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Isolation and Identification of *Xylella fastidiosa* that Cause Oleander Leaf Scorch

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ABSTRACT

During spring of 2022, leaf scorch symptom was observed in *Nerium oleander* plant in Erbil city. Surveys were carried out in three main Erbil streets (60-meter, 120 meter and Qasmlo) to determine the occurrence and distribution of oleander leaf scorch.

Oleander leaf scorch (OLS) is a disease caused by xylem limited bacterium *Xylella fastidiosa*. The percentage of plants with Oleander leaf scorch infection ranged usually between 23-91%, and the disease was assessed the highest disease rate based on disease rating scale was recorded in 60-meter street is 91.95%, Qasmlo street is 89.07% and the lowest disease rate was observed in 120-meter street is 23.17%. In this study the bacterium was detected after isolated in laboratory as Gram stain test was performed and the isolated bacterium was Gram negative, rod shaped, non-flagellate and non-motile also identified by PCR were performed with two primers forward primer 16S (AGAG TTTG ATCC TGGC TCAG) and reverse primer 16S (GGCT ACCT TGTT ACGA CTT), amplified a 1200 bp fragment from the isolated OLS. The Analysis of BLAST revealed that the highest identity number query sequences were (100%) identified and submitted in GenBank and have taken accession number *Xylella fastidiosa* subsp. Sandyi (ON131099) and *Xylella fastidiosa* subsp. Sandyi (ON131100) also the phylogenetic results show the each identified bacteria are grouped in one clade with other sequences were stored in NCBI GenBank. This is a first report of a '*Xylella fastidiosa*.in Erbil city.

Keywords: *Xylella fastidiosa*, *Nerium oleander*, ornamental plant, leaf scorch.

INTRODUCTION

Nerium oleander is an ornamental evergreen shrub or densely branched tree measuring 1 to 10 m in tall belongs to the dogbane family Apocynaceae (Frohne and Fander,1984). This plant is native to southwest Asia and Mediterranean region (Singh and Ferrin, 2010).

Oleander leaf scorch is a disease caused by bacterium *Xylella fastidiosa* because of the pathogen blocks the channels of xylem vessels of host plant it is called xylem limited bacteria (Grebus *et al.*, 1996). The disease can be appeared year-round but more evident in late spring and summer, in warm weather they grow swiftly (Wilen *et al.*, 2008).

Xylella fastidiosa is a Gram-negative bacterium belonging to Xanthomonadaceae family (Wells *et al*. 1987); however, this bacterium is separated from other common phytopathogenic bacteria of this family by having several distinctive molecular and pathogenic properties. Plant propagating materials are agents for spreading this bacterium for long distance, also it spread by xylem feeding insects for short distance and infect a wide range of plant species. Based on the literature search, the bacterium affects more than 500 plant species, mostly perennial species, notable modification of most infected plants are not shown (Loconsole *et al.*,2021).

The significant symptom of oleander leaf scorch is the scorching of leaves, which start from the tip and the edges of leaves and forward to leaf midrib (Singh and Ferrin, 2010). In severe plant infection discoloration are occurred at the marginal leaf tissue especially at the tip of leaves (Wilen *et al.*, 2008).

The symptoms of oleander leaf scorch on leaf are associated to those symptoms produced by drought but the leaf scorch by bacterium start from tips or margins and progress to midrib, whereas drought stressed leaves yellow along the central leaf veins uniformly, also the plant recovered when watered when drought is severe but the infection by *Xylella fastidiosa* do not recover because the xylem tubes of infected branches are clogged and limit the water flow. Also, marginal leaf browning of the disease confused with those symptoms caused by salt injury or boron toxicity (Wilen *et al.*, 2008). The Aims of this research are survey and identifying the pathogen of Oleander leaf scorch disease in the center in Erbil city, microscopically and confirming by PCR.

MATERIAL AND METHODS

Survey of Oleander leaf scorch:

This study was conducted at the laboratory of postgraduate of the Plant Protection Department at the College of Agricultural Engineering Sciences, in the Salahaddin University/ Erbil/ Iraq.

Surveys were made in *Nerium oleander* plants in the shoulders or the edge of three main streets (Qasmlo, 60 meter and 120 meter) of Erbil city, during the spring of 2022, from late May, plants showing symptoms similar to those caused by *Xylella fastidiosa* infection were surveyed as showed in Fig. (1).

Fig. 1: Oleander leaf scorch symptom in Erbil city.

The percentage of symptomatic plants in each location was calculated by measuring the number of symptomatic *N.oleander* plants per 100 plant, and up to 20 samples were randomly collected for further analysis. Twenty naturally occurring diseased plants (*N.oleander*). Samples of infected plants with various stages of infection were collected in three common streets of Erbil city. Symptomless *Nerium oleander* plants were also sampled as negative controls in that 3 main streets were surveyed. (Table 1).

Table 1: Survey of Oleander leaf scorch in Erbil city

	Location	**Number of samples**
1	120-meter street	725
2	Qasmlo street	119
2	60-meter street	87

Disease Assessment

The rating of OLS symptoms was between 0 to 3 rating scale, were 0=healthy tree, 1 = only one limb showing OLS symptoms, 2 = more than one limb showing OLS symptoms and 3 = all limbs within the tree were OLS symptomatic (Cao *et al*., 2011).

Bacterial Isolation

Infected leaves of Oleander were collected in Erbil city based on OLS symptoms and were taken in laboratory, then the bacterium was isolated by cutting the collected samples in to small pieces leaves and surface sterilized by immersion in 70% ethanol for 1 min followed by 2 rinses of distilled water to avoid the growth of saprophytes then dried after that the small pieces were taken in nutrient agar. The plates were incubated at 27°C for about 2 weeks (Schaad *et al*., 2001) after that time gram stain test was done for the isolated bacterium.

Molecular Identification

DNA Extraction:

Genomic DNA was isolated from isolated bacteria colony of different pure culture petri plate by PGA Bacterial DNA Extraction Kit was manufactured by Iran- No. PF230-050

Polymerase Chain Reaction (PCR) Amplification 16S ribosomal RNA (16S rRNA):

PCR amplification for 16S rRNA partial gene was done in 50 µl of reaction mixture containing; 2x Taq DNA Polymerase Master Mix (AMPLIQON A/S Stenhuggervej 22), 10 Picomol (pmol) of forward promer 16S (AGAG TTTG ATCC TGGC TCAG), 10 pmol reverse primer 16S (GGCT ACCT TGTT ACGA CTT), DNase free water and template DNA (Table 2) by Bioresearch PTC-200 Gradient thermocycler.

Table 2: 16S rRNA PCR Amplification Reagents

No.	**PCR components**	**Concentration**	**Volume (µl)**
1	Master Mix	2x	25
2	Forward Primer	20 Pmol	3
3	Reverse Primer	20 Pmol	3
4	DNase free Water	-	15
5	Template DNA	50ng/µl	4
Total			50

Temperature profile included step one is an initial denaturation at 95 °C for 5 min, step two followed by 35 cycles of a denaturation at 95°C for 35 second, a primer annealing at 58°C for 35 sec., an extension at 72°C for 1 min and final step is an extra extension at 72°C for 10 min.

1- Visualization of DNA fragments

An Ethidium bromide which is n intercalating dye added to1.5% melted agarose gel in 1X TBA buffer after 30minutes inside of electric field of electrophoresis, by running UV trans-illuminator on the gel electrophoresis tank the location of bands determined.

Sequencing of DNA

The samples of PCR product 16S rRNA partial gene have sequenced by ABI Prism Terminator Sequencing Kit (Applied Biosystem) at Microgen Center in Korea. Chromatograms of 16S gene were edited and base calls checked using Finch TV program software

2- Sequence alignment and submission

The 16S gene sequences were applied to Basic Local Alignment Search Tool (BLAST) is a searching tool that applies the sequence alignment method (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and is available at the NCBI (National Center for Biotechnology Information) website to comparing and alignment laboratory or query sequence with other biological sequence to find out more similarity with other targets.

RESULTS AND DISCUSSION

Survey of Oleander leaf scorch

Disease assessment for OLS were carried out and the highest disease rate was recorded in 60-meter st. by 91.95% and in Qasmlo st. 89.07% and the lowest disease rating was showed in 120-meter st. by 23.17% as illustrated in (Table 3).

Table 3: Disease assessment for OLS in three main street shoulders in Erbil city (60 meter, Qasmlo, 120 meter).

Disease rating	120-meter st.	Qasmlo st.	60-meter st.	Total
Healthy	557 (76.82%)	13 (10.92%)	7 (8.04%)	577 (59.82%)
1	49 (6.75%)	15 (12.60%)	13 (14.94%)	77 (8.27%)
2	95 (13.10%)	54 (45.37%)	18 (28.57%)	167 (17.93%)
3	24 (3.31%)	37 (31.09%)	49 (20.68%)	110 (11.81%)
Total OLS trees	168 (23.17%)	106 (89.07%)	80 (91.95%)	323 (38.01%)
Total trees	725	119	87	931

The result showed there are differences for disease incidence rate between 60-meter st. with two other streets, and there are a few significances difference between 120-meter st. and Qasmlo street. The result is agreement with (Cao *et al*., 2011) which the disease incidence rate of 120-meter st.

Bacterial isolation:

The isolated bacterium was gram negative**,** rod-shaped bacterium, non-flagellate, non-motile, also does not form spores (Davis *et al*., 1978; Wells *et al.,* 1987; Bradbury, 1991).

Molecular Identification:

Genomic DNA isolated

Genomic DNA was isolated from colonies individuals, each specimen was extracted by PGA Bacterial DNA Extraction Kit was manufactured by Iran- No.PF230-050. The isolated DNA was electrophorized in 1% Agarose gel Fig. (2).

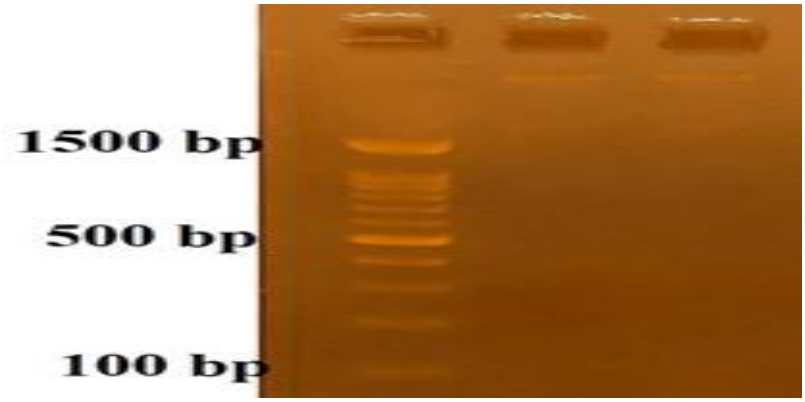

Fig. 2: Genomic DNA isolated from 10 colonies of bacteria

PCR amplification of partial 16S rRNA gene

Ribosomal gene specific primers were designed for the using the sequences of 16S rRNA Synthesized by Micro-gene Company (South Korea) the primers could yield a band ~1200bp. The PCR product was electrophoresed and visualized by 1.5% Agarose gel. Fig. (3).

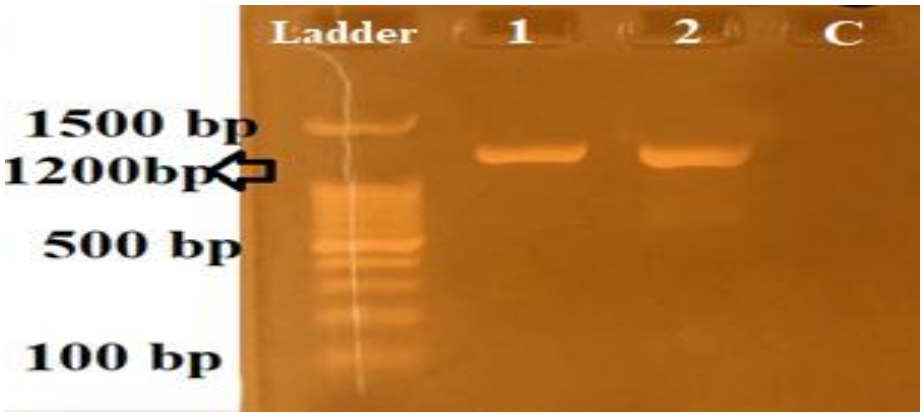

Fig. 3:Gel electrophoresis of PCR amplification of partial 16S rRNA gene from 10 bacterial colonies

Partial 16S rRNA Sequenced gene

DNA sequencing, using only forward primer 16S was performed separately by ABI 3130X genetic analyzer (Applied Biosystem). The PCR products of the 10 samples were used as a source of DNA template for sequence specific PCR amplification. Fig. (4)

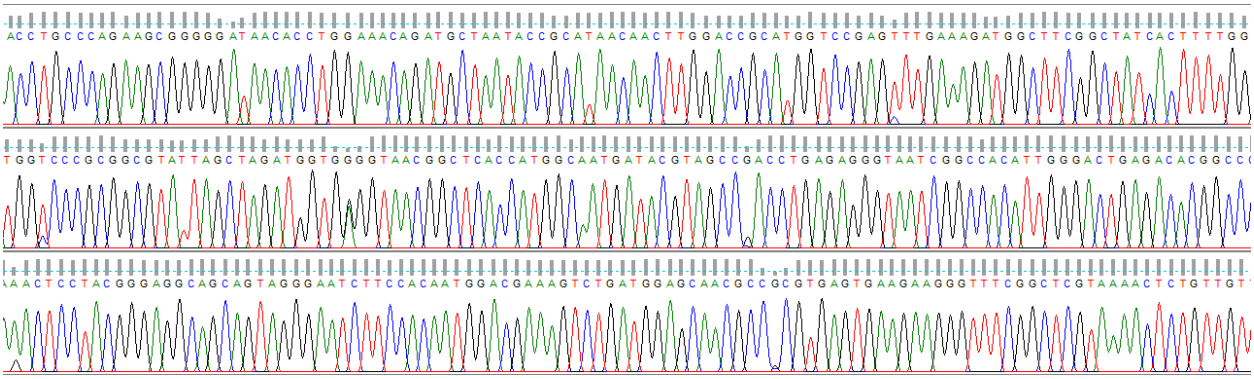

Fig. 4: The chromatogram sequences result of partial gene of 16s rRNA sequences.

Molecular Identification of Genus and Species of Bacteria

To compare our amplified sequences with other stored species of lactobacillus sequences the partial gene of 16S rRNA sequence samples with size 1000-1200 bp are alimented by using BLAST program from Gen bank (*http://blast.ncbi.nlm.nih.gov/*). The results obtained from the BLAST indicated that the highest identity number query sequences were 100% identified, submitted inside of NCBI GenBank and have taken accession numbers according to (Table 4).

Table 4: Molecular identification and submitted partial gene of 16S rRNA of 10 query sequences in NCBI GenBank

Samples	Bacterial Identified	Accession Numbers
1	*Xylella fastidiosa* subsp. Sandyi	ON131099
2	*Xylella fastidiosa* subsp. Sandyi	ON131100

Phylogenetic inferences

Phylogenetic analysis by MEGA 11 program based on partial 16s rRNA gene nucleotide sequence revealed grouping of 10 investigated or isolated species of bacteria on expected lines. From sequence divergence similarity data and phylogeny constructed, it was revealed that species belonging to respective genera were close to each other. The each identified Bactria samples are grouped or located with same subject species NCBI GenBank in one branch with high similarity (Table 5). The bacterial samples which relatedness with other GenBank bacterial species identified in GenBank were retrieved from Mega blast program of NCBI nblast. Fig. (5).

Table 5: Distribution of bacterial species % based on partial 16S of rRNA according to nblast in GenBank of NCBI

Samples	Bacterial Identified	Accession Numbers	Query Cover %	Identic Number %	GenBank Accession Number	GenBank Fungi Species Identification	Country Identification
1	*Xylella fastidiosa* subsp. sandyi	ON131099	100	100	CP090547	*Xylella fastidiosa* subsp. Sandyi	USA
					CP006696	*Xylella fastidiosa* subsp. Sandyi	USA
2	*Xylella fastidiosa* subsp. Sandyi	ON131100			HM125896	*Xylella fastidiosa* subsp. Sandyi	USA

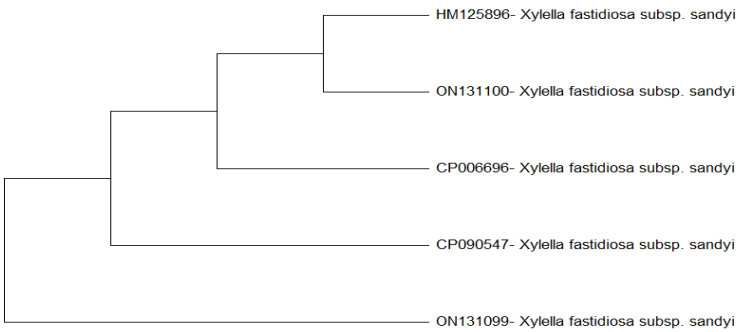

Fig. 5: MEGA 11 program show phylogenetic positioning of each sample according to sequences of 16S rRNA employing maximum likelihood available in GenBank sequence

CONCLUSIONS

Surveys were carried out in different parts of Erbil city to determine the occurrence and distribution of oleander leaf scorch. The percentage of plants with Oleander leaf scorch infection ranged usually between 23-91%, this study indicated that *X. fastidiosa*. Were isolated from infected Oleander plants and identified morphologically and PCR.

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عزل وتشخيص بكتريا *Xylella fastidiosa* المسببة لمرض حرق اوراق الدفلة

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الملخص

خلال ربيع 2022 تم ملاحظة اعراض مرض حرق الاوراق على نباتات الدفلة *Nerium oleander* في مدينة اربيل. تم اجراء مسح عن المرض لتحديد مدى انتشار المرض وتواجده. يتسبب مرض حرق اوراق الدفلة عن البكتريا المستوطنة في اوعية الخشب لنبات الدفلة *Xylella fastidiosa* . المسح شمل شجيرات الدفلة المزروعة على الارصفة والجزرة الوسطية لثلاثة شوارع رئيسية في مدينة اربيل وهي شارع (60م، 120م وقاسملو). تراوحت نسبة الاصابة بمرض حرق اوراق الدفلة بين 23–91%، واعلى نسبة إصابة على نباتات الدفلة سجلت في شارع 60 م والتي كانت 91.95% وتلتها شارع قاسملو والتي كانت 89.07% وأدنى نسبة اصابة بالمرض سجلت في شارع 120م والتي كانت 23.17%. في هذه الدراسة تم الكشف عن البكتريا المسببة للمرض، بعد عزلها في المختبر وتشخيصها مجهريا بعد اجراء اختبار صبغة غرام والتي كانت سالبة لصبغة غرام، شكلها عصوية، غير مبسوطة، غير متحركة. وتم تأكيد التشخيص للبكتريا عن طريق تقانة تفاعل البلمرة المتسلسل (PCR) باستخدام اثنين من البادئات الامامي 16S(AGAG TTTG ATCC TGGC

(TCAG، والعكسي (GGCT ACCT TGTT ACGA CTT) 16S. وتضخيم قطعة 1200 زوج قاعدة نايتروجينية من عينات البكتيريا. ومن خلال تحليل BLAST ظهر بأن العينات أخذ أعلى رقم تشخيصي هو (100%) و سجل في GenBank و اخذ رقم الانضمام (ON 131099) *Xyllela fastidiosa subsp. Sandy* و (ON 131100) *Xyllela fastidiosa subsp. Sandy* ، و أيضا يظهر من خلال نتائج النشوء والتطور أن كلا البكتريا المشخصة تقع ضمن تسلسل جيني واحد في بنك الجينات العالمي NCBI GenBank. ويعتبر هذا التشخيص تسجيل لأول مرة في مدينة اربيل.

الكلمات الدالة: *Nerium oleander*، نباتات الزينة، حرق الاوراق، *Xylella fastidiosa*.