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FIRST REPORT OF BACTERIAL ANGULAR LEAF SPOT CAUSED BY *PSEUDOMONAS SYRINGAE* PV. *LACHRYMANS* ON CUCUMBER IN IRAQ

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Abstract

The current study aimed to detect the spread of angular spot disease on cucumber leaves in different areas of Najaf province in 2017 agricultural season. Pathogenicity, biochemical and molecular diagnosis of pathogen were conducted in greenhouse and laboratory. Results of the field survey on the spread of disease were different in the infection rate ranged from 37% to 82% and the intensity of disease ranged from 30% to 60%. While the result of the hypersensitivity test on tobacco leaves and pathogenicity test on cucumber showed that 52 isolates out of 102 isolates tested were able to cause symptoms of the disease, P.s2 and P.s5 isolates were more virulence than most other isolates. The diagnostic tests were confirmed that the 52 isolates were *Pseudomonas syringae* pv. *lachrymans*. These isolates were grown on King's B agar; strains colure was initially white with a bright chromatic blue-yellowish, Gram stain negative. Oxidase, Arginine, Nitrate reduction, Indol, Aesculinhydrolys, Tartrate, Levan and Sugar fermentation tests showed that negative results. But KOH, Gelatine and Catalase and ammonia production of peptone showed that positive results. The diagnosis was confirmed by PCR for the two most virulence isolates that isolated from cucumber based on the 16S rRNA gene, which tow *P. syringae* pv. *lachrymans* strains were submitted to NCBI database and registered as new races nationally within accession number (MH279670 and MH 279671). These two strains were grouped in two different classes according to results of phylogenetic tree. The current study highlighted for the first time the incidence of bacterial angular leaf spot disease in Iraq.

***Key words*:** *P. syringae*, infection rate, pathogenicity, biochemical tests.

Introduction

Bacterial pathogens pose a serious threat to both agriculture and human health around the world in addition to the emergence of new pathogens and the continuous evolution of existing species. Pathogens of human and animals are often the major focus of research because of their adverse impact on universal health. However, it is also necessary to study pathogens that cause severe plant diseases that threaten agricultural production worldwide (Sheppard *et al*., 2013). In recent years, agriculture had suffered from the significant economic losses and the reduction of crop production due to outbreaks of diseases caused by bacterial plant pathogens (Borkar and Yumlemban, 2016). Therefore, as a result of the high humidity and moderate temperature in greenhouses and to provide suitable conditions for the maturity of cucumber crop, the spread of angular spot disease on cucumber leaves was increased. However, studies did not indicate the presence of the disease in Iraq according to the latest issue of plant diseases (Fayyadh and Abass, 2018) despite the fact that the disease is widespread in many greenhouses. The lack of knowledge in diagnosis the symptoms of disease on cucumber lead to the failure of the control of the pathogen. The infection process of *Pseudomonas syringae* pv. *lachrymans*, the causal of angular spot disease on cucumber leaves starts as small circular spots on the leaves then become large, irregular and in humid weather drops droplets of bacterial excretion, show to the bottom surface of leaf, finally dry and mutation into white slant and turn the affected area to gray, shrinks and dies, leaving large holes in the leaves. *P. syringae* is a varies according to the level of DNA, increasing its adaptation to environmental and host conditions, it was therefore a reason for its increased spread in the biosphere (Agrios, 2005; Woltman-Olczak *et al*., 2007; Słomnicka *et al*., 2015).

The study aimed to detect the bacteria that cause angular spot on cucumber leaves for the first time and to provide a foundation for future studies on the biology and ecology of *P. syringae* pv. *lachrymans* in Iraq.

Materials and Methods

Investigation of the spread of angular leaf spot disease on cucumber leaves in some areas of Najaf

The field survey was carried out in three areas of Najaf province (Qazwinia, Abbasiyah and Haidariyah), which is famous for cultivating the cucumber crop in green houses during the agricultural season 2017.

According to the method proposed by Naik and Lakkund, (1997), infection ratio was estimated in the areas covered by the survey of angular leaf spot disease on cucumber, and infection severity was estimated according to the method McKinney, (1923).

Leaf samples (102 leaves) were collected randomly and placed in plastic bags with proper labeling were brought directly to phytopathology laboratory and kept in the refrigerator at 4 ± 1°C until used for subsequent studies.

Detection of *P. syringae* pv. *lachrymans*

King's B agar: External surfaces of leaves samples for each cucumber plants were sterilized with 10% Clorox for 1 minute. Then the samples were washed with sterile water and the excess water was removed using blotter paper. Leaves parts of cucumber were placed on the King's B plate. Plates were incubated in an inoculation chamber at 27°C for 2 days to allow the growth of bacteria associated with the leaves.

Tobacco Hypersensitive Response (HR) Test: At 30-40 days old tobacco plants, bacterial suspension (5.7×10^8 CFU/ml) of isolate representing a group was injected in the leaves. Bacterial suspensions were injected into the intracellular space of the leaf with a hypodermal syringe. Hypersensitive reaction was observed daily and continued up to five days of injection. Necrotic lesions on the leaves were recorded positive.

Pathogenicity Test on Cucumber: Pathogenicity test was conducted using one month old cucumber var.Omiga according to method of Lelliot and Stead, (1987). Bacterial suspension (5.7×10^8 CFU/ml) was prepared from the 24 hrs old culture of different isolates of *Pseudomonas* sp. The suspension was sprayed onto the leaves of cucumber seedlings in 3-4 true leaf stage grown in pots by hand sprayer. Inoculated cucurbit seedlings were covered with a black polythene sheet to create 80-90% relative humidity for 48 hrs. Then black polythene sheets were replaced by a transparent polythene sheet to conserve moist condition. Then the seedlings were kept on the green house. The symptoms were appeared up to 10 days. During the experiments plants were watered regularly and wetted with hand sprayer. Control plants were sprayed with distilled water.

Biochemical Tests for Identification of *P. syringae* pv. *lachrymans*: Based on the results of tobacco hypersensitive response test and pathogenicity test on cucumber usingthe52 isolates in biochemical tests. *P. syringae* pv. *lachrymans* in the leaves of cucumber was identified by several biochemical tests as follows:. Gram staining (Shila *et al*., 2013), Potassium hydroxide solubility (Fahy and Parsley, 1983), Kovac's oxidase (Fingold and Martin, 1982), Levan (Lelliott and Stead, 1987), Sugar fermentation(Shila *et al*., 2013), Arginine hydrolysis activity (Thornley, 1960), Catalase (Crabtree *et al*., 1974), Gelatin hydrolysis (Skerman *et al.,* 1980), Nitrate reduction (Schaad *et al*., 2001), Tartrate test L. (Lelliott and Stead, 1987), Production of ammonia from peptone (McCance and Harrigan, 1976), Indol test (McCance andHarrigan, 1976), and Aesculin hydrolysis (Lelliott and Stead, 1987).

Molecular diagnosis of *P. syringae* pv. *lachrymans*

DNA extraction and PCR conditions: DNA was extracted using the adjusted procedure of Sujatha *et al*. (2012) form two different isolates of *P. syringae* which were showed more pathogenic on cucumber leaves (P.s2 and P.s.5), following the manufacturer's instructions (Genomic DNA Extraction Kit G-spin Total DNA). 16S rRNA was amplified using a forward primer (5'AGAGTTTGATCCTGGCTAG-'3) and a reverse primer (5'- GGTTACCTTGTTACGACTT '3) according to Ciantar *et al*., (2005) (Intron, Korea). PCR products were subjected to agarose gel electrophoresis at 100 V for 60 min on a 1% (w/v) agarose gel containing 0.1% GelRed™ Biotium Inc. (United States) and then visualized under UV light. Aliquots of PCR products of 1600 bp (30 µl of each) were sent to Macrogen Inc. (Korea) for sequencing.

DNA sequence manipulation alignment and analysis: DNA sequence manipulation alignment and analysis were conducted using Geneious v. 9.1.7 (Biomatters Ltd). All sequences were aligned using Geneious alignment with cost matrix of 70% similarity (IUB) (5.0/-4.5), gap open penalty: 13, gap extension penalty: 3 and global alignment with free end gaps as the alignment type. A neighbour-joining tree was constructed using the function 'Geneious Tree Builder' and adopting the Tamura-Nei genetic distance model and the bootstrap re-sampling method (random seed 520,500 number of replications 1,500, and support threshold higher than 70%). The sequence of ribosomal DNA (rDNA) gene of *Xanthomonas axonopodis* MF477906 from Gen Bank was used as the out-group.

Results

Infection and Severity Rate: The field survey showed that presence of angular spot disease on cucumber leaves in twenty seven greenhouses covered by the survey through the appearance of symptoms on the leaves. The incidence of infection rate in the greenhouses in the Qazwinia region ranged from 53% to 82%, Haidariyah region with a rate from 75% to 40%, but Abbasiyah region recorded the lowest infection rate from 37% to 52%. While, the severity of infection of

Qazwinia and Haidariyah regions were respectively from 32.51% to 60.56% and from 59.44% to 32.33%, but the lowest infection was in Abbasiyah region from 59.44% to 32.33% as in table (1).

Pathogenicity and Hypersensitive Response Tests: *P. syringae* pv. *lachrymans* detected from all isolates were tested for hypersensitive reaction in tobacco. Results of this test showed that the fifty two isolates were able to cause partial death of local cell of tissues within the infiltrated area of tobacco leaves, and the hypersensitive reaction was most distinctly manifested when 1-3 days old bacterial cultures. Fifty two of *P. syringae pv. lachrymans* from 102 isolates produced typical symptoms of angular leaf spot symptoms in seedlings of cucumber tested, 15 days after inoculation, causing greasy and individual spots, and P.s2 and P.s5 isolates were the highest in term of pathogenicity than most of other isolates (Fig. 1).

King's B agar and Biochemical Tests: Isolates of bacteria were grown on King's B agar, the colure of strainswas initially white with a bright chromatic blue-yellowish, gram stain was negative. Oxidase, Arginine, Nitrate reduction, Indol, *Aesculin hydrolys*, Tartrate, Levan and Sugar fermentation tests showed that negative results. But KOH, Gelatine and Catalase and ammonia production of peptone showed that positive results. These results clearly indicated that the 52 isolates were *P. syringae* pv. *lachrymans* (Fig. 3 and Table 2).

Molecular diagnosis of *P. syringae pv. lachrymans*: The diagnosis was confirmed by PCR for two most pathogenic isolates on cucumber. Races were recorded in this study as new races nationally, two strains belong to *P. syringae pv. lachrymans* were recorded inthe world. All of these strains have been registered at the National Center for Biotechnology Information (NCBI) within accession number MH279670 (P.s 2) and MH 279671(P.s 5).

DNA sequence alignment and analysis of *P. syringae pv. lachrymans*: Results of the phylogenetic tree showed that strains MH279670 and MH279671 were grouped into two different clads. The first strain was 100% identical with the KR708860 strain from China, while the second strain was isolated in different group and did not match any of the other strains of Japan, Spain, Iran, Russia and Pakistan used to build the phylogenetic tree (Fig. 2).

Table 1: Infection and Severity rate of *P. syringae pv. lachrymans* onon cucumber leaves

Region	Infection rate	Severity rate
Qazwinia	53% - 82%	32.51% - 60.56%
Haidariyah	40% -75%	33.32% - 59.44%
Abbasiyah	37% - 52%	30.33%- 36.33%

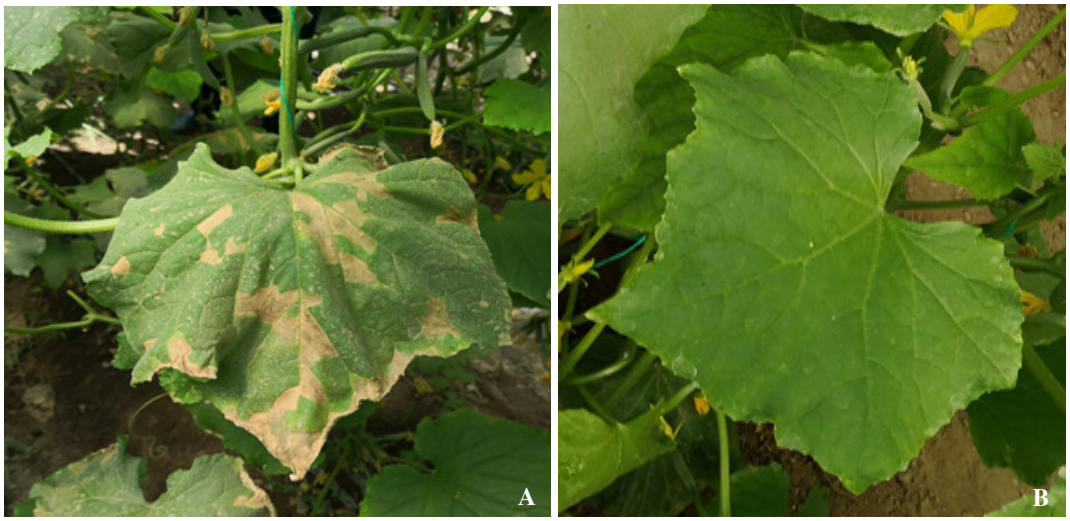

Fig. 1: Symptoms of angular spot disease on cucumber leaves.
(A) Infected leaf and **(B)** Uninfected leaf .

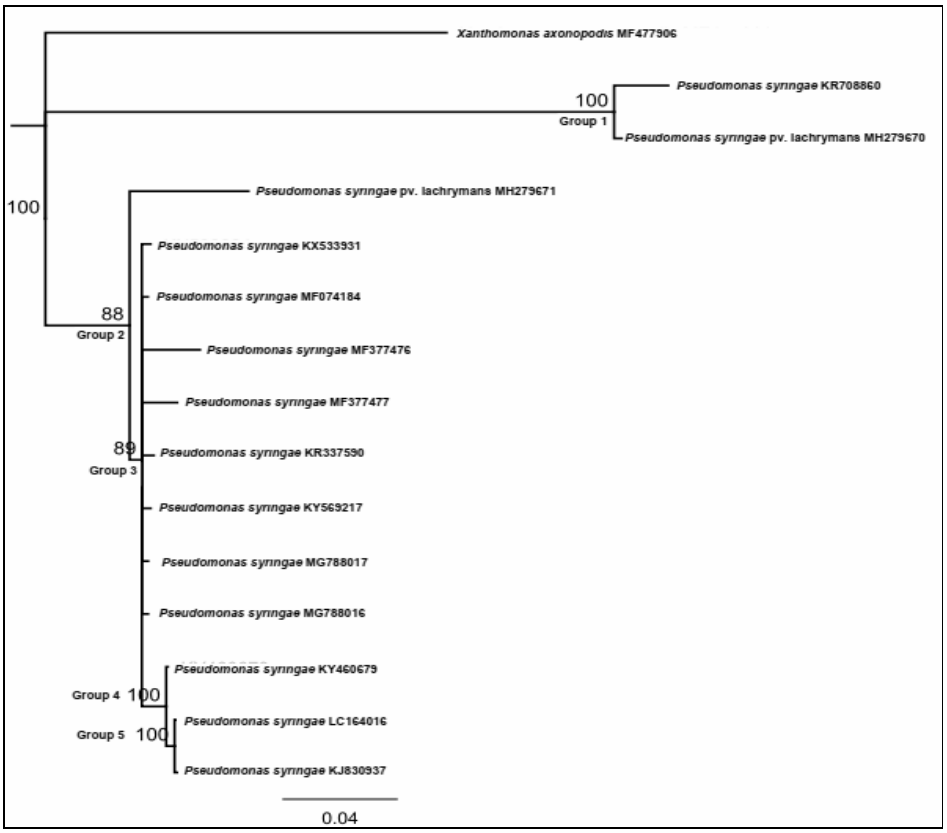

Fig. 2: Phylogenetic tree showed that strains MH279670 and MH279671

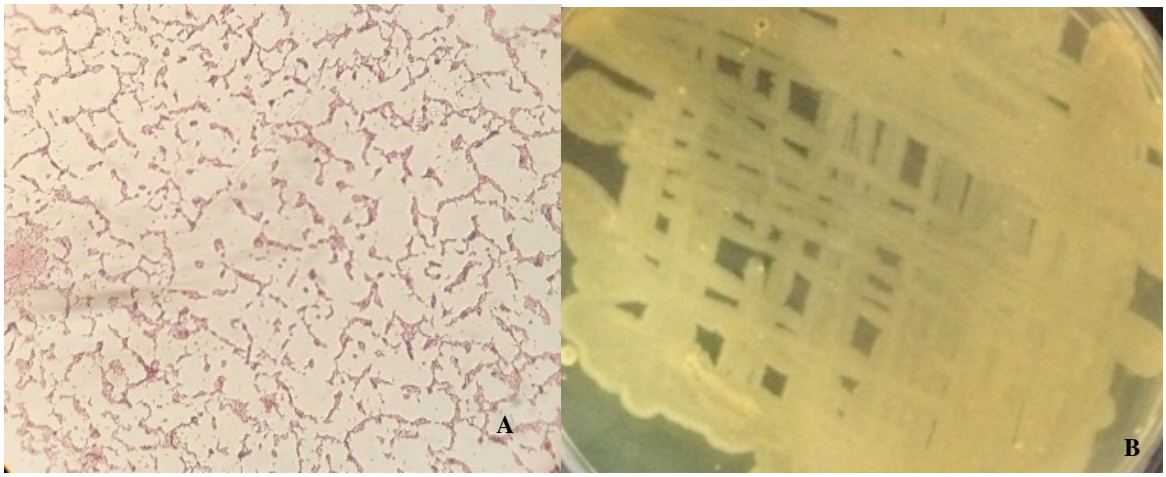

Fig. 3: (A) microscopic properties of *P. syringae pv. lachrymans* cells under the magnification of 1000 X of the bacteria, and **(B)** *P. syringae pv. lachrymans* was growth on King's B agar after 72 hours.

Table 2: Results of biochemical tests of *P.syringae pv. lachrymans*. (M) Mannitol, (F) Fructose, (S) Sucrose and (G) Glucose.

No. isolation	Oxidase test	Hydrolysis of Arginine	Catalase test	Hydrolysis of Gelatin	Nitrate reduction	Ammonia from peptone	Indol test	Aesculin hydrolysis	KOH solubility test	Sugar fermentation test				Levan sucrose (L) Production	Tartrate test L.
										G	S	F	M		
P.s1	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s2	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s3	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s4	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s5	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s6	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s7	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s8	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s9	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s10	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s11	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s12	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s13	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s14	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s15	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s16	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s17	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s18	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s19	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s20	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s21	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s22	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s23	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s24	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s25	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s26	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s27	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s28	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s29	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s30	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s31	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s32	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s33	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s34	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s35	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s36	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s37	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s38	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s39	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s40	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s41	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s42	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s43	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s44	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s45	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s46	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s47	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s48	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s49	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s50	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s51	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-
P.s52	-	-	+	+	-	+	-	-	+	+	+	+	+	+	-

(**+** = Positive Reaction and**-** = Negative Reaction).

Discussion

Current study highlighted for the first time the incidence of bacterial angular leaf spot disease in Iraq. The lack of knowledge about disease symptoms on plant parts and misdiagnosis of bacterial pathogen lead to inappropriate control of the pathogen. The wide spread in the Qazwinia region of the angular spot disease on cucumber leaves and evolution may occurred due to the heat and humidity in order to provide conditions which is suitable for the growth of cucumber were a major factor of the infection through the availability of warm temperatures and high humidity. This lead to fill plant tissues with water to develop the disease in greenhouses, this is consistent with Venette *et al*. (1996). This variation in the infection and severity of disease was due to that most of the fields surveyed were planted cucumber for several consecutive years, and the residues of the infected plants contributed to the accumulation of bacterial germs in soil and lead to an initial infection to the seedlings (Hansen, 2000). Age differences and nutritional status of cucumber may be another reason for this variation. The variation between isolates in the fast appearance of symptoms on tobacco leaves after inoculation was because the differences in penetration time and virulence of isolates on host, that lead to the partial death symptoms of tobacco leaf tissue and this is consistent with Shila *et al*. (2013). As for the pathogenicity test on cucumber, the52 different isolates have ability to cause the disease through the virulence of pathogen on the cucumber and this is consistent with Aksoy, (2006) and or that *P. syringae*pv. *lachrymans* reproduces in the interfaces in tissues of the leaves and produces enzymes to break the cell walls after 96 hours of inoculation and found abundantly in wood vessels (Yedidia *et al*., 2003). The cause of yellow spots is may be due to *P. syringae* pv. *lachrymans* releasing of syringomycins and syringopeptins, which inhibits glutamine synthesis, which is stimulated by T3SE (a bacterial virulence factor) (Gallo *et al*., 2000). The other reason is that *P. syringae*pv. *lachrymans* at the beginning of penetration into plant absorbs mineral elements and amino acids, lead to the production of active protein that causes freezing of water in plant tissue at low temperatures which affected on the plant defenses and ability of races to cause the disease on cucumber (Maki, 1974). Results of the biochemical tests on 52 isolates of *Pseudomonas*, showed that it had characteristics of *syringae* specie correspond to with previous studies (Elliot, 1966; Schaad, 1980; Kagiwata; 1990; Aksoy, 2006), as for the pathovar of lachrymans, results agreed with studies by Hossain *et al*., 2015. The result of molecular diagnosis by using PCR and the primer of 16S rRNA, was confirmed the biochemical diagnosis of *P. syringae*pv. *lachrymans* (Amirabad, 2017). Differences in phylogenetically extent of convergence and variability were due to two reasons. The first, *P. syringae* was spacious family range could infect many plants and their symptoms varied from crop to other (Hulin *et al*., 2018). The second, it may be due to variation isolation area or difference in nutrition status and age of cucumber.

Conclusion

Bacterial angular leaf spot on cucumber is an important disease in greenhouses and causes significant losses. The lack of know ledge about this pathogen in Iraq misdiagnosis of this disease enhanced the widespread of the pathogen particularly when a high level of humidity occurred. The current study highlighted for the first time the incidence of bacterial angular leaf spot disease in Iraq when several biochemical tests were carried out follow by molecular tests to confirm the findings. These results were beneficial to facilitate and solve the difficulties in the differentiation between species of this genus depending only on morphological and biochemical tests.

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