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Investigation and Detection of Fire Blight Disease on Pear Caused by *Erwinia amylovora* in Erbil Province

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ABSTRACT

Four isolates of *Erwinia amylovora* were isolated during (May, 2016) from different pear orchards of Erbil Governorate in Kurdistan/Iraq. Typical symptoms of fire blight such as shepherd crock and bacterial exudates were observed in pear trees, The bacteria morphologically similar to *E. amylovora* were isolated and characterized morphologically using selective media Crosse and Goodman, Biochemically using the API 20E and same biochemical tests (oxidase, catalase and indole) were also used for identification, and the result of API 20E kit (Biomérieux, France), demonstrated that the bacterium belongs to *E. amylovora*. Also all isolates were oxidase and indole negative, catalase positive and produced craters on the surface of colonies which permitted positive identification of the pathogen. A pathogenicity tests were positive on young pear shoots and immature fruits. PCR amplification was applied to identify a specific region of chromosomal pEA71, Results showed positive reaction to this specific primer. The first study conducted on pear fire blight caused by *E. amylovora* in Erbil Province.

Keywords: Fire blight disease, Pear, *Erwinia amylovora*, bacteria.

التحري والكشف عن مرض اللفحة النارية على العرموط (الكمثري) المتسبب عن بكتريا *Erwinia amylovora* في محافظة أربيل

الملخص

تم عزل أربع عزلات من *Erwinia amylovora* خلال (أيار، 2016) من مسح لبساتين العرموط في مناطق مختلفة من محافظة أربيل في كردستان / العراق. وقد لوحظت الأعراض النموذجية للفحة النارية والافرازات البكتيرية على أشجار العرموط، حيث تم عزل البكتيريا المشابه مظهريا للبكتريا *E. amylovora* وتميزت بالشكل المورفولوجي باستخدام الوسط الانتقائي Crosse و Goodman، وباستخدام الكيمياء الحيوية API 20E والاختبارات الكيميائية الحيوية نفسها (oxidase، catalase و indole) تم أيضًا تشخيص البكتريا، وأظهرت نتيجة مجموعة(API 20EBiomérieux ,France) أن البكتيريا تنتمي إلى *E. amylovora*. أيضا جميع العزلات كانت سالبة لاختبار أوكسيديز وإندول، وموجبة لاختبار الكاتاليز لوجود فقاعات على سطح المستعمرات والتي تسمح بالتشخيص الايجابي للمرض. كانت اختبارات الإمراضية إيجابية على براعم الكمثرى والثمار غير الناضجة. تم تأكيد التشخيص بواسطة تقنية PCR وإحداث التضاعف لمنطقة معينة من الكروموسومات pEA71 وأظهر تفاعلا إيجابيًا مع البادئ المحدد. على حد علمنا هذا هو التقرير الأول عن اللفحة النارية على العرموط الناجم عن بكتريا *E. amylovora* في محافظة أربيل.

الكلمات الدالة: مرض اللفحة النارية، العرموط (الكمثري)، بكتريا *Erwinia amylovora*.

INTRODUCTION

Fire blight disease is known as one of the most important plant bacterial diseases worldwide, causesd by the bacterium *E. amylovora* (Malika *et al*., 2011), it is the first bacterium illustrated as a causal agent of a plant disease by Burrill in 1883. Fire blight is a destructive necrotic disease affects *Malus domestica*, *Pyrus communis*, and other Rosaceae family plants (Norelli *et al*., 2003).

E. amylovora is determined as one of the important plant pathogenic bacteria in molecular plant pathology (Mansfield *et al*., 2012). According to (Van Der Zwet, 2006), fire blight disease recorded in more than 46 countries around the world. (Kaluzana *et al*., 2013), reported different methods for detection of the *E. amylovora* including; isolation of bacteria on selective or semi-selective media, tests of pathogenicity and DNA-based methods, especially Polymerase chain reaction, which offers the probability to overcome these constraints and allows fast detection of *E. amylovora* with high specificity and sensitivity.

MATERIALS AND METHODS

Field survey and samples collection

Samples of Pear trees (twigs including leaves and fruits) with suspected bacterial fire blight symptoms were collected from 24 orchards in different locations of Erbil Province during autumn 2015, and late spring of 2016. Samples were transferred to the laboratory of Plant Protection Dept. /Agriculture College in plastic bags as soon as possible and preserved in refrigerator at 4 °C. From each orchard samples, isolation was done from different plant parts.

A total number of 126 samples from symptomatic shoots, leaves and fruits were collected.

Isolation and purification of bacteria from infected plant parts

Isolates collected from infected pears (*Pyrus communis* L.) tissues (twigs, leaves, and fruits), showing the most suspected fire blight symptoms. Materials for processing are selected from the leading edge of disease lesions. Samples were washed in tap water. Infected tissues surface sterilized by soaking in 1% sodium hypochlorite for 3 min., then rinsed three times in sterile distilled water and blotted dry on sterilized tissue paper. Small pieces of diseased tissues were macerated in 1 ml of sterile phosphate buffer saline (PBS) in a plastic petri dish. The tissues were left for 30 min. to allow the bacteria to diffuse out of the tissue. With a sterile a loop, 30 µl of macerated tissue was streaked (EPPO,2004) onto King's medium (KB) agar (King *et al*., 1954).

Phenotypic Identification

To bacteria morphological and microscopic identification the following key tests were performed; gram reaction, oxidase and catalase tests (Jones and Geider, 2001), colony on 5% sucrose nutrient agar (Billing *et al*., 1961) and Crosse and Goodman (CG) media (Crosse and Goodgman, 1973). 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 °C for 48 h. Before starting a quick oxidase test for cytochrome enzyme was done according to manufacturer's instructions.

Pathogenicity Test

Pathogenicity test was performed on immature pear fruits and young shoots. In order to perform infections, branches with healthy shoots (25-30 cm length) were placed in conical flask with water. Infections were carried out by injecting the apical bud and four stings in upper leaves with 0.1 ml of bacterial suspension approximately 10^7 to 10^8 colony forming units (CFU/ml) with a hypodermic needle in the apical bud and four stings in upper leaves. For bacterial isolate, three shoots were used. For negative control shoots were inoculated only with sterile distillated

water. The relative humidity close to 90% and the temperature in range from 27 to 30 °C were established for 8-10 days during the experiment time. Symptoms observation was recorded (Severin and Cornea, 2009). Immature pear fruits were surface sterilized with 70% ethanol then cut in transverse slices about 1.0 cm thick. Three slices were placed on a sterile moist filter paper in sterile petri dishes for bacterial isolate. By using hypodermic needle, 50 µl of bacterial suspension was placed at the center of each slice. SDW was used as negative control. The slices were kept under humid conditions at 27 °C for five days (Beer and Rundle, 1983).

Genomic DNA extraction

Genomic DNA was extracted directly on the sub-cultured colonies of the identified bacteria by Presto™ Mini g DNA Bacteria Kit from Geneaid/ UK with minor modifications.

DNA amplification by (PCR)

The specific DNA primer (pEA71) for *E. amylovora* was used to amplify a targeted *E. amylovora* DNA by PCR. The primers pEA71; G1-F: 5′-CCT GCA TAA ATC ACC GCT GAC AGC TCA ATG-3′ and G2-R: 5′-GCT ACC ACT GAT CGC TCG AAT CAA ATC GGC-3′ were obtained from specific chromosomal DNA of *E. amylovora* (Taylor *et al*., 2001), and used to amplify DNA. The pEA71 primer and components were mixed in the same amplification reaction (Table 1). Appropriate thermocycling program was set on thermocycler according to the Go Taq Green Master mix protocol for pEA71 chromosomal DNA as pre-denaturation step at 95 °C for 3 min., Thermocycling (30 cycles): Denaturation 95 °C for 30 seconds, Annealing 67.5 °C for 30 sec., Elongation 72 °C for 30 sec., Final Extension 72 °C for 5 min. At the end of the process, amplified products were removed and stored at -20 °C until used for electrophoresis.

Table 1: The components required for primer pEA71 amplification

Components	Volume per reaction (µl)
Go Taq Green Master Mix 2x	12.5
G1-F Forward primer	2.5
G2-R Reverse primer	2.5
Sample DNA	5
Deionized distilled water	2.5
Total reaction volume	25 (volume of a single PCR reaction)

Electrophoresis

PCR products were separated on a 1% agarose gel in TBE buffer (1 h at 80 V), stained with ethidium bromide, the gel was viewed by UV Transilluminator and the result was photographed with digital camera (Sambrook *et al*., 2000).

RESULTS AND DISCUSSION

Occurrence of Bacterial Fire Blight Symptoms

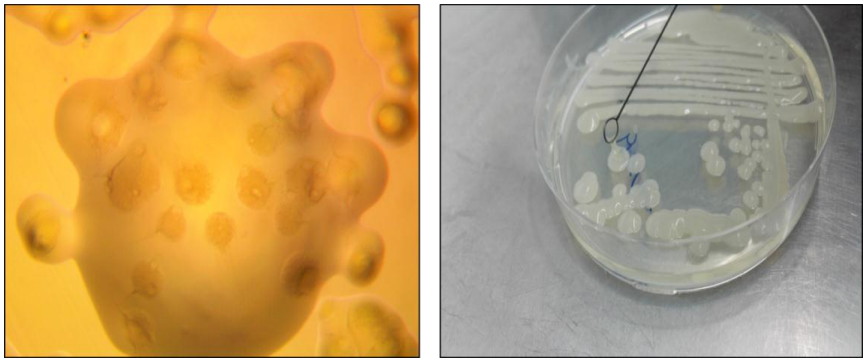
Symptoms were found on fruits, stems, leaves and exudates of Pear trees in one orchard. In late spring, small droplets of sticky bacterial ooze were observed on the surface of blighted shoots Fig.(1; A). Foliage parts of pear trees were observed as scorched or burned by fire Fig.(1; C). Shepherd's crook which is a characteristic symptom of fire blight was observed in the infected shoots Fig.(1; D). Dark small and shriveled fruits were also found on diseased branches

Fig.(1 B). These symptoms were also observed on pear trees in Iran (Kazempor *et al*., 2006), Turkey (Bastas, 2014), Syria (Ammouneh *et al*., 2008**)** and Egypt **(**Ashmawy *et al*., 2015).

Fig. 1: Different symptoms of fire blight observed in pear orchard from Choman area of Erbil Province during collection of samples. A: Blighted shoots. B: Dark small and shriveled fruits. C: Foliage parts of pear trees. D: Shepherd's crook.

Isolation and identification of *E.amylovora*

Only four isolates were observed, a characteristic craters on the surface of colonies when examined at 10X magnification by light microscopy Fig.(2A), which permitted positive identification of the pathogen. The isolates plated on sucrose nutrient agar formed only one morphological type of colonies typically white, domed, shiny, mucoid (levan type) Fig.(2B).These morphological characteristics are compatible with (Atanasova *et al*., 2005; Crosse and Goodman, 1973). Fire blight, caused by the *Erwinia amylovora*, is a common and very serious bacterial disease. The disease is also referred to as blossom blight, spur blight, fruit blight, twig blight (Anonymous, 2014).



A: CG plates B: 5% sucrose nutrient agar

Fig.2: Colonies of *E. amylovora*. A: Characteristic crates observed on the surface of colonies, using (10X) magnification, B: The colonies shape of *E. amylovora* grown on SNA, typically yellow-white, domed, shiny and mucoid.

Biochemical characteristics

The identification system API 20E was applied to all the isolates. The results were interpreted after 48 h. at 26 °C. The isolates showed an identical API 20E profile number which was 0005522 Fig.3, this code number belong to *E. amylovora* according to (Mergaert *et al*., 1984). The same result was also recorded in Moropcco by (Ameur *et al*., 2014). They collected 402 strains of *E. amylovora* from different regions of pome fruit. Biochemical characterization of selected strains by API 20E system displayed quite homogeneous API 20E results, 80% of them exhibited the profile number 0005522.

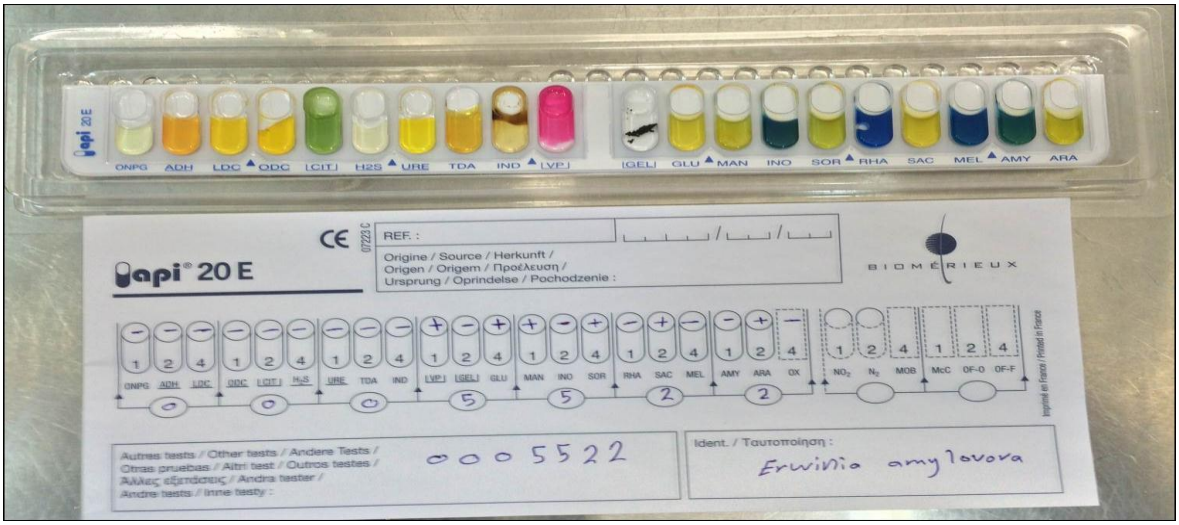


Fig. 3: The API 20E tests. Strip containing 20 tests and the profile sheet (0005522) code numbers indicated that pathogen belong to *E. amylovora*

Pathogenicity of *E.amaylovora*

All isolates showed typical symptoms of the disease upon infection to immature pear fruits such as production of ooze on pear slices after five days with brownish Fig. (4 B), necrosis of leaves and shoots within 8-10 days. Necrotic spots appeared on the leaves starting from midribs and enlarged getting a triangle shape (Fig. 4 A). No symptoms were recorded in the case of control. The similar observations were also reported in Iran by (Kazempour *et al*., 2006).

Fig. 4: Pathogenicity test for *E. amylovora*. A: Pear shoot inoculated with *E. amylovora*, necrosis of leaves and shoots started in the wound sites after 10 days. B: Bacterial ooze drop on pear slices after five days.

Molecular identification of *E.amylovora* isolates

The results revealed that all *E. amylovora* isolates gave a positive response to the specific primer pEA71, which amplified a DNA fragment with expected size of 187 bp during electrophoresis process within 1h., as it's illustrated in the figure the ladder is running 100bp for each band. Fig. (5).Our result was in agreement with (Taylor *et al*., 2001) who proved that pEA71 was universal for all known *E. amylovora* strains to date. These results were also reported in Morocco by (Ameur *et al*., 2014), where they identified 402 strains of *E. amylovora*. All strains reacted positively to specific primer (pEA71).

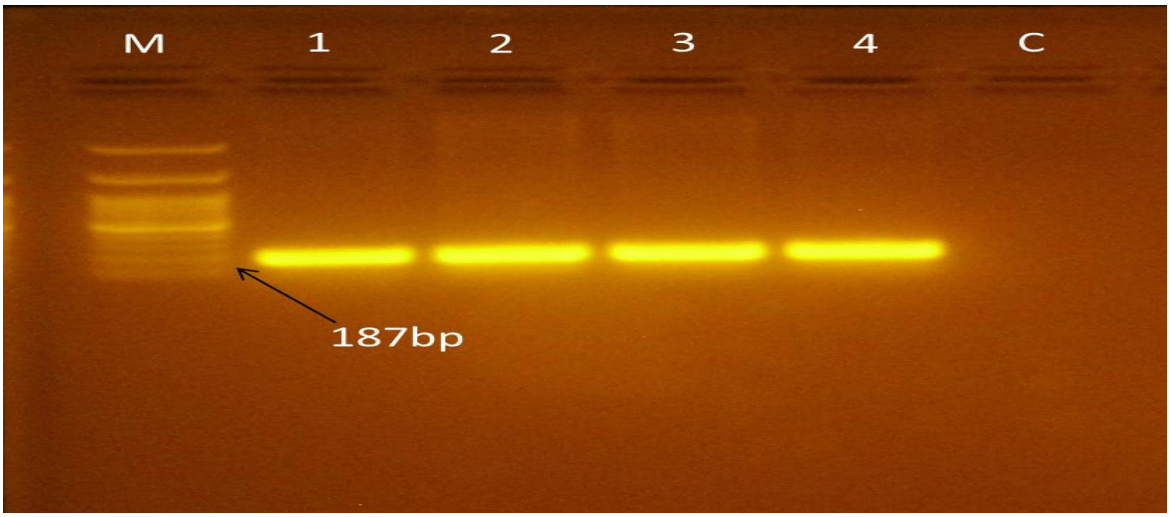


Fig. 5: Agarose gel (1%) electrophoresis illustrating PCR products based on specific primer for *E. amylovora*. Lane M: 100 bp DNA Marker. Lanes 1 to 4; DNA of *E. amylovora*. Lane C: Negative control (PCR mixture without DNA).

CONCLUSIONS

Our study was carried out to investigate fire blight disease of pear and its causal pathogen, *Erwinia amylovora*. Symptoms were recognized and bacterial fire blight is not a widespread disease in Erbil province. Out of 9 localities, four isolates from one location in Choman area of different plant parts were identified. Detection of *E. amylovora*, applying PCR and the primer pEA71 is dependable method. The current study is the first identification and characterization of *E. amylovora* isolated from pear in Erbil Province.

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