First report of (*Enterobacter cloacae*) causing the brown leaf spot disease on Pomegranate (*Punica granatum* L) in Erbil province

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ABSTRACT

This study was carried out to isolate and identify the pathogen causing spots on pomegranate leaves in Erbil province, Kurdistan/ Iraq. It started with sample collections during July to October 2021. Among twenty-eight orchid's surveyed areas within nine locations, field observations showed that the disease occurred on 24 orchids within 6 locations. Of two hundred and five isolated bacterial strains from one hundred and forty leave samples, 57% were *Enterobacter cloacae*. The *in vivo* pathogenicity test and biology analysis resulted in *Enterobacter cloacae* as the main cause pathogen of yellowish to brownish spot symptoms on infected leaves. These results were confirmed by API20E test and also by DNA sequencing of the 16S rRNA gene. To our best knowledge, this is the first study to identify *Enterobacter cloacae* as a causal agent of bacterial leaves spot disease in pomegranate.

Key words: Bacteria, *Enterobacter cloacae*, pomegranate, spot

INTRODUCTION

Pomegranate (*Punica granatum*) belong to the family *Punicaceae* (Munhuweyi *et al*. 2016) is a perennial fruit crop which has been cultivated for over 5000. years (Chandra *et al*. 2010). Traditionally used as a medical therapy as all parts of this plant have several bioactive metabolites (Vučić *et al*. 2019). The species is native to the Iran and neighboring countries, where a rich diversity of genetic resources and genotypes exist (Stone 2017). Plant pathogens and diseases caused by them are a major reason for crop losses which are occurring worldwide (Dhakate and Ingole 2015). A wide range of diseases affects negatively pomegranate production and their permanency in the various producing areas (Mondal and Mani 2012;Sharath *et al*. 2019; Jayaprakasha, Negi and Jena 2006; Chikte *et al*., 2019). *Enterobacter cloacae* bacterium belonging to the *Enterobacteriaceae family* i gram-negative facultative anaerobic rod shaped bacterium. . This bacteria was described for the first time in 1890 (Nigro and Hall 2011) and has been reported as important opportunistic and multi resistant bacterial pathogens for humans recently in hospital wards (Davin-Regli and Pagès 2015). These bacteria were mainly described in Europe and principally in France (Emeraud *et al*. 2022) during several outbreaks of hospital acquired infections.

More recently this bacteria has increased in importance as a plant pathogen (García-González *et al*., 2018). For example it has been associated with onion decay (Spies, Stücker

and Reichelt 1999), internal yellowing disease of papaya (García-González *et al.*, 2018). Moreover *E. cloacae* and other species of this complex are reported as pathogen in mulberry in China (Wang *et al*. 2008), dragon fruit Malaysia, macadamia in Hawaii (Masyahit *et al*., 2009), lucerne seed in China (Zhang and Nan 2013), odontoid orchids in Japan (Spies *et al*., 1999), and rice seedling in China (Cui *et al*., 2020).There is no incidence that this bacterium cause disease in pomegranate.

The aim of this work was to characterize and identify the causative agents responsible for leaves spot disease on pomegranate trees in Erbil province of Kurdistan region/ Iraq.

MATERIAL AND METHODS

Disease Survey

One hundred and forty leave samples were collected from twenty-four orchids within nine different locations in Erbil providence from July to October 2021. Pomegranate leaves with spot and light symptoms were collected and carried in paper bags in cooler box then were transferred to the laboratory of Plant Protection Department/ Agriculture College/ Salahaddin University and preserved in refrigerator at 4℃ until use.

Isolation of *Enterobacter cloacae*

Leave samples were washed under tap water and surface sterilized in 70% ethanol for one mint, followed by washing twice with sterilized distilled water then dried on sterilized sterile filter paper before cutting in small pieces (2 – 5 mm) and culturing on petri dishes with nutrient agar (NA) medium and incubating at 26℃-28℃ for 2-5 days.

Pathogenicity test

Depending to morphological characters (color and shape) the isolates were separated into different groups. Pathogenicity test was performed for (20) bacterial isolates on young leaves. Bacterial colonies were cultured in nutrient broth (NB) medium (Oxoid Ltd., Basingstoke, Hampshire; England) and incubated at 150 rpm of rotary shaker for 48h at room temperature. The bacterial suspension was adjusted by serial delusion to approximately $10^7 – 10^8$ colony forming units per mL (CFU/ mL-1). Infections were carried out by spraying three healthy leaves of young seedlings with 48h old 10 ml bacterial suspension. Artificial wounds approximately 2 mm deep were aseptically made on tested leaves using sterile needle before spraying with bacterial suspension. Negative control leaves were sprayed only with 10ml sterile distillated water. The relative humidity close to 90% was managed by covering the plants with plastic bags for 72h. The temperature ranging 27 – 30 ˚C were established for 8-10 days during the experiment time and symptoms observation was recorded daily. Re-isolation from symptomatic treated leaf tissue was carried out as described above and were identified by morphological characters, by using API 20E strips and by DNA sequencing of the 16S rRNA gene (Patil *et al*. 2017).

Identification methods

Biochemical reaction API20E

For bacteria morphological and microscopic identification, the following key tests were performed; gram reaction, oxidase and catalase tests (Jones and Geider, 2001), colony forming on 5% sucrose nutrient agar (Billing *et al*. 1961) and Crosse and Goodman (CG) media (Crosse and Goodman 1973) were used. The biochemical characterization of the isolates was carried out by analytic profile index (API 20E) (BioMerieux/France). The API strip was used according to the manufacturer's indications, except the temperature of incubation that was established at 26℃ for 48 h. Before starting a quick oxidase test for cytochrome enzyme was done according to manufacturer's instructions.

Molecular identification

The genomic of bacteria were extracted according to the protocols of BLUMENTAL GERMANY DNA kit based on gram negative samples. The quantity and quality of the extracted genomics were confirmed by Nano Drop technique. The extracted genomics of the bacteria were amplified using polymerase chine reaction (PCR) technique and using one of the 16S rRNA gene universal primers. The forward primer -5-GTGACACGTACACGT-3- The reverse primer -5-ATCGCACGTACACGT-3- (Brons and Elsas, 2008). PCR products were visualized on a 1% agarose gel stained with ethidium bromide under UV light to confirm the size of amplified genes. PCR products were purified using EXOSAP-IT (Ambion-AC) prior to bi-directional sequencing using primers 16S rRNA. The generated sequences were analyzed by chromosome pro amplification. Appropriate thermocycling program was set on thermocycler according to the Go Taq Green Master mix protocol .

Pre-denaturation step at 95 ^{O}C for 5 min., Thermocycling (35 cycles): Denaturation 95 ^{O}C for 40 seconds, Annealing 56 ^{O}C for 45 sec., Elongation 72 ^{O}C for 42 sec. Final Extension 72 ^{O}C for 10 min.

At the end of the process, amplified products were removed and stored at -20 ^{O}C until used for electrophoresis.

RESULTS

Disease survey

From a total of 205 bacterial strains isolates from 140 pomegranate leave samples, 57% were *E. cloacae*. These samples were identified by traditional methods according to the classification Keyes (Cao *et al*. 2020). The isolates plated on sucrose nutrient agar (NA) formed one morphological type of colony (Fig1).

Figure 1. *Enterobacter cloacae* subculture. Greyish to white-colored large, circular, and convex colonies.

Pathogenicity test

Symptoms appeared within three days to one week after inoculation and control plants were heathy. All tested bacterial isolates were pathogenic to pomegranate. Inoculated seedlings showed irregular small spots on leaves. Later, these spots became necrotic with a chlorotic halo. Also, brown necrosis at margins tips were observed and, in the end, the seedlings were defoliated while control seedlings remained healthy (Fig. 2). Bacterial strains were re-isolated from symptomatic inoculated seedlings to represent a completion of Koch's postulates.

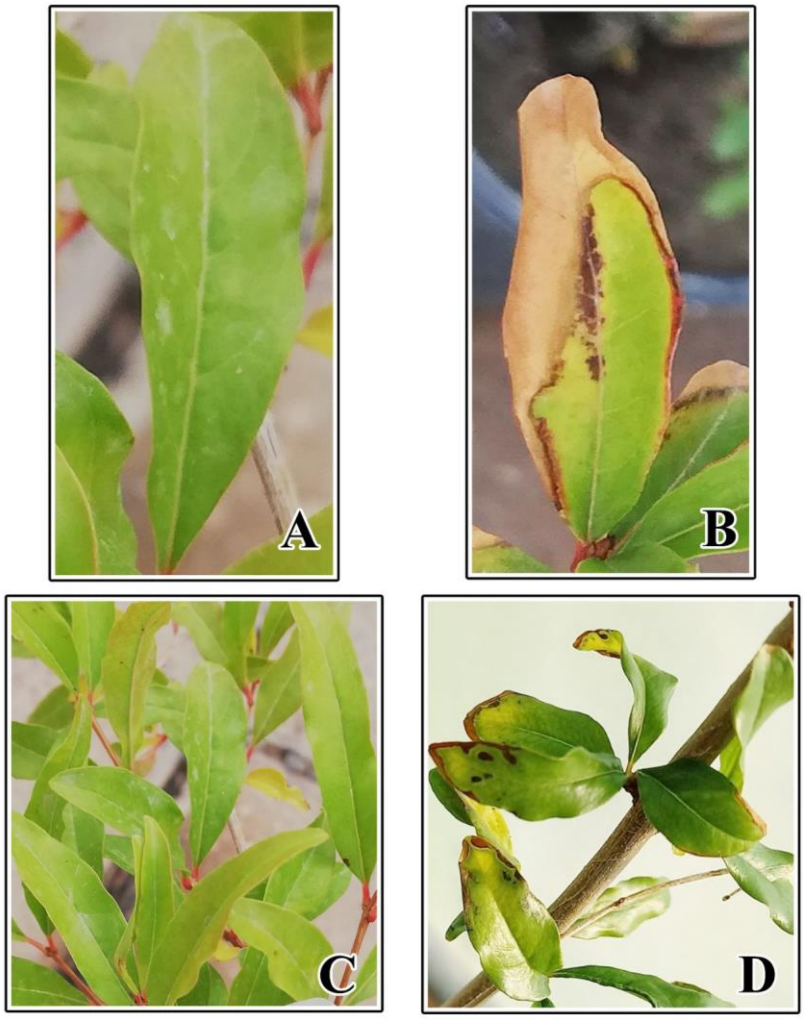

Figure 2. Symptoms on inoculated pomegranate leaves. A & C. healthy control and B & D. Brown necrosis at margins tips and irregular brown spot with yellow halo

Identification

Biochemical identification

The identification system API 20E was applied to all isolates. The results were interpreted after 48 h. at 26 °C. The isolates showed an identical API 20E profile number which was 3306773 (Fig.3).

Figure 3. The API 20E tests. Strip containing 20 tests and the profile sheet (3306773) code numbers indicated that the bacteria belong to *E. cloacae*

Molecular identification

The results revealed that *Enterobacter cloacae* isolates gave a positive response to the Universal Primer 16S rRNA, which amplified a DNA fragment with expected size of 1024 bp during electrophoresis process within 1h (Fig. 4). Our bacterial accession numbers in gen bank are OP565142, OP565146 and OP565149.

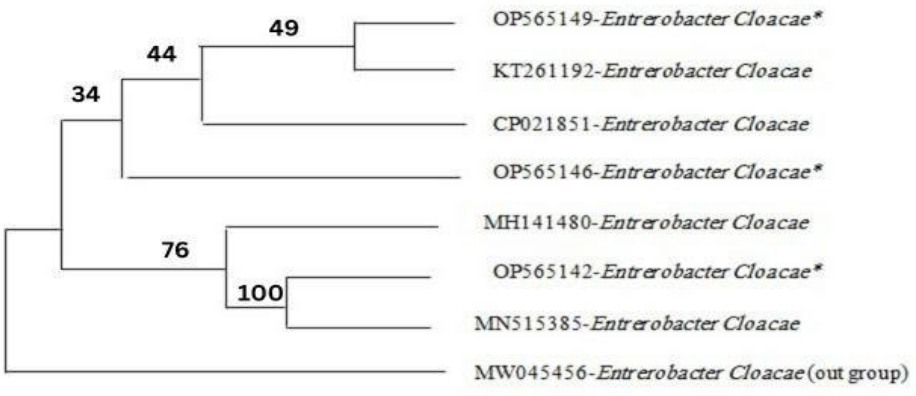

Figure 4. Phylogenetic tree of *Enterobacter cloacae* isolates. Sequences were identified via BLAST matches in the NCBI database. Our three isolates with stars are compared with other isolates from gene bank

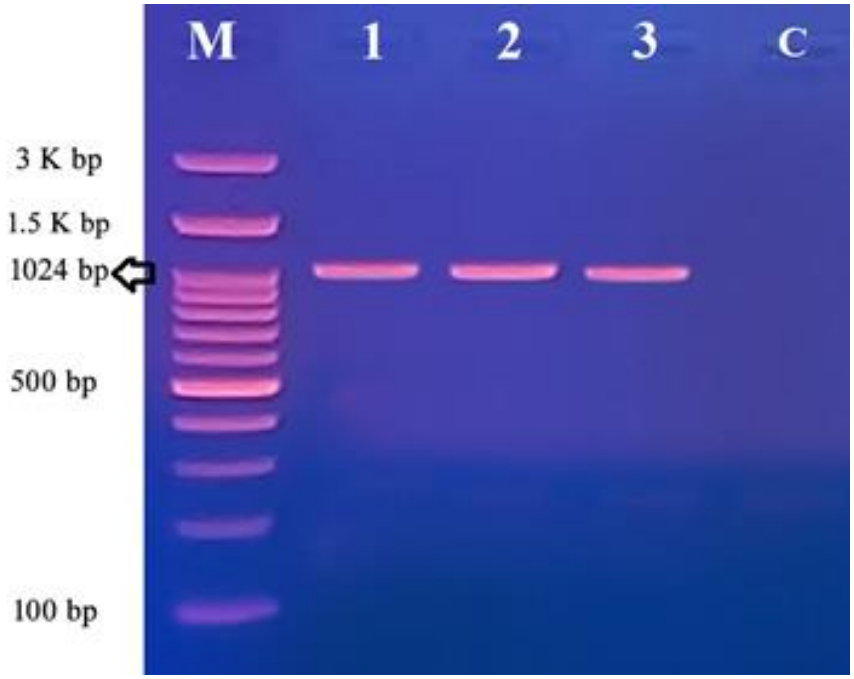

Figure 5. PCR amplification of partial 16S rRNA gene from representative three bacterial isolate. M; indicate: ladder (3000bp-100 bp), lane number 1- 3: 1024 bp of PCR products of bacteria and C is negative control

DISCUSSION

In our study, we describe a new bacterial disease on pomegranate leaves caused by the bacterium *E. cloacae*. Biochemical, and molecular analyses were performed for identification of bacterial isolates. To fulfill Koch's postulates, pathogenicity tests were performed for bacterial isolates and re-isolated bacteria.

The isolates showed an identical API 20E profile number which was 3306773 (Fig.3). This code number belongs to *Enterobacter cloacae* according to (Khalifa *et al.* 2016; Hayek and Willis 1976; Amin, Zafar and Ejaz 2013).

Our result is in agreement with (Taylor *et al.*, 2001) who proved that pEA71 was universal for all known *E. cloacae* strains to date. Similar results were also reported in Morocco by (Kohsaka *et al.*, 2014). This is the first report of *E. cloacae* isolation from pomegranate leaves in Kurdistan Region and whole Iraq.

Bacteria of the genus *Enterobacter*, including members of the *E. cloacae* complex are adapted to multiply and survive in diverse environmental conditions (Sanders Jr and Sanders, 1997). Besides that *E. cloacae* is recognized principally as causing harmful diseases affecting humans (Mezzatesta *et al.*, 2012), it cause several plant diseases. Since 1922, *E. cloacae* has been reported to cause diseases on a widespread variety of plants such as maize (Rosen, 1922), elm tree (Carter, 1945; Murdoch and Campana, 1983), coconut (George *et al.*, 1976). First report of spot root disease on Dargon fruit caused by *E. cloacae* in Malaysia (Masyahit *et al.*, 2009). García-González *et al.*, (2018) reported *E. cloacae* as emerging plant pathogenic bacterium affecting Chili Pepper seedling. Pathogenicity of *E. cloacae* on rice seedling in Heilongjiang province in China was reported by (Cao, *et al.*, 2020). Some reports mention that *E. cloacae* can be present in symptomless kernels (Nishijima *et al.*, 2007), thus the emergence of an infection can be latent until environmental conditions are favorable for the onset of the disease (Bishop and Davis, 1990).

The way of pomegranate leaves infection by *E. cloacae* is still unknown. Therefore, more research is needed to understand the epidemiology of this new disease and to develop management strategies. Peng Cao *et al* (2020) hypothesized that *E. cloacae* invades rice seedlings through hydathodes at the leaf tip and leaf margin.

CONCLUSIONS

- Until now there was no incidence that the bacterium *Enterobacter cloacae* cause disease in pomegranate.
- *Enterobacter cloacae* as a pathogenic bacterium on pomegranate trees is widely spread and occurred in several surveyed areas in Erbil region.
- This report could be considered as the first scientific documentation of pomegranate leave spot disease infection caused by the bacterium *E. cloacae* in Kurdistan region and whole Iraq.

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Conflict of interest:

There is no conflict of interest

Color should be used for figures 1,2,3 and 5 in print.

أول تقرير عن (*Enterobacter cloacae*) *المسببه* لمرض تبقع أوراق الرمان في محافضة اربيل

الملخص

أجريت هذه الدراسة لعزل و تشخيص المسبب المرضي لتبقع اوراق الرمان في محاقضة اربيل / اقليم كوردستان/ العراق. ا لدراسه بدأت بجمع العينات خلال الفترة من تموز ألى تشرين الاول 2021 . من بين ثمانية وعشرين بستان ضمن تسع مواقع, مراقبة الحقول (البساتين) أضهرت ان المرض منتشر في اربع وعشرون يستان في ستة مواقع. من بين 205 عزلة بكتيرية 57% كانت (*Enterobacter cloacae*). نتج عن ألاختبارات الحقلية و التخليل البايلوجي ان (*Enterobacter cloacae*) هو المسؤل الاول عن ضهور اعراض بقع صفراء الى بنية اللون على اوراق الرمان المصابة. تم تأكيد هذه النتائج بواسطة الفخص الكيميائي ((API20E test و تشخيص بتقنية ال PCR)). على حد علمنا هذا هو اول تقرير عن (*Enterobacter cloacae*) المسببة لمرض تبقع اوراق الرمان في محافضة اربيل. اقليم كوردستان و العراق بأكمله.

كلمات الدالة: بكتريا, *Enterobacter cloacae*, الرمان, تبقع