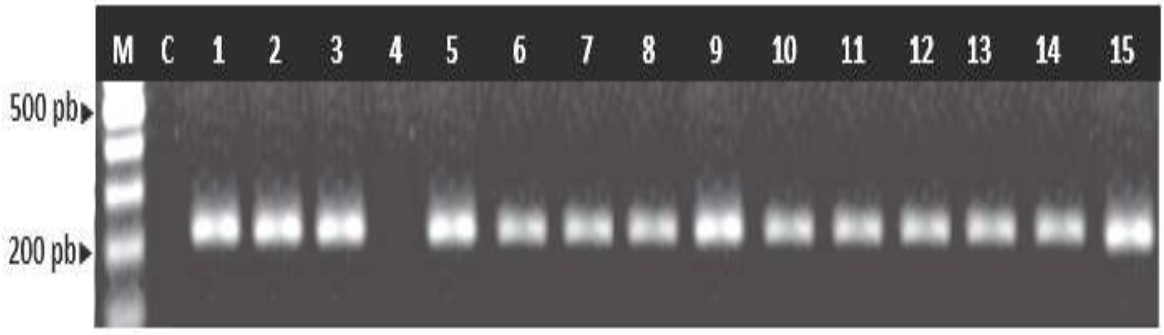

Fig. 9. Agarose gel electrophoresis of PCR amplification products from fifteen *X. citri* subsp. *citri* isolates with the 2-3 primer pairs. LaneM,1 00-bpDNAladder marker (Promega); lane C, negative control (PCR mixture components without DNA); lanes 1 to 15, isolates of *X. citri* subsp. *citri* (designated XCC 1 to 15). The sizes of the targeted bands were 222 bp.

Isolates aggressiveness

Differences in aggressiveness between the isolates were evaluated by artificial inoculation into attached leaves of sweet orange and grapefruit using two inoculation methods. Generally, all isolates elicited typical symptoms of citrus canker on both hosts with both inoculation methods. Nevertheless, some differences in symptoms expression and its progress were detected in the duration of the test (Fig. 10).The values of aggressiveness of each isolate markedly correlated within both methods. Isolates designated XCC 4, XCC 5, XCC 6, XCC 8, XCC 9, XCC12 and XCC 15 were the highest in aggressiveness level(Table 3).

In the pricking method, all isolates caused water-soaked lesions at the inoculation sites within 5 days and the erumpent tissue reaction was observed 7 days after inoculation on grapefruit leaves while both reaction were delayed 2 to 3 days therefrom in sweet orange leaves. The phenotype and the number of the resulted canker lesions significantly differed among the isolates in both hosts.

In the Carborundum method, all isolates elicited the initial canker lesion within 7 days on grapefruit and 8 days on sweet orange and no differences in the time of lesion emergence among the bacterial isolates were observed. However, lesion number and expansion significantly differed among the isolates on both hosts.

Fig. 10. Differences in symptoms expression (number and phenotype) of the canker lesions resulted from different isolates of *Xanthomonas citri* subsp. *citri*.

Table 3. Aggressiveness of fifteen isolates of *Xanthomonas citri* subsp. *citri* on sweet orange and grapefruit leaves using two inoculation methods.

	Aggressiveness					
	Pricking method		Carborundum method			
	* Mean of lesion diameter (mm)		**Mean of lesion number /cm^2 (nearest hale number)		*** Mean of lesion diameter (mm)	
Isolate	Sweet orange	Grapefruit	Sweet orange	Grapefruit	Sweet orange	Grapefruit
XCC1	1.3	1.38	9	16	0.48	0.56
XCC 2	1.5	1.95	17	25	0.67	0.64
XCC 3	1.2	1.73	13	19	0.54	0.61
XCC 4	1.9	2.45	23	31	0.89	1.08
XCC 5	2.2	2.67	23	29	1. 14	1.26
XCC 6	2.1	2.76	26	32	1.11	1.24
XCC 7	1.4	1.89	16	24	0.59	0.63
XCC 8	2.3	2.77	24	29	1.21	1.38
XCC 9	1.9	2.54	22	28	0.93	1.22
XCC10	1.3	1.63	13	20	0.61	0.79
XCC 11	1.9	2.25	21	29	0.98	1.23
XCC 12	2.5	2.83	27	35	1.28	1.43
XCC 13	1.3	1.72	15	22	0.51	0.82
XCC 14	1.8	2.14	25	32	0.87	1.04
XCC 15	1.9	2.21	26	29	0.88	1.11
LSD at 5%	0.4		11		0.31	

***: Mean measurements of lesions expansion resulted in six inoculation sites per each leaf of each treated plant.**

****: Calculated from the mean number of lesions in four squared placements per each inoculated leaf in which, the area of each square equals 4 cm2.**

*****: Mean lesion diameter of 1/10 of the total counted lesions in each square. Five plants of each citrus type were use and six leaves from each plant were inoculated by each strain and the assays were repeated three times.**

Disease occurrence within citrus groves

Citrus canker disease was occurred in all surveyed localities regardless to the number of diseased groves (Table 4). Among a total of 82 surveyed groves, there were 26 groves contained symptomatic trees equivalent to percentage of 31.70 %. Disease occurrence significantly differed between Al-Salhia locality and the other localities. The highest disease occurrence recorded in Wadi El-natroun locality (38.46%) followed by Noubaria (34.61%),Markaz Bader (33.33%),and Wadi El-mollak while Al-Salhia locality was the lowest at all (18.18).

Disease incidence within trees in diseased citrus groves

Off 41504 surveyed trees within 26 diseased citrus groves located in 5 different localities, the recorded symptomatic trees were 3524 corresponding to 8.49% of disease incidence (Table 4).The incidence of the disease significantly differed between the surveyed groves which fluctuated from 4.03to 16.07%. Among the surveyed localities, Markaz Bader, Wadi El-Mollak and Wadi El-natroun were the highest in disease incidence levels (16.07, 10.49 and 8.06 % respectively) while Al-Salhia and Noubaria were the lowest (4.03 and 5.74 % respectively).

Disease severity in diseased citrus groves

Severity of citrus canker was evaluated on 520 diseased trees by a shear of 20 trees representing each grove that were recorded in disease incidence survey. As illustrated in Table (4), the severity rating within localities fluctuated from 1.06 to 2.13(26.5:53.25 %)by a total mean equivalent to 1.43(35.75%). Except Wadi El-mollak locality, the mean of severity level did not significantly differ within localities.

Table 4. Occurrence, incidence and severity of citrus canker in main Egyptian citrus cultivation areas.

Governorate and Locality		Disease occurrence [A]			Disease incidence [B]			Disease severity [C]		
		Number of surveyed groves	Diseased groves		Number of surveyed trees	Symptomatic trees		*Assessed trees	**Means of severity rating	
			No	%		No.	%		(0 - 4)	***%
Al Sharqia	Wadi El-mollak	14	4	28.57	8258	867	10.49	80	2.13	53.25
	Al-Salhia	11	2	18.18	7190	290	4.03	40	1.06	26.5
AL-Beheira	Noubaria	26	9	34.61	10500	603	5.74	180	1.63	40.75
	Markaz Bader	18	6	33.33	6356	1022	16.07	120	1.52	38.00
	Wadi El-natroun	13	5	38.46	9200	742	8.06	100	1.12	28.00
Total		82	26	31.70	41504	3524	8.49	520	1.43	35.75
LSD at 5%				11.7			8.3			16.4

A: Proportion of groves contains diseased trees per total number of surveyed groves. B: Proportion of symptomatic trees in diseased groves. C: Severity level of each diseased tree was rated as following: 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. *: Proportion of 20 diseased trees representing each diseased grove. **: Mean of severity ratings of assessed trees within each locality. *: Mean of severity ratings /4 x 100**

DISCUSSION

Discovering the occurrence of disease in new places is very critical to paying attention to the risks of this disease on the host crops, hence, subjection of the disease to phytosanitary regulation. Thence, this work was accented on the detection and diagnosis of the citrus canker disease and studying its main epidemiological features to standing on the current status of the disease in Egypt.

Isolation trials were considerably successful which conduce to occupancy of pure isolates from the pathogen. Whereupon, the merited step was to ascertain that the isolates are *Xanthomonas citri* subsp. *citri*. For this purpose, these isolates were subjected to numerous diagnostic approaches such as the routine identification protocol (morphological, physiological and biochemical characteristics), automated identification (Biolog system), pathogenicity testing and PCR technique.

In regard to the routine identification, the tested isolates shared the morphological, physiological and biochemical characteristics with *X. citri* subsp. *citri*. Although, some more common approaches are much accurate and preferable, physiological and biochemical tests are still meaningful for identification of plant pathogenic bacteria to genus and species and have been used to study and characterize the phenotypic variation among *Xanthomonas* species isolated from various host plants including citrus plants (Schaad *et al*., 2001).

The second diagnostic approach which has been followed to the identification of the putative isolates was Biolog system. Using this system, almost the tested isolates showed high degree of congruence in their responses to this test which increases the likelihood that these isolates are *X. citri* subsp. *citri*. Which is noteworthy, isolate designated XCC4 showed less similarity which draws attention to the fact that something has to be taken into account. However, the Biolog system is be considered as complementary test, it has proved to be very convenient to identification of different bacteria including xanthomonads (Schaad *et al*., 2006 and Verniere *et al*., 1998). This automated identification system which is based on the diversity among microorganisms for their ability to utilization of carbon source could be used effectively for the identification of pathotypes of citrus bacterial canker (Yong *et al*., 2008). It has been successfully used for providing the full metabolic profiles of the bacterial spot's xanthomonads (MariyaStoyanova *et al*., 2014).

The molecular approaches proved to be powerful techniques and have been mostly used to improve the identification and detection of plant pathogens due to their accuracy, specificity and sensitivity. Unlike isolate XCC4, all isolates reacted positively with conventional PCR assay, in the sense that, primer pairs 2-3 specifically amplified the targeted DNA from14 out of 15 isolates. These results are consistent with the results of Biolog analysis and interpreted to a quite extent the less similarity in the case of isolate XCC 4 in Biolog test. This supports the possibility that this isolate be a different pathotype of *X. citri* subsp. *citri*. This does not mean that, there is some other reasons that led to this negative result. It is formerly known, the primer pair 2-3 directed the specific amplification of target DNA from pathotype A but not from other pathotypes of *X. citri* subsp. *citri* (Hartung *et al*,1993). Traditionally, the DNA-based methods have been used for characterization and study of genotypic variation among bacterial strains. Characterization of molecular features of *X. citri* subsp. *citri* strains has been carried out using different techniques to demonstrate the type of strains, to trace the new outbreaks and to illustrate the genetic variation within strains (Hartung *et al*., 1993, 1996; Cubero & Graham, 2002; Mavrodieva *et al*., 2004; Coletta-Filho *et al*., 2006; Golmohammadi *et al*., 2007).

Aggressiveness of the obtained isolates were evaluated on the susceptible hosts grapefruit and sweet orange using two inoculation methods. Differences between the isolates in symptoms expression (lesion number and expansion) and its progress were detected. There is a wide range of pathogenic variation among strains of bacteria associated with citrus canker. Moreover, new strains are regularly originating as a result of mutation (Gottwald *et al*., 1993and Schubert *et al*., 2001). Graham and Gottwald (1990) studied the variation in aggressiveness amongst strains of *Xanthomonas campestris* pv. *citrumelo* (the causal agent of citrus bacterial spot) in citrus nurseries. They found that, the strains varied in aggressiveness based on the extent and persistence of water-soaking and the development of necrosis and the incidence, severity, and spatial distribution of the disease was related to strain aggressiveness. Das (2002) reported the existence of pathogenic variability within the 'A' strain of *X. citri* subsp. *citri*. Madhuri *et al*, (2016) studied the pathogenic variability between different isolates of *X. citri* subsp. *citri* on four different varieties of acid lime and proved the existence of variation in the symptoms development.

Distribution of citrus canker in main citrus-growing areas in Egypt was quantitatively determined in terms of occurrence and incidence of the disease. The citrus groves contained symptomatic trees were approximately 32 % from the total of surveyed groves. These diseased groves were distributed in all surveyed localities which signify the presence of inoculum source of the disease which constitute an important source of primary inoculum for disease free or newly established citrus groves in such areas. The spread of citrus canker, caused by *X. citri* subsp. *citri* strain "A", through groves has been previously reported (Danos *et al*., 1981 and Gambley *et al*., 2009). After citrus canker had been introduced into a new geographical area, its spread was reported as "rapid" (Doidge, 1916 and Dopson,1964).

The incidence of the disease within citrus tree varied among the surveyed groves which fluctuated from 4to 16% approximately. This variation can be attributed to several factors, including susceptibility of citrus type, trees age, propagation source of the trees, copper compound spraying and many other cultural practices such as pruning and irrigation.Measuring the disease incidence is more meaningful in quantifying the seriousness of the disease and much easier method for

determination of disease development than assessing the severity of the disease on individual trees or groups of trees (Danos *et al*., 1981).In the study conducted by Agostini *et al*. (1985)there was a strong correlation between incidence and severity, hence they suggested that, the disease development could be usefully and more simply followed by determining disease incidence.

Severity of citrus canker was assessed on 520 diseased trees in 26 citrus groves distributed in major citrus cultivation localities in Egypt. The total mean of severity rating reached 35.75% and there was no significant difference in mean of severity level within localities. Although this evaluation is not a real measure of disease severity because it is performed on infected trees only, it gives a general overview about the extent of damage caused by the disease on infected trees. Severity assessment is one of the most important measurements practiced in epidemiological studies such as comparisons of the control measures(Das and Singh, 2001)and also genetic resistance for citrus canker (Agostini *et al*, 1985; Stapleton and Lopez, 1988 and Das and Singh,1999, 2001).Gottwald *et al* (1989)analyzed the disease progress (disease increase over time) of citrus canker in nurseries by measuring either the disease severity (proportion of leaves infected per plant) or disease incidence (proportion of infected plants).Serizawa and Inoue (1983), examined the percentage of diseased leaves and the severity of lesions as a standard for forecasting its occurrence of citrus canker in Japan.

The results reported in this work draws attention to the presence of the citrus canker disease in citriculture regions in Egypt. Thus, there is a need to follow the correct cultivation practices to reduce the seriousness of the disease on citrus cultivation and minimize its incidence in citrus nurseries and groves, moreover, to prevent the introduction of *X. citri* subsp. *citri* to new groves.

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ملامح المسبب وبعض ملامح الوبائية لمرض التقرح البكتيرى على الموالح فى مصر

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شوهدت أعراض مرضية متطابقة مع أعراض مرض تقرح الموالح البكتيرى على الأشجار فى العديد من بساتين الموالح المتواجدة فى أماكن زراعة الموالح فى مصر و بالتالى أجريت هذه الدراسة للتعرف على المتسبب فى حدوث هذه الأعراض و هل هى بالفعل أعراض مرض تقرح الموالح البكتيرى أم لا؟ , ثم دراسة بعض السمات المرتبطة بعوامل وبائيته تحت الظروف المصرية.هذا" و كانت خطوات الدراسة و أهم نتائجها كالتالى:أولاً: الدراسات المتعلقة بأسباب المرض (Etiological studies):بإجراء عملية العزل من العينات المشتبه فى إصابتها بالمرض تم التحصل على خمسة عشر عزلة مشابهة فى خواصها المزرعية للجنس *Xanthomonas*.وفقاً للخصائص الميكروسكوبية و الإختبارت الفسيولوجية و البيوكيماوية التى تم تطبيقها لتعريف هذه العزلات, كانت النتائج متناغمة مع الصفات المسجلة للمسبب المرضى: *Xanthomonas citri* subsp. *citri*.بإخضاع هذه العزلات للنسخة الحديثة (OmniLog ID System) من نظام التعريف بيولوج (Biolog GN microplate system) كانت تعريفاتها متطابقة مع *Xanthomonas citri* subsp. *citri*. وبنسبة تشابه تدور حول 90% ما عدا العزلة صاحبة الكود XCC 4 حيث كانت أقل فى نسبة التشابه (74 %).تم إجراء إختبار القدرة المرضية لهذه العزلات على الأوراق المنفصلة و المتصلة و كذلك على الأغصان و الأفرع و أيضا على الثمار لنباتات من البرتقال الصيفى و الجريب فروت حيث كانت جميع العزلات لديها المقدرة على إحداث أعرض على جميع الأجزاء النباتية المعداه متطابقة مع الأعراض التى تظهر على النباتات المصابة طبيعياً.تم تعريف هذه العزلات على المستوي الجزيئي بإستخدام بادئ متخصص للبكتيريوم *Xanthomonas citri* subsp. *citri*حيث كانت النتائج إيجابية مع جميع العزلات ما عدا العزلة صاحبة الكود XCC 4 حيث أعطت نتيجة سالبة.ثانياً: الدراسات المتعلقة بعوامل الوبائية (Epidemiological studies): تم دراسة نسبة الشراسة المرضية للعزلات إعتماداً على الشكل الظاهرى وعدد البقع الناتجة على أوراق نباتات من البرتقال الصيفى و الجريب فروت بعد العدوى الإصتناعية بالمعلق البكتيرى لهذه العزلات حيث كان هناك بالفعل تباين فيما بينها فى نسبة الشراسة .بدراسة نسبة البساتين المتواجدة فى المناطق الرئيسية لزراعة الموالح فى مصر و التى تشتمل على أشجار تظهر عليها أعراض مرض تقرح الموالح تبين تواجد المرض فى جميع هذه المناطق مع الإختلاف فيما بينها فى نسبة البساتين المصابة فى كل منطقة على حدة .تم دراسة نسبة حدوث الإصابة بالمرض و ذلك بإحصاء نسبة الأشجار المصابة إلى السليمة فى البساتين التى سجل تواجد المرض فيها حيث تراوحت نسبة الحدوث ما بين 4.03% إلى 16.07% بمتوسط عام بلغ 8.49% .تم تقدير شدة الإصابة بالمرض فى البساتين المصابة حيث تم إختيار20 شجرة مصابة بطريقة عشوائية تمثل كل بستان من البساتين المصابة وتقدير شدة الإصابة لكل شجرة على حدة ثم حساب متوسط شدة الإصابة لكل بستان و من ثم تقدير المتوسط لكل منطقة على حدة ثم حساب المتوسط العام لشدة الإصابة على جميع الأشجار المفحوصة بغض النظر عن مكان تواجدها سواء فى البستان أو المنطقة حيث تراوح متوسط شدة الإصابة بين المناطق ما بين 26.5 % إلى 53.25% بمتوسط عام لشدة الإصابة يساوى 35.75%.