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(Original Article)

Isolation and Characterization of *Enterobacter cloacae* Associated with Snake Cucumber Leaf Spots in Minya, Egypt

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Abstract

Seven bacterial isolates viz Cu1, Cu2, Cu4, Cu5, Cu6, Cu7 and Cu8 were isolated from leaves of snake cucumber (*Cucumis melo. Flexuosus.cv.* Balady) plants grown during summer growing season 2019 showed water-soaked spots that developed to small specks then enlarged to form irregular spots.

All bacterial isolates tested were virulent to snake cucumber. Bacterial isolates Cu1, Cu2 and Cu4 were the most virulent than those Cu5, Cu6, Cu7 and Cu8. The most virulent bacterial isolates were identified as *Enterobacter cloacae*. Analysis of their DNA profile confirmed that the three pathogenic isolates have DNA patterns with 99 – 100% similarly to *Enterobacter cloacae*. Only isolate Cu1 was selected to confirm its identification through phylogenetic based on 16s rRNA that showed 99.90% identity with *Enterobacter cloacae* strain 279-56.

Enterobacter cloacae was reacted as leaf spotting (Cantaloup, cucumber, melon, squash, watermelon) and soft rot in fruits of (cucumber, melon). Neither leaves nor fruits of watermelon infected by any of *Enterobacter cloacae*.

Keywords: *Snake cucumber, Cucurbitaceous plants and Enterobacter cloacae.*

Introduction

Cucurbits plants are considered as the most important fresh consumed vegetables worldwide. The global production of cucurbits cultivated in the world is 151,212,210 tons (FAOSTAT, 2012). Cucurbits are minimal in calories, carbs, sodium, fat, and important minerals including vitamin K **(**Istúriz-Zapata *et al.*, 2020**)**. Because of its subtropical origin, cucurbit fruit is susceptible to harm at low temperatures, with chilling injury (CI) occurring at storage temperatures below 10°C (Kader, 1995).

Cucurbit crops are often threatened by diseases of fungal, bacterial, and viral origin (Sitterly, 1972). Many bacterial diseases attack cucurbit plants, such as angular leaf spot caused by *Pseudomonas syringae pv. lachrymans* (Galal *et al*, 2003; Newberry *et al*., 2016), bacterial wilt disease that caused by *Erwinia tracheiphila* (Rojas *et al.*, 2015), bacterial leaf spot caused by *Xanthomonas campestris pv. cucurbita*, bacterial soft rot caused by *Erwinia carotovora* subsp.

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carotovora, and by *Pectobacterium carotovorum* subsp. *brasiliense* (Meng *et al.*, 2017). Brown spot caused by *Erwinia ananas* (Kido *et al.*, 2008).

Gram-negative, rod-shaped, motile by peritrichously-flagellated, and facultatively anaerobic phytopathogenic bacteria were originally classified as Erwinia. Several species were transferred to the genus Enterobacter after taxonomic changes. However, twenty-two Enterobacter species have been identified (Euzeby, 2009). To be mentioned, *Enterobacter cloacae* has been reported as an important pathogen that can cause diseases in different plant species. The infection of *E. cloacae* has been reported in several fruits, for example, in papaya fruits (Nishijima *et al.*,1987); in onions (Bishop 1990; Schroeder *et al.,* 2010) and in the Odontoid orchids (Takahashi *et al.,* 1997); in ginger plant (Nishijima *et al.*, 2004); *Macadamia spp* (Nishijima *et al.*, 2007); in the dragon fruit (Masyahit *et al.*, 2009); in mulberry (Wang *et al*., 2010); in cassava (Santana *et al*., 2012); in lucerne (Zhang and Nan, 2013); in potato tubers (Abd-Elhafeez *et al.,* 2018); in chili pepper (García-González *et al.*, 2018); in rice seedling (Cao *et al.,* 2020); in *Aloe vera* (Nguyen *et al.,* 2021); in apple(El-Safty *et al.,* 2021) and in melon (Ozdemir, 2021).

According to the available literature, *Enterobacter cloacae* has been reported as a pathogen causing potato soft rot (Abd-Elhafeez *et al.,* 2018) and apple fire blight like disease (El-Safty *et al.,* 2021) in Egypt.

The present work was aimed to, 1) isolate the original pathogenic bacteria associated with snake cucumber spots grown during summer of 2019, 2) run pathogenicity and identification tests for obtained bacterial isolates and 3) test the response of some of Cucurbitaceous plants to infection.

Materials and Methods

Samples

Infected snake cucumber (*Cucumis melo. Flexuosus*) leaves were brought to the Department of Plant Pathology, Faculty of Agriculture, from different districts in Minya governorate in summer 2019. These leaves showed water-soaked areas, small specks and enlarged to irregular white spots were pre-mature defoliated and declined.

Isolation and purification

Surface sterilized samples were washed with tap water, then surface sterilized with 0.3 percent sodium hypochlorite for 30 seconds before being washed three times with sterile distilled water (SDW). The leaves were then drained and dried before being sectioned aseptically. Small leaf pieces (3-mm) were removed from the diseased tissue's edge and macerated for 90 seconds in 3 ml SDW in a sterile mortar and pestle. The suspension was incubated at 28°C for 20 minutes before one bacterial loopful was collected and streaked onto nutritional agar (NA) plates. To get pure bacterial isolates, single bacterial colonies were chosen and re-streaked at least twice (García-González *et al.*, 2018).

Pathogenicity tests

Pathogenicity tests were conducted on snake cucumber cv. Baladi. Healthy apparent seeds were surface sterilized by immersing them in 0.3% sodium hypochlorite for 30 s. Then washed thoroughly three times by SDW. These seeds were sown in soil sterilized with formalin containing pot 30 cm diameter (Galal *et al.*, 2006**)**. Completely randomized experiment was conducted with three replicates (5 pots for replicate) for each isolate and the experiment was repeated two times.

Preparation of bacterial suspension

Twenty-four hour-old bacterial cultures of obtained isolates viz cu1, Cu2, Cu4, Cu5, Cu6, Cu7 and Cu8 were grown on Nutrient Agar (NA) medium and kept for 24 h at 28°C. Plate-grown bacteria were suspended in 30 ml of SDW and adjusted to an Optical Density (OD) of 0.1 at A600, on Spectrophotometer (Milton Roy, Spectronic, 1201) corresponding to approximately 107 colony-forming units (CFU) ml^{-1}, for control SDW was used.

Twenty-thirty-day-old healthy snake cucumber plants cv Balady, with four to five leaves. Surface of leaves sterilized with 70% ethanol alcohol. Inoculation was carried out with seven bacterial isolates individually through spraying bacterial suspension until it run off. Three replicates with five samples of each replicate. Five plants were inoculated per bacterial isolate and SDW was used for negative control inoculations. Inoculated plants were covered with plastic bags for 24h in a greenhouse. Plants were watched daily for presence or absence of symptoms (Meng *et al*., 2017). Disease severity (DS) was measured 10 days after inoculation.

As for fruits of snake cucumber, surface sterilized with 70% ethanol alcohol. Fruits were inoculated by injected 50µl of bacterial suspension of each fruit. Inoculated fruits put in plastic bags. Three replicates with five samples of each replicate.

Disease assessment

Disease severity was rated as follow, - = no infection, + = 1- 20% infection (weak infection), ++ = 21- 40% infection (moderate infection) and +++ = mor than 41% infection (high infection).

Identification of the pathogenic bacterial isolates

Studying their morphological, physiological, biochemical and molecular characters to identify three bacterial isolates following recommended in the Bergey's Manual (Bergey and Holt, 1994), also the determinative scheme of the taxonomy of the genus, Enterobacter (Grimont and Grimont, 2006).

Morphological characterization

To study the morphological characters of each, isolate a dilute cell suspension was prepared by suspending loopful of bacterial culture in sterilized

distilled water and streaked on NA plates. These plates were incubated at 28°C for 3-5 days and colony characters of bacteria color, size and shape were record.

Physiological and biochemical characterization

Cu1, Cu2, and Cu4 bacteria were cultivated on NA plates for 24 hours at 28°C. Physiological and biochemical tests were performed on selected bacterial cultures. Bergey's Manual of Determinative Bacteriologytr was used to determine Gram staining. Hydrogen sulphide, gelatin liquefaction, indole synthesis, citrate utilization, Voges Proskauer, arginine dehydrolase, lysine decarboxylase, and ornithine decarboxylase are among the enzymes involved in these reactions. D-glucose, mannitol, l-rhamnose, l-arabinose, D-mannose, D-maltose, D-lactose, raffinose, and glycerol acid synthesis). Other examinations (esculin). These tests were carried out as described by Beiderbeck (1991).

Molecular techniques

The most pathogenic isolates viz Cu1, Cu2 and Cu4 were subjected to PCR analysis to confirm their identification. A PCR procedure was kindly applied by Dr. Y.E. Ebrahim, King Saud University (KSA) and only isolate Cu1 was selected to confirm its identification through phylogenic analysis. The PCR procedure was done as following:

DNA extraction amplification and sequencing of 16S-rDNA

Total DNA was extracted according to Llop *et al*.,(1999).

The 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and the 1492R (5′ GGTTACCTTGTTACGACTT-3′) primers were used to amplify 16S-rDNA fragments from bacterial strains (Lane, 1991). The PCR procedure consisted of an initial denaturation step of 95°C for 15 minutes, 30 cycles of denaturation at 95°C for 20 seconds, annealing at 50°C for 40 seconds, and extension at 72°C for 90 seconds, followed by a final step at 72°C for 5 minutes.

After electrophoresis in a 1 percent (w/v) agarose gel stained with ethidium bromide, the PCR products were examined under UV light. The PCR products were cleaned and sequenced at Macrogen Inc.'s sequencing service unit (Macrogen Inc., Seoul, Korea). BioEdit software (http://www.mbio.ncsu.edu/Bioedit/bioedit) was used to clean, edit, and align DNA sequences. The cleaned genomes were compared to already deposited bacterial species in the GenBank database Scanning electron microscope (SEM)

Bacterial suspension 24h old culture of isolate Cu1 was examined with a scanning electron microscope SEM (JSM- IT 200, Jeol) in the Central Laboratory for Microanalysis, Minia University. Following methods described by Feng *et al*., (2014).

Response of some cucurbitaceous plants to infection

Foliar and fruits from various cucurbitaceous plants, cantaloup, cucumber, melon, squash and watermelon were inoculated with each of the three *E. cloacae*

isolates viz Cu1, Cu2 and Cu4. Inoculation, inoculum preparation, inoculation and disease assessment were conducted as described above.

Results

Pathogenicity tests

All bacterial isolates tested showed ability to infect foliar and fruits of snake cucumber plants (Fig. 1, Table 1). Isolates Cu1, Cu2 and Cu4 were mor aggressive than other bacterial isolates tested, bacterial isolate cu1 provided the most infective isolate, followed by Cu2 and Cu4. Bacterial isolate Cu8 exhibited the weakest pathogen, followed by Cu7, Cu6 and Cu5.

Table 1. Infectivity of bacterial isolates isolated from leaves of snake cucumber plants. Diseases severity (DS) on snake cucumber. cv. Balady on foliar and fruits was assessed 10 days after inoculation

isolates	Districts	Foliar infection	Fruits infection
Cu1	Beni-Mazar	+++	+++
Cu2	Mattay	+++	++
Cu4	Samalott	+++	++
Cu5	Minya	++	+
Cu6	Minya	++	+
Cu7	Mattay	++	+
Cu8	Beni-Mazar	+	+

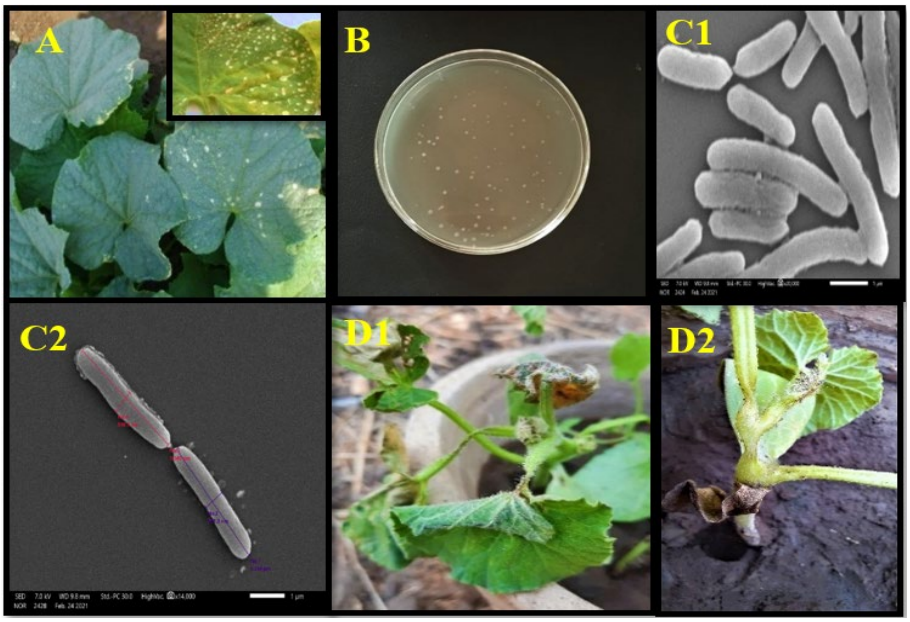

Fig. 1. Natural infection of A= snake cucumber (*Cucumis melo. Flexuosus*) plants that showed symptoms on leaves as water-soaked areas, small specks and enlarged to irregular white spots, leaves were pre-mature defoliated and declined, B= bacterial culture 24h old, C1 and C2= Scanning electron microscope (SEM) of isolate Cu1 (short rode shaped, 1.0 -3.6 µm in length and 0.3 - 0.6 µm in width with peritrichous flagella) and D= artificial infection on snake cucumber plants (D1= inoculation of leaves, D2= inoculation of stems).

Table 2. Physiological and biochemical characteristics of *Enterobacter cloacae* strains

Characteristics	[a]Isolates			[b]*Enterobacter. cloacae* from:	
	Cu1	Cu2	Cu4	Bergey's manual, (1994)	Santana *et al*., (2012).
Cell size	0.3 -0.6 µm × 1.0 - 3.6 µm			ND*	ND
Gram staining	-	-	-	-	-
Rod shaped cell morphology	+	+	+	+	+
Enzymatic activities:					
Arginine dehydrolase	+	+	+	+	+
Lysine decarboxylase	-	-	-	-	-
Ornithine decarboxylase	+	+	+	+	+
Citrate utilization	+	+	+	+	+
Hydrogen sulfide	-	-	-	-	-
Indole	-	-	-	-	-
Voges-Proskauer	+	+	+	+	-
Gelatin liquefaction	-	-	(±)	ND	+
Acid production from:					
D-Glucose	+	(±)	+	+	+
Mannitol	+	+	+	+	+
L-Rhamnose	+	+	+	+	+
L-Arabinose	+	+	+	+	+
D-Mannose	+	+	+	+	ND
D-Maltose	+	+	+	+	ND
D-Lactose	-	(±)	-	+ slow	ND
Raffinose	+	+	+	+	ND
Glycerol	+	+	+	(±)	ND
Other tests:					
Esculin	+	+	+	(±)	ND

+ (positive reaction), - (negative reaction) and (±) weak reaction, *ND= Non detected.
[a]Three bacterial isolates (cu1, cu2 and cu4) were tested. [b]Used reference strains; *E. cloacae* from Bergey's manual (Bergey and Holt, 1994) and data for *E. cloacae* pathogenic in cassava (Santana *et al*., 2012).

Physiological and biochemical characterization

The most virulent isolates viz Cu1, Cu2 and Cu4 were selected for identification. Physiological and biochemical characters summarized in (Table 2), showed that all the bacterial isolates were identical as Gram negative and were positively reacted to produce acid from D-Glucose, Mannitol, L-Rhamnose, L-Arabinose, D-Mannose, D-Maltose, Raffinose and Glycerol, and negatively reacted to produce acid from D-Lactose.

Also, bacterial isolates were positively reacted with catalase, Arginine dehydrolase, Ornithine decarboxylase, Voges-Proskauer, Esculin and Citrate utilization. And negatively reacted with Lysine decarboxylase, Hydrogen sulfide, Indole, Gelatin liquefaction and oxidase. Based on their physiological and biochemical characters the three isolates could be identify as *Enterobacter cloacae* (Bergey and Holt, 1994; Santana *et al.,* 2012).

Molecular identification

DNA sequences of partial 16S-rDNA obtained from the three bacterial isolates were 99 –100% identical to the homologous 16S-rDNA sequences deposited in GenBank. The 16S- rDNA primers amplified a 1378 bp fragment from *Enterobacter* strains. Comparison between the partial sequences of 16S rDNA of the snake cucumber strains and the other sequences of 16S rDNA deposited in GenBank showed that those isolates were *Enterobacter cloacae.* The three isolates were recorded in GenBank and each isolate take sequence ID as follow, Cu1 (MZ087940.1), Cu2 (MZ087941) and Cu4 (MZ087942.1). The phylogenetic characteristics of Cu1 isolate (Fig. 2) that showed 99.90% identity with *Enterobacter cloacae* strain 279-56.

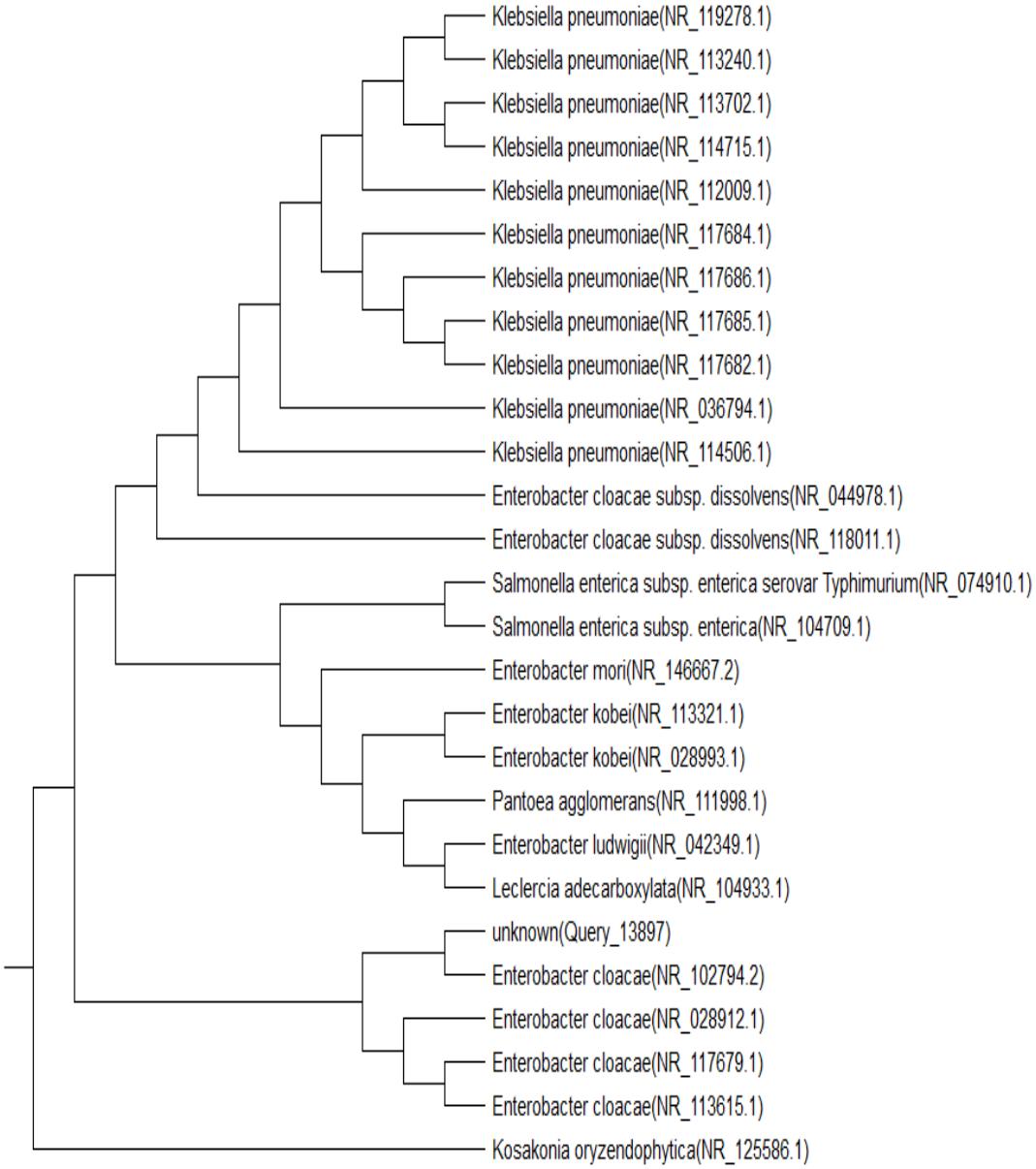

Fig. 2. Phylogenetic tree based on partial sequence of 16s ribosomal RNA of bacterial isolate Cu1 that showed 99.90% identity with *Enterobacter cloacae* strain 279-56.

Cell morphology

As visualized by scanning electron microscope that *Enterobacter cloacae* cells were short rod shaped, 1.0 -3.6 µm in length and 0.3 - 0.6 µm in width with peritrichous flagella. Colonies are round, 2–3 mm in diameter, and slightly iridescent or flat with irregular edges (Fig. 1. C).

Response of some cucurbitaceous plants to infection

Infectivity of *E. cloacae* was varied with different plant species and bacterial isolates (Table 3). All the bacterial isolates were infected leaves of cantaloup, cucumber, squash and melon, and fruits of cucumber and melon they showed soft rot symptoms. Meanwhile, fruits of cantaloup and squash were not infected by any bacterial isolates. Neither foliar nor fruits of watermelon infected by any of *E. cloacae.*

Table 3. Ability of *Enterobacter cloacae,* to infect foliar and fruits of cucurbitaceous plants. Diseases rating on foliar was evaluated ten days after inoculation under greenhouse

Plant	Foliar infection by,			Fruits infection by,		
	Cu1	Cu2	Cu4	Cu1	Cu2	Cu4
Cantaloup (*Cucumis melo var. cantalupensis).*cv Shahd El-doqy	+++	+++	+++	-	-	-
Cucumber (*Cucumis sativus L.*) cv Madaen.	+++	+++	+++	++	+	+
Melon (*Cucumis melo)* cv. shahd el-doqy.	++	+++	++	+	+	+
Squash *(Cucurbita sp*) cv. Escander el-deib.	++	++	+	-	-	-
Watermelon (*Citrullus lanatus*) cv Balady.	-	-	-	-	-	-

- = no infection, + = weak infection, ++ = moderate infection and +++ = high infection.

Discussion

Several plant disease control measures, along with many agronomic practices used in contemporary agriculture, have resulted in unintended consequences, such as loss of biodiversity and other natural resources (Lucas, 2011; Gonthier *et al.* 2014), environmental deterioration (Enserink *et al.* 2013), and accelerated evolution in pathogens (Zhan and McDonald, 2013). Leaves of snake cucumber plants grown under hot weather conditions during summer of 2019 showed water-soaked spots that developed to small specks then enlarged to form irregular spots leaves were pre-mature defoliated and plants were then declined. Early symptoms were confused between bacterial and fungal pathogens. However, isolation trails resulted bacterial isolates were dominant. Seven bacterial isolates viz Cu1, Cu2, Cu4, Cu5, Cu6, C7 and Cu8 were isolated from snake cucumber leaves.

Pathogenicity tests proved that all bacterial isolates tested were infect snake cucumber plants. Isolates Cu1, Cu2 and Cu4 were the most virulent than those Cu5, Cu6, Cu7 and Cu8. The three bacterial isolates were tested for identified through morphological, physiological and biochemical characteristics which they could be identified as *Enterobacter cloacae* (Bergey and Holt, 1994; Santana *et al.,* 2012). Furthermore, isolates were subjected for DNA profile analysis and confirmed that the three pathogenic isolates have DNA patterns with 99 – 100%

similarly to *Enterobacter cloacae.* The phylogenetic tree of isolate Cu1 confirmed its identification as *Enterobacter cloacae* strain 279-56.

Enterobacter cloacae strains occur as commensal microflora in the intestinal tracts of humans, animals and in many plants as endophytic bacteria (Mezzatesta *et al.,* 2012; Moreira *et al.,* 2015**),** also it pathogenic to plants and insects. This diversity of habitats is mirrored by the genetic variety of *E. cloacae* (Mezzatesta *et al.,* 2012)**.**

The three bacterial isolates of *E. cloacae* were exhibited leaf spots to cantaloup, cucumber, melon and squash. Meantime, they reacted as soft rot bacteria towards fruits of cucumber and melon, in contrast, *E. cloacae* isolates were not able to infect fruits of cantaloup and squash. Neither foliar nor fruits of watermelon infected by any of *E. cloacae.* There is currently no evidence of pathogenicity mechanisms or virulence genes in Enterobacter species that cause plant diseases. Only that *E. cloacae* may emit some hazardous biochemical compounds to cause plant diseases is known. **(**Zhu *et al.,* 2010). Bacteria belonging to the genera when changes in host physiology occur as a result of high temperatures, Enterobacter can become an opportunistic pathogen (Cother and Dowling, 1986; Sanders and Sanders, 1997). Also *E. cloacae* can be present in symptomless kernels (Nishijima *et al.,* 2007), As a result, an infection can remain dormant until environmental conditions are favorable for disease manifestation. A prior study, for example, found a link between diseased onion plants in the field and a time of high heat (40-45°C) (Bishop and Davis, 1990).

This association has also been reported for mung bean sprouts (Wick *et al*., 1987) and for internal yellowing in papayas (Nishijima *et al*., 1987). Accordingly, the present work is in line with those reported ells by (Cother and Dowling, 1986; Bishop and Davis, 1990; Sanders and Sanders, 1997). Which showed that the sickness produced by *E. cloacae* was severe at 38°C and 80% RH, but no symptoms were found at 25°C and 30% RH. No symptoms were seen on plants in a preliminary field study done in Mexican chilli pepper fields to discover *E. cloacae* symptoms on plants, possibly due to the comparatively low humidity in this location of 20 to 30%. However, meteorological data during summer were showed mean averages of temperature was Op 25ºC and 80% RH which favorite for *Enterobacter cloacae* infection.

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عزل وتوصيف بكتيريا *Enterobacter cloacae* المصاحبة لتبقع أوراق القثاء في المنيا، مصر

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الملخص

أمكن الحصول على سبع عزلات بكتيرية ممرضة Cu1 وCu2 وCu4 وCu5 وCu6 وCu7 وCu8 من أوراق نباتات قثاء مصابة (صنف بلدي) أثناء موسم النمو الصيفي لعام 2019 والتي أظهرت بقع مائية وبقع صغيرة والتي تكون منها بقع غير منتظمة الحافة، وكانت جميع العزلات البكتيرية المختبرة ممرضة لنباتات القثاء. أظهرت العزلات الثلاثة Cu1 وCu2 وCu4 أعلى قدرة مَرضية عن باقي العزلات Cu5 وCu6 وCu7 وCu8. تم تعريف العزلات الأكثر قدرة مَرضية على أنها بكتيريا *Enterobacter cloacae* وتأكد تعريفها بتحليل بصمة الحمض النووي وكانت العزلات متشابهة مع *Enterobacter cloacae* بنسبة 99.9%، وتم انتخاب العزلة Cu1 فقط لتأكيد تعريفها من خلال تحليل 16s rRNA والتي أظهرت 99.9% تطابق مع البكتيريا *Enterobacter cloacae* للسلالة (56-279) وأوضحت الدراسة أن البكتيريا *Enterobacter cloacae* ذات قدرة على إحداث تبقع أوراق الكانتالوب و الخيار والشمام والكوسة كما أحدثت العزلات البكتيرية المختبرة عفن طري لثمار الخيار والشمام فقط بينما أوراق وثمار البطيخ لم تصب بأي من عزلات *Enterobacter*.