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BIOLOGICAL AND MOLECULAR CHARACTERIZATION OF DIFFERENT EGYPTIAN ISOLATES OF *SPIROPLASMA CITRI*

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Abstract

Citrus Stubborn Disease (CSD) is a se serious disease of citrus caused by *Spiroplasma citri*, which is a helical motile, wall-less and cultivable microorganism belongs to class mollicutes. *Spiroplasma citri* was isolated from samples collected from lime trees representing symptoms of CSD from 8 Egyptian lime, lemon and volkamere trees. The presence of *S. citri* was checked by examination of cross sections stained with Dienes' stain as blue stained phloem, indicated the presence of Spiroplasma in infected phloem tissues. Culturing on C3-G specific liquid and solid media was also achieved. The isolated Spiroplasma was identified as *S.citri* by DAS-ELISA using specific polyclonal antibodies. By transmission electron microscopy the identity and morphology of *S.citri* was confirmed. *S.citri* units were wall-less, spiral filamentous and/or branched long helices in liquid cultures and rounded segments forming the Fried-egg shape colonies on solid culture. Examination of ultrathin sections prepared in infected plants revealed the presence of elementary helices inside the phloem sieve elements. Necrotizaton and clumping of the cytoplasm of phloem parenchyma in infected volkamer lemon leaf samples was obvious. Damaging of nucleoli in companion cells was also an effect of *S.citri* infection. A rep-PCR DNA fingerprinting study with BOX-A1R oligonucleotide primer matching REP sequences *S.citri* showed that among 8 *S.citri* isolates, only three isolates (EL Lux, EL1 and EL2) were shown to be in one group. Real-time PCR amplification from 8 *S.citri* liquid cultures showed differential specificity using Php-orf1 primer pair. Total DNA was isolated successfully from five fresh samples and used as a template for conventional PCR using the Spiralin, P58 and P89 primers pairs. The results of BLASTn analysis confirmed that the PCR product of isolate (EL K) represented the Spiralin protein coding gen and the results of the nucleotide sequence were uploaded in the GenBank with accession number "MN599050" as *Spiroplasma citri* EL K isolate. Furthermore, using DNAMAN sequence analysis software, the generated phylogenetic tree provided an indication that the ELK isolate was evolutionarily more related Egyptian isolates; Few (Acc. No. AM157770.1) and Qualubia isolate (Acc. No. AM157771.1) via 99.4%.

Key words: *Spiroplasma citri*, C-3G, DAS-ELISA, Dienes' stain, TEM, rep-PCR, PCR, real-time PCR, Sequence.

Introduction

Spiroplasma is a helical motile, wall-less and cultivable microorganism which belongs to class mollicutes (Yokomi *et al*., 2008). *Spiroplasma citri,* the causal agent of citrus stubborn disease (CSD), has been isolated from almost all species which belongs to the citrus genus (Salehi *et al*., 2005). Also, other non-rutaceous plant species like periwinkle plants (*Catharanthus roseus*) (Nejat *et al*., 2011), carrot (Lee *et al*., 2006) and China aster (Nejat *et al*., 2004).

Citrus stubborn disease was first observed in California 1915 (Fawcett *et al*., 1944) before the nature of Spiroplasma was elucidated, Then the disease was recorded in many countries in North Africa, west of Asia and Florida (Wang *et al*., 2015). CSD was recorded in Egypt by Abou-Zeid *et al*., (1988), Omar (1999), El-Banna *et al*., (2005), Omar *et al*., (2006 and 2008) and Abd El-

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Fatah *et al*., (2016) and by El-Banna (1995) in the western region of Saudi Arabia and by Abou Kubaa *et al*., (2009) in Syria.

Citrus stubborn disease is difficult to be diagnosed specially in early stages of its development as symptoms resemble that of mineral deficiency. But during fruiting the symptoms on fruits are characteristic as small, acorn shape and curved columella (Nour El-Din, 1967; Abou-Zeid *et al*., 1988; El-Banna, 1995; Omar, 1999 and Shi *et al*., 2014).

Spiroplasma citri the causal organism of CSD is a wall-less helical cultivable microorganism belongs to class mollicutes. As it is cultivable isolation on specific media was successful in almost all attempts (Abou-Zeid *et al*., 1988; El-Banna, 1995; Omar, 1999 and Shi *et al*., 2014). Methods of detection and characterization of *S. citri* varies from preliminary methods including the use of Dienes' stain of free hand sections in the phloem of infected samples (Musetti, 2013), isolation on specific media and investigation of the helical motile units by light microscopy. On the other hand, more advanced methods of detection and characterization are applied including electron microscopy of the cultivated *S. citri* by negative staining (Ammar *et al*., 2004).

The concentration of Spiroplasma in woody plants like citrus remains very low and is unevenly distributed within the infected plants (Lee *et al*., 2000). So electron microscopy is used to check the presence of Spiroplasma units in phloem tissues of infected plants and clear the effect of the disease on infected tissues (El-Banna and El-Deeb, 2007; Rocchetta *et al*., 2007 and El-Banna *et al*., 2015), *S.citri* infection result in ultrastructure changes especially in sieve elements, as obliteration and necrosis are observed. The ultrastructure changes also expand to the cell wall, chloroplast and then nucleus.

The use of molecular biology based methods like PCR in which the spiraline and adhesion genes (Yokomi *et al*., 2008 and Alfaro-Fernandez *et al*., 2017) are amplified, represents good method for detection of *S. citri* in liquid cultures and plant tissues as well.

Because of erratic distribution and low titers of *S. citri* DNA, it may be not reliably detectible by using primers to sequences of low-copy genes in infected citrus like Spiralin, adhesion gene and, 16r RNA for finger print detection (Lee *et al*., 2006 and Yokomi *et al*., 2008).

Spiroplasma contain a circular, extra chromosomal DNA that may be present in the form of plasmids and/ or viruses. Spiroplasma viruses were observed initially by electron microscopy of *Spiroplasma citri*. These viruses were classified into three groups on the basis of their morphology and their biochemical and biophysical properties (Cole, 1979); SpV1 (rods), SpV2 (long-tailled polyhedrons) and SpV3 (short-tailed polyhedrons). These viruses are critical for horizontal gene transfer and contribute to the Spiroplasma environmental adaptation (Ku *et al.*, 2013).

To improve sensitivity of PCR detection, phage or prophage sequences of *S. citri* which has the largest genome were detected by real-time PCR (Shi *et al.,* 2014; Wang *et al*., 2015 and Alfaro-Fernandez *et al*., 2017). In previous study of Carle *et al*., (2010), partial chromosomal sequence of *S. citri* GII3-3X was reported and showed that phage-related prophage sequences accounted for 20.5% of the sequenced *S. citri* genome.

Over the last years, the use of sequence analysis of nucleotide fragments and comparison with different Spiroplasma isolates all over the world, have significantly facilitated the task of Spiroplasma' detection (Foissac *et al*., 1996). In 2006, *Spiroplasma citri* has first been identified by a sequence analysis in two governments in Egypt, and has been considered a new member of the genus *Spiroplasma* attached citrus (Omar *et al*., 2006).

The present study focuses on; (1) Biological detection of *S. citri* from lime leaves, flowers and fruits exhibiting typical symptoms of CSD, (2) Improvement of the molecular detection methods of citrus stubborn disease by setting up real-time PCR targeting prophage gene of *S. citri* among different Egyptian lime/ lemon and volkamer lemon isolates, (3) Study the effect of *S. citri* infection on the infected tissues by electron microscopy and (4) sequence analysis of a fragment of spiraline gene of one *S. citri* isolate.

Materials and Methods

Sampling

Samples were collected from different citrus orchards included lime (*Citrus aurantiifolia* (Christm.) Swingle), volkamer lemon (*Citrus volkameriana* Tan & Pasq.) and lemon (*Citrus limon* L. Osbeck) showing typical symptoms of citrus stubborn disease (CSD). Sampling was carried out between July 2018 and March 2019 from eight Egyptian governorates; Ismailia, Damietta, Faiyoum, Giza (Al Mansouria and Kirdasah), Cairo (Maadi and Cairo University, Faculty of Agriculture experimental station), Luxor (Al-Odeisat), Qalyubia (Benha University, Faculty of Agriculture experimental station and Tersa) and Beheira. These symptoms included stunted trees, compressed growth branches, with chlorotic mottle leaves and acorn-shaped fruits with curved columella in different growth stages Fig. 1.

Isolation and culturing

Leaves, flowers and fruits were collected from symptomatic and asymptomatic citrus trees. The candidate samples were surface sterilized by immersion in ethanol 70% and sodium hypochlorite 1% for five min. Then, they were rinsed in sterile distilled water.

Isolation of *S. citri* was achieved by culturing on C-3G medium suggested by Whitcomb (1983) and adapted by EL-Banna (1995 and 1999), EL-Banna *et al*., (2005) and Abd El-Fatah *et al*., (2016) after filtration of the ground tissue samples through 0.45, then 0.22 Millipore filters as 1 ml of the filtrate was added to screw capped tubes containing 5 ml of C-3G media and incubated at 30 ± 2°C.

The cultures were observed for color change from red to yellow indicating Spiroplasma growth. The presence of Spiroplasma (as spiral motile helicses) was checked by examination by phase contrast light microscopy of 5 days old liquid cultures using light microscope (Lica ICC50 HD). The identification of the isolated Spiroplasma was confirmed using ELISA as the ready-made ELISA kit (SANOFI, Sante Animale, Paris, France) was used according to (Saeed *et al*., 1992 and El-Banna *et al*., 2000).

To check the growth of the isolated spiroplasma on the solid medium 0.1 ml of the liquid cultures (5 day old) was dispensed on the surface of C-3G solid medium in9ml diameter Petri dishes. The plates were incubated at 30 ± 2ºC for 7 days and checked for the formation of fried-egg shape colonies as carried out by El-Banna (1999), Sidaros *et al*., (2000) and Mello *et al*., (2008). The different isolates were coded as illustrated in table 3.

Detection and characterization of *Spiroplasma citri*

Detection of Spiroplasma using Dienes' stain

Dienes' stain was used as a preliminary method to detect Spiroplasma in infected plant tissues. Petioles of freshly collected leafy samples were used, as free hand cross sections were prepared and transferred to Dienes' stain for 5 min as described by (Musetti, 2013). The stained sections were later washed in distilled water and examined immediately by light microscope (LEICA ICC50 HD). Photos were captured using the built in microscope camera.

Electron microscopy

Transmission electron microscopy was carried out to detect Spiroplasma units in 5 days old liquid cultures. The cultures were mixed with 2% gluteraldehyde (v/v) and left for 2 hours for fixation of the motility of Spiroplasma and facilitate the examination. Then, the preparation was negatively stained with 2% phosphotungstic acid and mounted on 400 mesh carbon coated grids (Ammar *et al*., 2004 and Rocchetta *et al*., 2007). The grids were examined using transmission Electron microscope JEOL (JEM-1400 TE, Japan) at the candidate magnification. Images were captured using CCD camera model AMT. On the other hand, smears of the fried-egg shaped colonies were examined with the same method but without fixation.

Fig. 1: Naturally infected lime leaves showing typical symptoms of citrus stubborn disease collected from different governorates. Symptoms ranged from yellow mottling (A and B). A malformed lime fruits with curved columella (C and D). Lemon fruits representing malformation and reduction in size (E).

Preparation of plant tissue for examination of electron microscope

The effect of *Spiroplasma citri* on the infected tissues was studied by electron microscopy of ultrathin sections of both volkamere lemon and periwinkle leaf midrib tissues. Pieces of about 2×2 mm of the leaf midribs and small part of the lamina were transferred to a separate vial to be fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for one hour. Then refixed in 1% of Osmium-tetroxide (OsO_4) for 1.5 h at 4^0C. Then the samples were dehydrated in an ethanol series. The subsequent steps were carried out according the method described by EL-Bannna and EL-Deeb (2007) and El-Banna *et al*., (2015). Samples were then sectioned (90-100 nm thick) with the ultra-microtome (Leica model EM-UC6, Japan) mounted on copper grids (400 mish). Sections were double stained with 2% Urany1 acetate and 10% lead citrate. Stained sections were examined by transmission Electron microscope JEOL (JEM-1400 TE, Japan) at the candidate magnification. Images were captured using CCD camera model AMT. This part of the work was carried out in TEM lab, Faculty of Agriculture, Cairo University. Research Park (FARP).

Molecular characterization

DNA Extraction

For molecular characterization ,Spiroplasma, DNA was extracted by two different methods: First, DNA was extracted from infected fresh samples of lime, volkamer lemon and lemon trees exhibiting citrus stubborn diseases symptoms using the modified Dellaporta extraction method (Dehestani and Kazemi Tabar, 2007). Second, DNA was purified from 5 days old liquid cultures using the 'Thermo Scientific GeneJET Genomic DNA Purification Kit #K0721 according the company steps.

Finger print

The whole units suspensions of 5 days old Spiroplasma liquid cultures (EL Lux, EL1, EL2, EL3, EL4, EL K, EL S and EL Be) were used to provide template DNA used for Rep-PCR Using the BOX-A1R oligonucleotide primer (CTA CGG CAA GGC GAC GCT GAC G) according to Martin *et al*., (1992). Each 25 µl PCR reaction mixture contained 0.2 µM primer, 2.5 m*M* each of the 2mM dNTPs, 3.75 mM of the 25 mM $MgCl_2$, 1.25 U of EP-TaqPolymerase in a buffer containing 5% DMSO and 1 µl of extracted DNA.

The finger print conditions were; initial denaturation at 94°C (7 min), followed by 30 cycles of 94°C (1 min), 53°C (1 min), 65°C (8 min) and 65°C (16 min). PCR products were electrophoresed in 1.5% TBE-agarose at 100 V. Bands were compared with a 1 kb plus DNA ladder (Thermo fisher). Gels were stained with ethidium bromide and visualized using a gel documentation system (Bio rad).

Polymerase Chain Reaction (PCR)

The amplification of DNA from five freshly infected samples (EL G, EL K, EL M, EL B and EL S (T-L)) was used for PCR according to the Yokomi *et al*., (2008). The types and structure of the used primers are illustrated in table 1.

PCR was performed in 25µl reaction mixture containing: 3µl of template DNA, 1.5 µM forward primer, 1.5 µM reverse primer, 5 µl Master mix (Ultra-Pure Master mix) and 14 µl H_2O.

The PCR was carried out for 40 cycles on Labnet (MULTIGENE MINI) thermal cycler as following conditions were applied: Denaturation for 3 min at 95ºC, followed by 40 cycles of 95ºC for 10 s. Annealing for 10 s at 58ºC. Primer extension was for 45 s at 72ºC, followed by 5 min of elongation at 72°C.

The PCR products were compared with a 100 bp DNA ladder (BIOMATIK). Gels were stained with ethidium bromide and visualized using a gel documentation system (TR201 UV Trans illuminator, acculab, USA).

Table 1: Primers sequences, target gene used for PCR and expected amplification size.

Primer	Target gene	Primer sequence (5' to 3')	Expected amplification size (bp)	Reference
Spiralin-f	Spiralin	GTCGGAACAACATCAGTGGT		Yokomi *et al*.,
Spiralin-r		TGCTTTTGGTGGTGCTAATG	675	(2008)
P89-f	Putative P89	ATTGACTCAACAAACGGGATA		Yokomi *et al*.,
P89-r	adhesion gene	ACGGCGTTTGTTAATTTTTGGTA	707	(2008)
P58-6f	Putative P58	GCGGACAAATTAAGTAATAAAAG		Yokomi *et al*.,
P58-4r	adhesion-like gene	AGCGCACAGCATTTGCCAACTACA	450	(2008)

Table 2: Sequences, target gene and expected size of primers used for q PCR.

Primer name	Target gene	Primer sequence (5'-3')	Expected amplification size (bp)	Reference
Php-orf1-F	SpV1-	TGGCAGTTTTGTTTAGTCA		Wang *et al*.,
Php-orf1-R	ORF1	TCCGGGTCTAAACGCCGTTAAAGT	190	(2015)

Real-time PCR

The extracted DNA of eight Spiroplasma liquid cultures (EL Lux, EL1, EL2, EL3, EL4, EL K, EL S and EL Be) was used as a template for real-time PCR according to Wang *et al*., (2015) using Php-orf1 primer targeting Spv1-ORF1 detection table 2.

The reaction cocktail consisted of 20 µl of reaction mixture containing: 4 µl of 5 × HOT FIREPol® EvaGreen® qPCR Mix Plus, 0.5 µM each primer and 5 µl of DNA template from *S. citri* liquid cultures. Amplification, detection, and data analysis were performed with a One Step Plus Real-Time PCR System AB Applied Biosystems (California, USA). The thermal profile consisted of one step at 95°C for 15 min, followed by 35 cycles of 95°C for 15 s, 60-65°C for 20 s, and 72°C for 20 s. Data was analyzed using DROMHASHEM program.

Nucleotide sequencing analysis

The PCR product of the Spiralin gen of the Kirdasah (EL K) isolate was purified with Gel/PCR DNA Fragments Extraction kit (Geneaid, Taiwan) according to the manufacturer's instructions. The purified Spiralin gen was sequenced by Sigma company. The sequence analysis of Kirdasah isolate (EL K) was submitted in the Gen Bank.

Phylogenetic analysis

The nucleotide sequencing was assembled and analyzed using DNAMAN sequence analysis software (Lynnon Corporation, Canada) *ver.* 7. Then it was compared with the other nineteen sequences of different *Spiroplasma citri* isolates from different geographical regions deposited in the GenBank.

Results

Symptoms of citrus stubborn disease (CSD)

Samples were collected from different citrus orchards included lime (*Citrus aurantiifolia* (Christm.) Swingle), volkamer lemon (*Citrus volkameriana* Tan & Pasq.) and lemon (*Citrus limon* L. Osbeck) showing typical symptoms of citrus stubborn disease (CSD). Sampling was carried out between July 2018 and March 2019 from eight governorates. *Spiroplasma citri* was isolated from all samples of young leaves, flowers, fruits and seeds of volkamer lemon (*Citrus volkameriana* Tan & Pasq.), lime (*Citrus aurantiifolia* (Christm.) Swingle) and lemon (*Citrus limon* L. Osbeck) showing typical symptoms of disease, except Ismalia isolate (EL I) which gave negative results. The different isolates were coded as illustrated in table 3.

Isolation and culturing of Spiroplasma

The organism was cultured in C-3G medium and incubated at 30±2ºC for 5 days. A change in the color of the inoculated media was observed. The color gradually changed from red to yellow, which indicated the presence

Table 3: Incidence of citrus stubborn disease (CSD) in citrus samples evaluated by Isolation and by DAS-ELISA.

Serial N.	Name	Location	Governorate	Citrus species	Type of sample	Isolation on liquid medium*	Absorbance**
1	EL1	Damietta	Damietta	Lime	Fruit	+	0.288
2	EL2	Faiyoum	Faiyoum	Lime	Fruit	+	0.275
3	EL3	Al Mansouria	Giza	Lime	Fruit	+	0.302
4	EL4	Al Mansouria	Giza	Lime	Fruit	+	0.298
5	ELK	Kirdasah	Giza	Lime	Fruit	+	0.272
6	ELG	Faculty of Agriculture experimental station	Giza	Volkamer lemon	Leaves	+	0.206
7	ELBe-Fl	Beheria	Beheria	Lemon	Flower	+	0.255
	ELBe-F				Fruit	+	0.288
	ELBe-L				Leaves	+	0.284
8	ELLux	Al-Odeisat	Luxor	Lime	Leaves	+	0.318
9	ELM	Maadi	Cairo	Lime	Fruit	+	0.254
10	ELI	Ismalia	Ismalia	Lime	Fruit	-	0.138
11	ELB	Benha University	Qalyubia	Lime	Leaves	+	0.206
12	ELT-F	Tersa	Qalyubia	Volkamer lemon	Fruit	+	0.304
	ELT-L				Leaves	+	0.233
	ELT-S				Seeds	+	0.301

* + = Positive, - = Negative. ** A mean of three reading.
Negative Control: 0.143

of Spiroplasma in the cultured samples.

On the other hand, the presence of Spiroplasma was confirmed by examination of drops of liquid culture by light microscope (LM), helical motile units were detected Fig. 2A. The presence of Spiroplasma was further confirmed by the appearance of typical fried-egg shape colonies on C-3G liquid medium containing 0.8% 7-8 days of incubation 30±2°C. The colonies were very small in size Fig. 2B, appeared as pinheads and clear in color when photographed directly on the surface of the plates. These colonies were circular in form when examined under dark field light microscopy Fig. 2C.

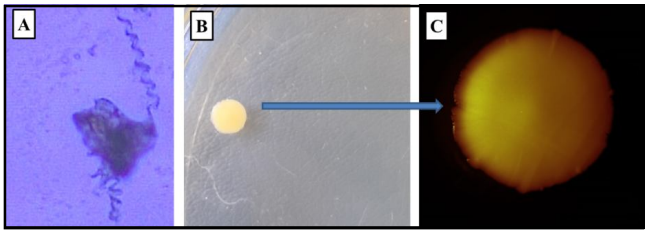

Fig. 2: Morphology of *Spiroplasma citri* in C-3G liquid medium by light microscope; the spiral shape is very obvious in (A) x1000. Fried shape colonies on solid medium 7-8 days of incubation at 30 ± 2°C in (B). The colony shape by dark field light microscope (C) (400 X).

Identification of the isolated Speroplasma

The identification of the isolated Spiroplasma was confirmed by ELISA as all cultures were positive in ELISA except Ismalia isolate (EL I) which gave negative results table 3. *S. citri* presence was checked in leaf midribs of citrus plants naturally infected with citrus stubborn disease. It was detected in different parts of flowers, fruits, leaves and seeds taken from plants showing typical symptoms of citrus stubborn disease. Positive reactions were also obtained when the purified *S. citri* liquid cultures were used as antigens table 3. Generally ELISA was applied to check the presence of *S.citri* in all the subsequent experiments.

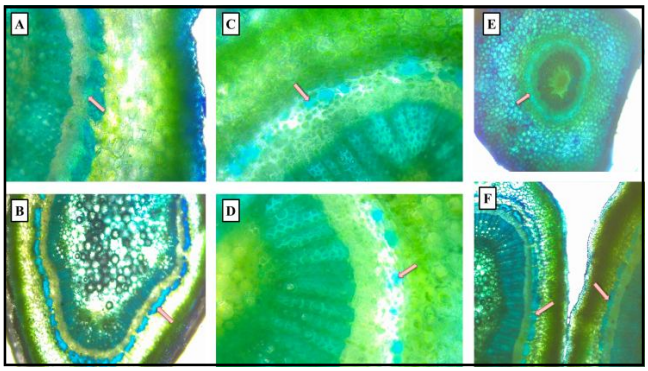

Fig. 3: Free hand sections in petioles of (EL K) isolate (A and B), (EL Lux) (C & D) and volkamer lemon (E & F) leaves immerging from infected seeds stained with Diense' stain (400 X).

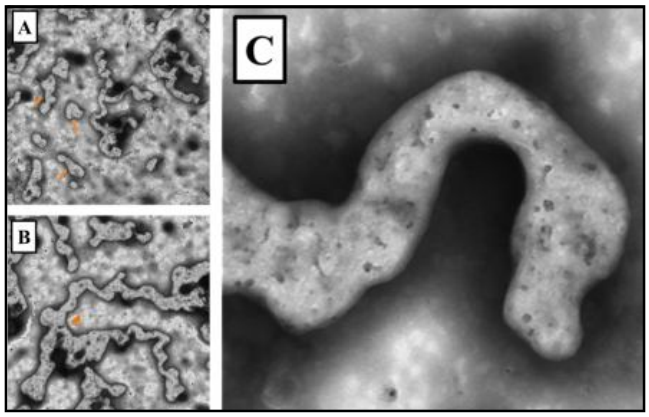

Fig. 4: An electron micrograph representing different phases of *Spiroplasma citri* growth in liquid culture: (A) Elementary helices (arrows) (20000 X), (B) Branched long helices (head arrows) (25000 X), (C) The blunt end of *Spiroplasma citri* unit at high magnifications (100.000 X).

Detection of *Spiroplasma citri* using Dienes' stain

Free hand sections taken from infected volkamer lemon leaves immerging from infected seeds (EL T-S) and leaves of lime trees (EL K and EL Lux), showed blue stained phloem tissues Fig. 3, indicating the presence of Spiroplasma in infected phloem tissues.

Transmission electron microscopy (TEM) of *Spiroplasma citri*

Electron microscopy of Spiroplasma units in dip preparation from liquid culture (5 days old) (EL2) and smears of the fried egg shape colony was carried out after negative staining with 1% phosphotungstic acid (PTA). The examination revealed the presence of Spiroplasma units which were filamentous to helical with

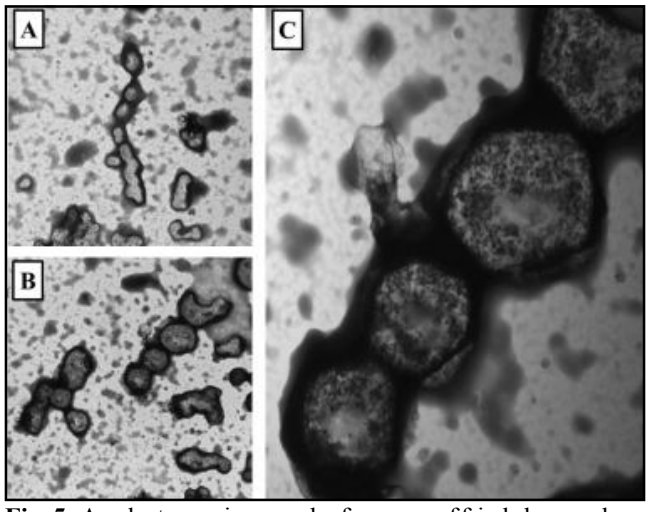

Fig. 5: An electron micrograph of a smear of fried shape colony of *Spiroplasma citri* representing the different steps of fragmentation of the helix into rounded segments forming the Fried-egg shape colonies (A: 15000 X, B: 25000 X and C: 40.000 X).

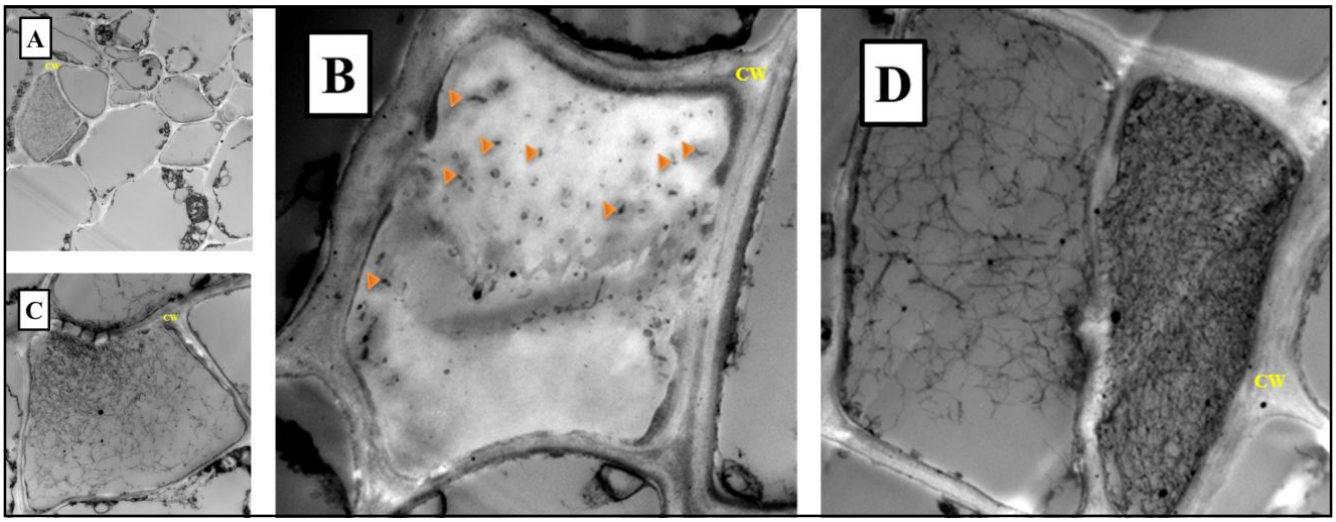

Fig. 6: An electron micrograph of phloem sieve elements in infected Periwinkle leaf. (A) Overview of phloem tissue of infected periwinkle leaf as disorganization is obvious (5000 X). (B) Numerous *Spiroplasma citri* units in elementary form (head arrows) (20.000 X) in infected periwinkle leaf. (C) Sieve elements of healthy phloem tissue (10.000 X). (D) An uneven division of sieve elements of infected perwinkle leaf with *Spiroplasma citri* (15000 X).

elongated or branched segments and elementary helices Fig. 4A & B. The end of the spiral shape helix appeared as blunt at high magnification Fig. 4C. Inspection of smears of the fried egg shape colony showed the steps of forming the colonies by fragmentation of the helix into small rounded unites which aggregate to form the characteristic colony shape Fig. 5.

The effect of *Spiroplasma citri* on infected tissues was studied by electron microscopy of ultrathin sections of both volkamer lemon and Periwinkle phloem tissues in midribs of infected leaves. The investigation revealed the disorganization of the phloem tissue Fig. 6A. The sieve elements contained numerous Spiroplasma units in the elementary form Fig. 6B if compared with those of healthy ones Fig. 6C, which no Spiroplasma units were observed. On the other hand, dilatation of the sieve pores was observed as Spiroplasma units pass through Fig. 7, the cell walls of phloem cells were clearly thick and the cytoplasm was necrotized and vacuolated. The effect of CSD infection was obvious on the components of phloem parenchyma cells, as the nucleus was greatly damaged Fig. 8A and B if compared with that in cells of healthy tissues Fig. 8C. The effect also expanded into the chloroplast in which appeared as miss-shaped, laysed and clumped Fig. 9A and B if compared with that in healthy tissue Fig. 9C. Uneven division of sieve element was also obvious Fig. 6D.

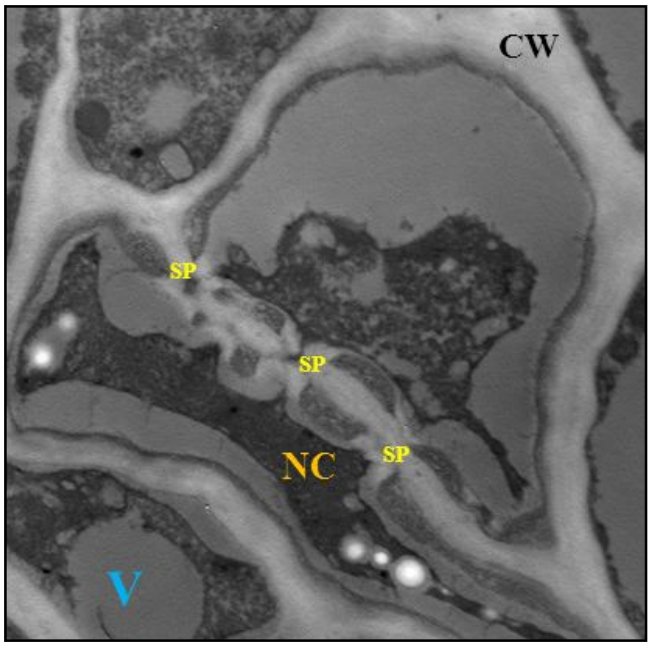

Fig. 7: An electron micrograph of phloem in infected volkamer lemon leaf with dilation of sieve pores (SP) and necrotization (NC) and vacuolation (V) of the cytoplasm (15000 X).

Molecular characterization

Finger print

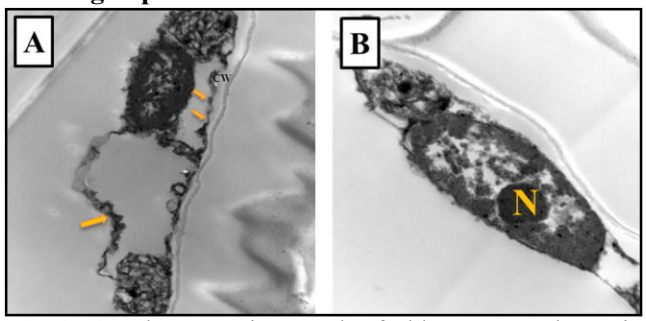

Fig. 8: An electron micrograph of phloem parenchyma in infected Periwinkle leaf. (A) Destroyed chloroplast and invaginations of cell wall (cw), (arrows) are obvious (10000 X). (B) Completely damaged nucleolus (N) (20000X).

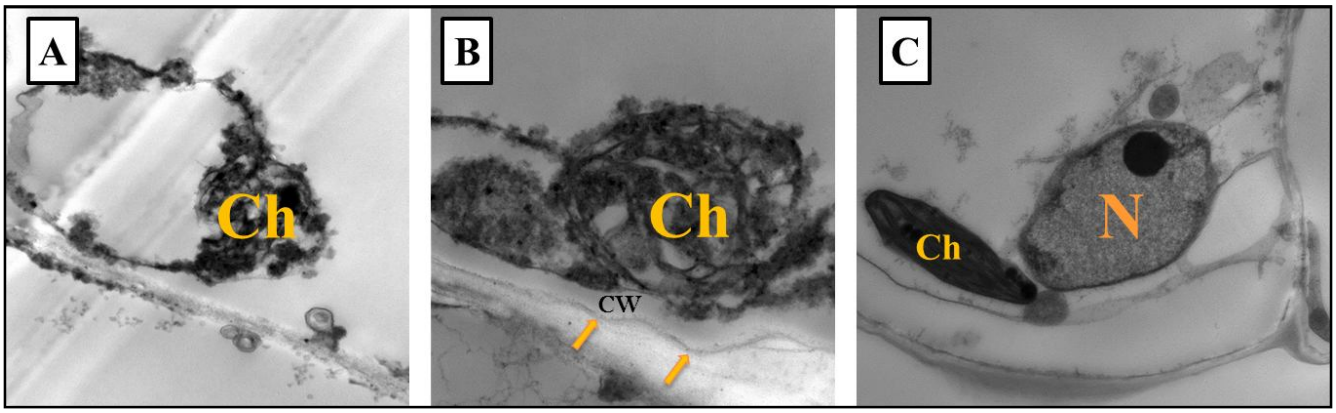

Fig. 9: An electron micrograph of phloem of infected periwinkle leaf with completely lazed chloroplast (Ch) (2000x) (A), miss-shaped, laysed chloroplast and waving (arrows) of cell wall (CW) (30000 X) (B), normal chloroplast and nucleus in healthy tissue (6000 X) (C).

A rep-PCR DNA fingerprinting study with BOX-A1R oligonucleotide primer matching REP sequences in *Spiroplasma citri* revealed 6 different PCR-based DNA fingerprint groups among 8 Egyptian Spiroplasma isolates. Only three Egyptian isolates (EL Lux, EL1 and EL2,) were shown to be in one group according to rep-PCR Fig. 10.

Polymerase Chain Reaction (PCR)

Total DNA was isolated successfully from five fresh samples (EL G, EL K, EL M, EL B and EL S (T-L)) which used as a template for conventional PCR using the P89 Fig. 11A P58 Fig. 11B and Spiralin Fig. 11C, primers pairs.

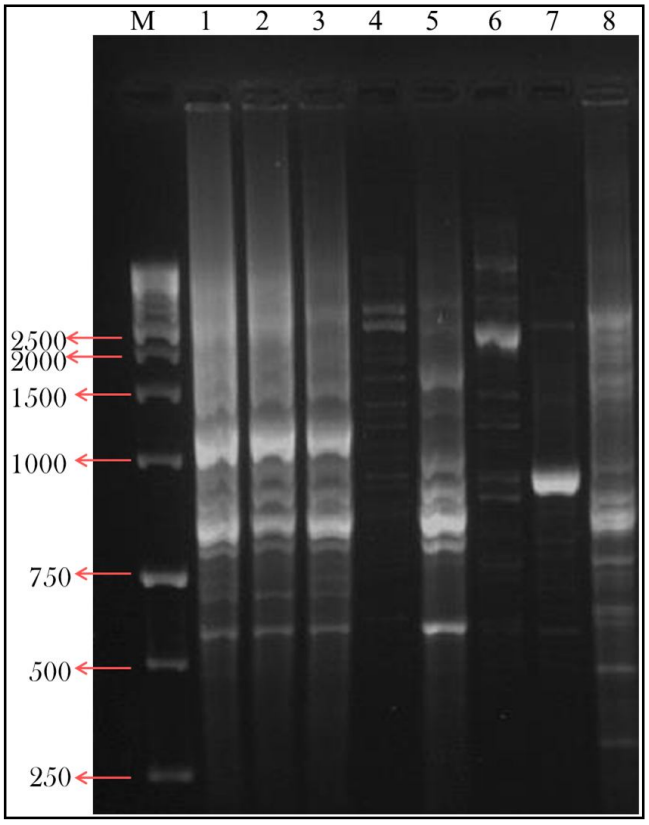

Fig. 10: Finger print products of *Spiroplasma citri* for BOXAIR primer. Lane 1: EL Lux, Lane 2: EL1, Lane 3: EL2, Lane 4: EL3, Lane 5: EL4, Lane 6: EL K, Lane 7: EL S, Lane 8: EL Be and M: DNA molecular weight marker (1 kb).

Real-time PCR

DNA was purified from liquid cultures using DNA Purification Kit and measured using Nano-drop machine. DNA concentration of the different Spiroplasma isolates was measured as illustrated in table 4. It was remarkable that DNA concentration varied from one isolate to another as it was higher in EL lux isolate being 80.2 ng/µl while it was very low in both EL G, EL B and EL M (0.6, 2.2 and 3.2 ng/µl).

The real-time PCR assay was used to detect prophage of *S. citri* were utilizing both Php-orf1-F/ Php-orf1-R primer pairs tested. Data obtained only four *S.citri* Egyptian isolates; EL K, EL Lux, EL 1 and EL T-S had prophage of *S. citri*. All melting curves of *S.citri* cultures showed one peak from DNA analyzed. Also, melting

Table 4: Nucleic acid concentration (ng/il) of *Spiroplasma citri* isolates.

Seri-al N.	Name	Nucleic acid concentr ation(ng/µl)	260 A	280 A	260/ 280	260/ 230
1	EL 1	26.2	0.524	0.284	1.85	10.40
2	EL 2	26.7	0.535	0.303	1.76	8.54
3	EL 3	3.6	0.072	0.042	1.72	-0.46
4	EL 4	23.4	0.469	0.311	1.51	0.82
5	EL K	12.5	0.251	0.178	1.41	0.83
6	EL G	0.6	0.011	-0.004	-3.03	-0.05
7	EL Be	10.8	0.216	0.104	2.09	-1.24
8	EL Lux	80.2	1.604	0.890	1.80	2.25
9	EL M	3.2	0.065	0.016	3.93	0.10
10	EL B	2.2	0.043	0.020	2.21	-0.20
11	EL T-S	12.6	0.251	0.144	1.74	-7.36

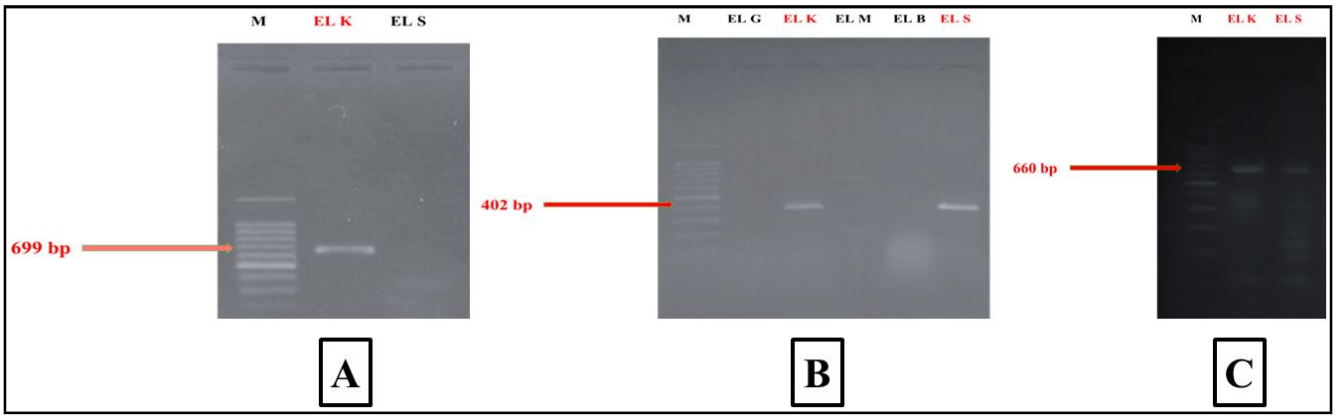

Fig. 11: Agarose gel electrophoresis stained with gel star showing the PCR products for Putative P89 adhesion gen (A), Putative P58 adhesion-like gen (B) and Spiralin gen (C) after amplification**.** M: DNA molecular weight marker (100 bp).

curves of *S. citri* cultures from volkamer lemon leaves immerging from infected seeds (1S) was with one peak. No-template controls were always negative (flat line), indicating that nonspecific reactions occurred. Data obtained showed that among 8 *S. citri*, isolates only four isolates (EL K, EL Lux, EL 1 and EL T-S) had prophage gene in their genome. All melting curves of infected Spiroplasma cultures showed a one peak from DNA analyzed. The other 3 isolates were negative (flat line), indicating that nonspecific reactions occurred.

Nucleotide Sequence

The Purified PCR product of ELK isolate was directly sequenced in both directions and compared with the corresponding sequences of other available Spiroplasma isolates in GenBank, the obtained data were assembled with MEGA sequence analysis software. The nucleotide sequence was submitted to the GenBank with accession number "MN599050" as *Spiroplasma citri* strain ELK.

Multiple sequence alignment and phylogenetic analysis was conducted to compare the Egyptian strain (*Spiroplasma citri* strain ELK) with the corresponding sequences of other Spiroplasma isolates from different geographical regions available in GenBank Fig. 14. Results showed that *Spiroplasma citri* strain ELK was 99.7% and 39.6% similar to *Spiroplasma citri* (Acc. No. JN974242.1, KT834818.1, JN974243.1, KP148817.1 and KP148818.1) and *Spiroplasma* sp. MUF0901 (Acc. No. HM641859.1) from Iran and China respectively table 5.

On the other hand, the highest degree of similarity with spiralin gene of *S. citri* of Egypt isolate was found

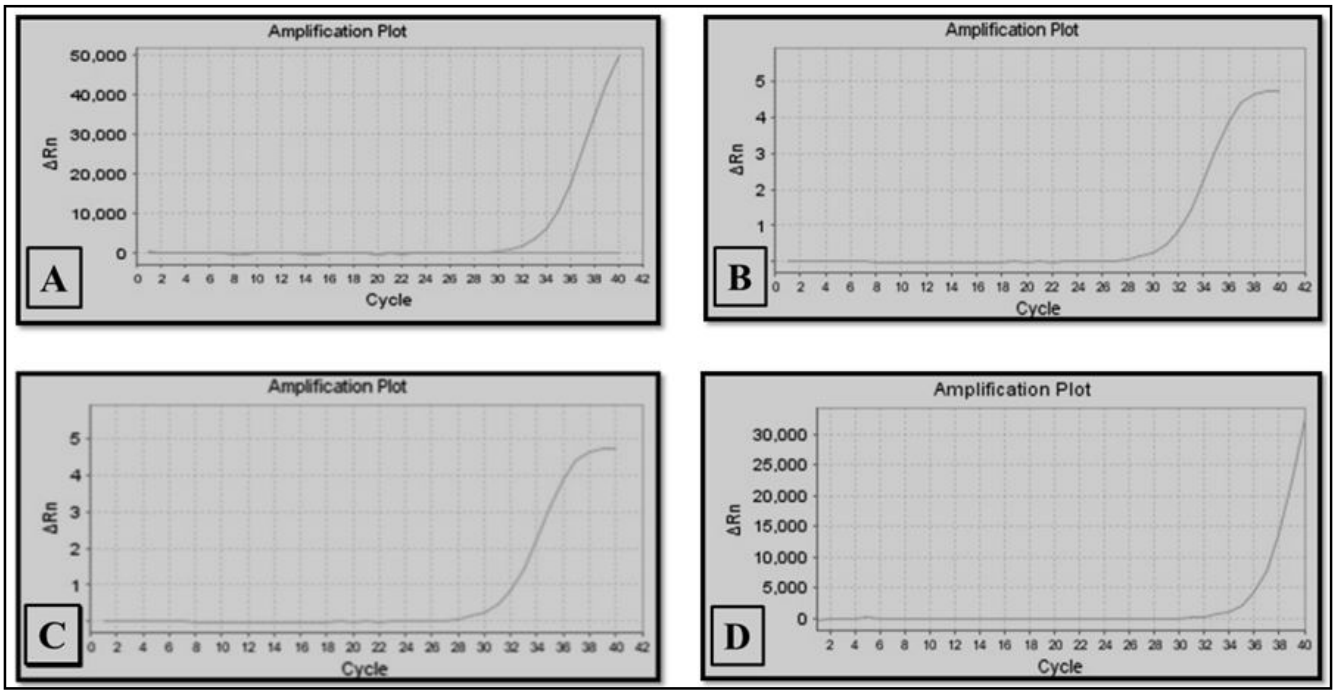

Fig. 12: Real-time polymerase chain reaction (PCR) amplification from *S.citri* cultures (A: ELK, B: EL Lux, C: EL1 and D: EL T-S) showing differential specificity between the primer pair Php-orf-1.

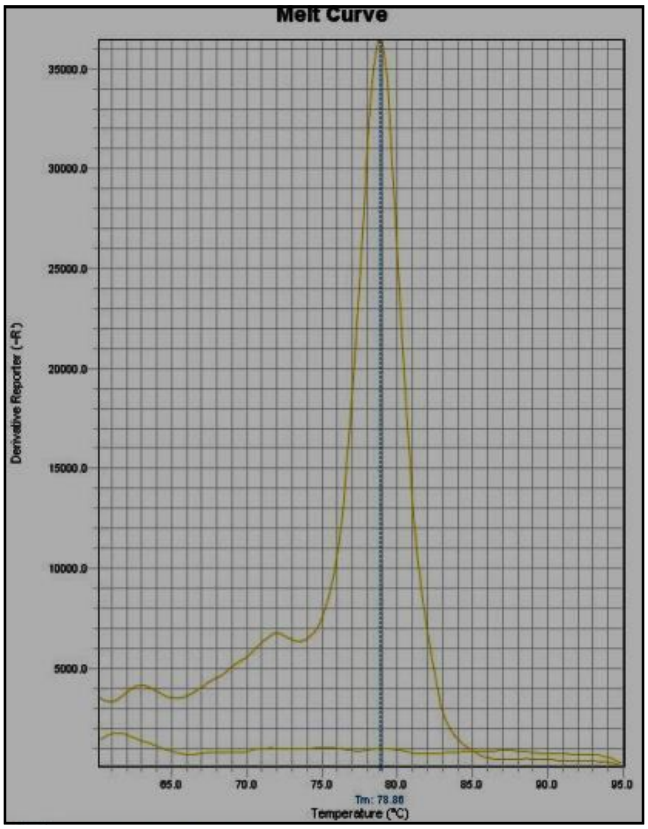

Fig. 13: Real-time polymerase chain reaction (PCR) product analyses. Melting peak obtained using primer Php-orf-1showing a single peak in the infected sample (EL Lux) and the absence of peaks in the healthy and non-template controls.

with Few (Acc. No. AM157770.1) and Qualubia isolate (Acc. No. AM157771.1) from Egypt via 99.4%.

Discussion

In Egypt, *Spiroplasma citri* has been considered as a major threat and devastating for lime/lemon plants either grown as trees in the fields or as seedlings in the greenhouses. In the present study naturally infected lime trees exhibited symptoms of citrus stubborn disease (CSD) as yellow mottling, yellowing and fruit deformation symptoms. Samples including leaves and fruits were collected from eight different Egyptian governorates: Ismailia, Damietta, Faiyoum, Giza, Cairo, Luxor, Qalyubia and Beheira. Lime and lemon fruits showed symptoms characteristic to CSD as they were malformed with curved and sometimes swollen columalla. In some cases, seed abortion and /or seed redness was also remarkable. Such described symptoms are similar to those induced by *Spiroplasma citri* infection on citrus species as have been described worldwide (Nour El-Din, 1967; Abou-Zeid *et al*., 1988; El-Banna, 1995; Omar, 1999 and Shi *et al*., 2014).

Preliminary detection of CSD was carried out by

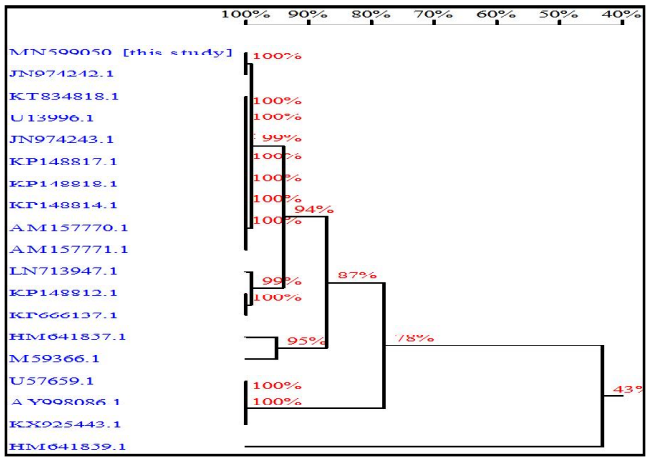

Fig. 14: Phylogenetic tree constructed by MEGA, showing the phylogenetic relationships for the partial *Spiroplasma citri* nucleotide sequences for Egyptian Spiroplasma isolate (*Spiroplasma citri* strain ELK), compared with 19 representatives from other available Spiroplasma isolates in the GenBank.

investigation of free hand sections of petioles of leafy samples. The samples were considered positive when blue color was observed in the phloem tissues. Dienes' stain was used for location of the aggregates of Spiroplasma in the phloem tissues of diseased plants since sieve tubes are nucleate cells (Omar, 1999 and Sidaros *et al*., 2000).

Spiroplasma citri, was isolated from almost all citrus samples by culturing on C3-G liquid and solid media. The presence of Spiroplasma was indicated by color change of liquid medium from red to yellow and by inspection of the helical motile units. On the other hand the formation of fried egg shape colonies on solid medium was also evidence. These observations were in line with those obtained by several workers (Sidaros *et al*., 2000). The change in color was attributed to the increase in acid production resulted during growth (Yokomi *et al*., 2008; Nejat *et al*., 2011 and Shi *et al*., 2014). The morphology of the spiral shape helices and fried egg shape colonies was confirmed by electron microscopy (El-Desouky *et al.,* 2004). The isolated organism was identified as *Spiroplasma citri*, by DAS- ELISA, as the ready for use ELISA kit was used for identification and detection in all steps of Spiroplasma culturing. EL-Banna *et al*., (2000) used ELISA for detection and identification of *S.citri* infecting strawberry for the first time in Egypt. Wang *et al*., (2009) used ELISA for detection and identification of commercially exploited crustaceans from China. They compared the sensitivity of the test against Spiroplasma isolated from plants and honeybee. It was indicated that it was simple and applicable assay for diagnosis of Spiroplasma.In the present work the obtained

data showed that the high concentration of *S. citri* was detected in fruits and seeds than in flowers and leaves. Lime fruits contained high concentration of *S. citri* than lemon fruits. This result is resembling those obtained by Nejat *et al*., (2007).

Infected Spiroplasma isolates stained with Dienes'stain, showed that trakawaz blue color of the phloem tissue indicates the presence of Spiroplasma compared with sections of healthy tissues. This result might be due to the high levels of *Spiroplasma citri* in the phloem cells, which reflect a defect of these cells in leaves for the support of the replication or the movement of Spiroplasma. Similarly, same results were obtained by Musetti (2013) and Abd El-Fatah *et al*., (2016).

The effect of on infected tissues was studied by electron microscopy of ultra-thin sections prepared in leaf tissues. The most remarkable ultrastructural changes were observed in the phloem area as the mollicutes including Spiroplasma are restricted. Spiroplasma units were observed in the sieve tubes in the form of elementary helices. This finding was also suggested by Igwegbe *et al.,* (1970), who mentioned that it is tempting to speculate that mass production of these small forms and their subsequent dissemination within the plant may be of enormous adaptive value to this phloem-limited organism. However, it is tempting to speculate that mass production of these small forms and their subsequent dissemination within the plant may be of enormous adaptive value to this phloem-limited organism. The sieve elements showed uneven division and the cytoplasm of phloem parenchyma cells was clumped, necrotized and vacuolated. On the other hand, the chloroplasts and the nucleus of the same cells were dramatically affected. The layses and degradation of the chloroplasts reflects the yellowing and chlorosis of the infected citrus leaves.

Molecular biology based methods were applied in the present investigation to confirm the identification and for detection of *S.citri* and identification. Finger print, which amplifies DNA sequences between repetitive sequences on the bacterial chromosome, was demonstrated as a useful tool for assess genetic diversity between Egyptian *S.citri* isolates obtained from different governorates. Data obtained revealed the presence of band patterns among *S. citri* isolates, which suggested that the repetitive elements varied in number and distribution in different location in the Spiroplasma genomes. This indicates that there are 8 different Egyptian isolates generated by repetitive element PCR (rep-PCR). These results were similar to that reported by Mello *et al*., (2008), who mentioned that rep-PCR has been successfully used for typing and differentiation in assessing genetic diversity of *S. citri* using the common rep primers ERIC and BOX.

Polymerase chain reaction (PCR) was used for the detection of *S. citri* by implication of certain segments of the Spiroplasma genome. Three different primer pairs : P89-f and P89-r corresponding for both ends of the *S. citri* P89 gene (Putative P89 adhesion gene), Spiralin-f/ Spiralin-r detecting for Spiralin gene and P58-6f/ P58-4r used to detect P58 gene (Putative P58 adhesion-like gene). Only EL K gave positive result with P89-f/ P89-r. This result might be due to the high levels of *S. citri* in the phloem cells. Yokomi *et al*., (2008) and Alfaro-Fernandez *et al*., (2017) used the same primer for detection and identification of several isolates of Spiroplasma. Detection of DNA common region of the Spiralin gene was also achieved, as a PCR product of size 675 bp in the DNA extracted from five freshly infected samples (EL G, EL K, EL M, EL B and EL S (T-L)) was amplified from DNA extracted from isolates (EL K and EL S) leaves gave positive results. The high levels of Spiroplasma in EL S indicates the presence of Spiroplasma in lemon seedlings leaves immerging from seeds collected from infected fruits, leaves from infected seeds which support of the replication movement of *S. citri* in seeds.

As *S.citri* is typically low in titer and unevenly distributed in citrus making reliable detection is challenging. Genome sequence of *S.citri* revealed several copies of prophage genes to improve the sensitivity of PCR detection of Spiroplasma gens, real-time PCR assay was used to detect prophage of *S. citri* utilizing Php-orf1-F/ Php-orf1-R primer pair. Melting curve analysis was done to verify amplicon specificity after 35 cycles. Data obtained showed that among 8 *S. citri* isolates, only four isolates (EL K, EL Lux, EL 1 and EL T-S) had prophage gene in their genome. All melting curves of infected *S.citri* cultures showed a one peak from DNA analyzed. The other 3 isolates were negative (flat line), indicating that nonspecific reactions occurred. This report is result was obtained for the first time in Egypt as none of studies on *S.citri* dealt with this point of view which was studied by Wang *et al*., (2015).

The nucleotide sequence of amplified product of Spiralin gene was compared with 18 other strains of *Spiroplasma* sp., such as the strain E 275 and strain asp1 (Foissac *et al*., 1996). The similarity value between sequences of EL K was 99.7% with Iranian isolates. The ELK isolate also has 99.4% similarity to the Fewa isolate and Qualubia isolate (Omar *et al*., 2006), and 77.3% to the Brazil and Cuba. The relatively lower value of similarity for China 39.6% to the ELK isolate.

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