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Isolation and Identification of *Spiroplasma citri* Associated with Citrus Stubborn Disease in Egypt.

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Abstract

Stubborn disease of citrus is one of the main causes of quality deterioration of citrus fruits in Egypt. The early detection and the molecular characterization of the causal agent (*Spiroplasma citri)* are vital for revealing its real distribution and for management. Citrus included sweet orange (*C. sinensis*) and mandarin (*C.reticulate Planco*) showing typical symptoms of stubborn disease were detected from different fields located at El-Qualubia, Ismailia and Kafr El-Sheikh governorates in Egypt. The detected *S. citri* from diseased samples were cultured in the artificial C-3G liquid medium and the color gradually changed from red to yellow, an indication of the presence of *Spiroplasma* in the cultured samples. The *Spiroplasma citri*, when growing on low-agar medium, forms a fried-egg , fuzzy colonies with occasional surrounding satellite colonies due to the ability of the *Spiroplasma* cells to move through the agar matrix. DNA extracted from symptomatic samples were used as a template for amplification of products of 675 bp using primer pair Spiralin- f / Spiralin- r by PCR. Furthermore The spiralin gene was cloned, sequenced and the obtained isolate was characterized molecularly by sequence analysis showing close relationship with Qualubia isolate (AM157771), Fewa isolate from Egypt (AM157770), SPF1 isolate from Iran (KT834818) and (U13996) isolate from France.

Keywords: *Spiroplasma citri*, Stubborn Disease, C-3G media, PCR, spiralin gene, sequence analysis.

1. Introduction

Citrus considered the main fruit crop in Egypt, which represents about 15% of the total citrus production in the Mediterranean Basin and is considered the ninth largest citrus producer in the world **(FAO, 2013)**. Citrus is belonging to the genus citrus L., Family: Rutaceae, Sub family: Aurantioideae. Citrus trees are subjected to invasion by several bacterial, fungal, virus and virus –like diseases. In recent years, trees of citrus have been seriously affected by stubborn disease caused by a prokaryotic pathogen *Spiroplasma citri* **(Gumpf *et al*., 1981)**.

Spiroplasma citri are a Pleomorphic, phloem-limited, cell-wall-less bacterium in class Mollicutes, which multiplies and moves slowly through the tree, requirement of cholesterol for growth, absolute resistance to penicillin, low guanine and cytosine content of cellular deoxyribonucleic acid (DNA), small genome size, and several cultural properties **(Raju *et al*., 1981)**. *S. citri* cannot be transmitted mechanically but transmitted in a circulative-propagative manner by several phloem-feeding homopteran insects including the leafhoppers *Circulifer tenellus* in citrus-growing areas of California and Arizona and *C.haematoceps* (syn. *Neoaliturus haematoceps*) in the Mediterranean region **(Calavan and Bové, 1989)**. *S. citri* are also transmitted by grafting **(Gaurivaud *et al*., 2000a)** and

dodder **(Lee *et al.*, 2000)**. *S. citri* can be reliably detected by culturing in cell-free liquid medium and observing the organism by dark field microscopy to confirm its typical helical morphology and motility **(Yokomi *et al.*, 2008)**.Among the major constraints, Stubborn Disease of citrus (CSD) is a very serious disease in most citrus growing regions and dramatically decreases citrus yields **(Bove, 1986).** Severely affected trees are stunted and have short leaf internodes, leaf mottling, unseasonal blossoms, and lopsided fruits **(Shi *et al.*, 2014)**. Field diagnosis of CSD, however, is difficult and often inaccurate as symptoms can be confused with those of other citrus pathogens or nutritional problems **(Polek *et al.*, 2007)**. In addition, detection in field samples is erratic due to low titer and uneven distribution of the pathogen. Since *S. citri* grows well at warm temperatures, stubborn diagnosis may be most reliable in the summer months. Further genetic studies have led to the identification of Spiroplasmal genes associated with biological functions such as motility, insect transmission, and pathogenicity **(Bové *et al.*, 2003)**. *Spiroplasma citri* detection methods were based previously on somewhat tedious procedure of culturing the mollicute in liquid media and confirmation of the presence of the *Spiroplasma* cells by dark-field microscopy **(Tully 1983)**. Polymerase Chain Reaction (PCR) is useful method for *Spiroplasma* detection in infected plant phloem or insect vectors with 100-1000 times of sensitivity greater techniques and culturing **(Fletcher *et al.*, 2006)**. PCR detection of S. citri has been used with primers based on gene sequence for spiralin**(Foissac *et al.*,1996)**, 16 S rRNA gene in particular *Spiroplasma* infection **(Lee *et al.*, 2006)**, Putative P89 adhesin and Putative P58 adhesion-like genes **(Yokomi *et al.*, 2008)**.

The aim of the present investigation is to detect *Spiroplasma citri* associated with disorders observed on citrus trees in different locations in Egypt, isolate and cultivate it on C-3G modified media, identify by PCR, sequence and analysis the spirallin gene of Egyptian isolate.

2. Materials and Methods

Source of samples

Columella, young shoot and leaves were collected from seedy citrus trees [sweet orange (*C. Sinensis*), mandarin *(C. reliculate Planco*)] showing typical symptoms of citrus stubborn disease were collected from different fields located at El-Qualubia, Ismailia and Kafr El-Sheikh governorates in Egypt. These symptoms include trees compressed and stunted, branches show compressed growth, with smaller leaves similar to those in the young budded nursery tree, leaves show a chlorotic mottle and the fruit does not colour properly, small (be acorn-shaped) and the stylar end retains its green color.

Culturing and Isolation *Spiroplasma citri* on C-3G Medium

Columella, shoots and leaves were excised, surface disinfested and then diced with a sterile razor blade in 5ml of C-3G medium and leave five min **(Bove *et al.*, 1983)**, then it passed through a 0.45µm filter membrane. A volume of 1ml of filtered medium was then transferred into culture tubes containing 10 ml of C-3G medium each. Tubes were incubated at 30°C - 32°C without shaking. The growth of *S. citri* in C-3G medium is not inducing turbidity but the *Spiroplasma* growth should be followed by the turning of phenol red pH indicator colour; the cultures were examined approximately after 5 to 14 days of incubation. When color change appeared, 0.1 ml was taken from the culture and dispensed on the surface of solid medium in Petri dishes. The plates were incubated at 32 °C and checked for the formation of fried- egg shape colonies.

Molecular biology studies

Molecular biology studies were carried out to detect the suspected *Spiroplasma citri*

DNA Extraction and PCR Amplification

DNA was extracted from fresh samples of orange, mandarin trees exhibiting stubborn symptoms using the modified Dellaporta extraction method **(Dellaporta *et al.*, 1983)**.

The extracted DNA was used as a template for PCR. The primer pair Spiralin- f / Spiralin- r illustrated in Table (1) **(Yokomi *et al.*, 2008)** was used in the amplification of 675bp product of spiralin gene. The amplified DNA was electrophoresed in 1% agarose gel in 1XTBE buffer at 120V for 1 hour, stained with ethidium bromide (0.5µl/ml) and photographed using gel-documentation system (Bio-Rad, GelDoc XR). VC 100bp Plus DNA ladder (Vivantis) was used as PCR Markers.

Table 1. Sequence, Target gene and size for primer used for DNA amplification.

Primer	Target gene	Primer sequence (5' to 3')	Expected amplicon size (bp)
Spiralin- f	spiralin	GTCGGAACAACATCAGTGGT	675
Spiralin- r	spiralin	TGCTTTTGGTGGTGCTAATG	

Cloning, Sequence and analysis of spiralin gene:

PCR amplified fragments of spiralin gene were cloned using the TOPO TA Cloning® Kit (Invitrogen) according to manufacturer instruction manual. Isolation of recombinant plasmid was done using mini-preparation protocol **(Sambrook *et al.*, 1989)**. The isolated recombinant plasmid was sequenced by Macrogen Inc., Korea. Nucleotide sequences were analyzed using blast NCBI database and DNAMAN software.

3. Results

Diseases symptoms

Stubborn infected citrus tree showed of small chorortic rectangular leaves with reduced blade. The infected trees appeared stunted and compressed with multiple sprouting (Fig. 1B), when compare to the healthy tree **(Fig.1A).** Shoots included small, upright leaves with chlorotic mottle resembling nutritional deficiencies **(Fig.1C)**.

Fig. (1): The characteristically appearance of stubborn infected tree and healthy one, 1(A) Normal healthy tree; 1(B) Stunted and compressed tree; 1(C) typical symptoms of *S. citri* UP right leaves.

Fruits collected from healthy, and infected trees showed various symptoms as observed in **(Fig.2)**. Healthy fruit with normal colour and size **(Fig. 2A)**; symptoms are abnormal small size and color, lopsided, with immature acorn-shaped **(Fig.2B)**; Fruit were rough touch and wrinkle **(Fig. 2C)** and stylar-end breakdown, albedo layer was thick in the inner part of the orange fruit **(Fig. 2D)**. Severely-affected fruit can be insipid or bitter flavored. Seeds produced are often aborted. Infected plants appeared bushy because of shortened internodes, small leaves and unseasonal flowering.

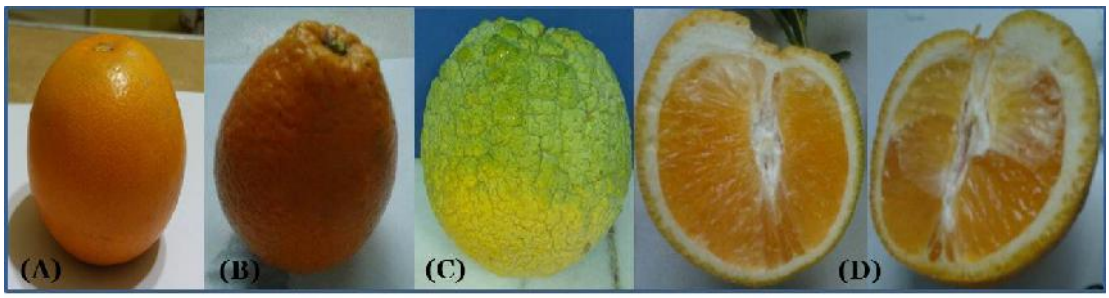

Fig. (2): Fruit symptoms of healthy and infected plant, 2(A)Normally developed fruit from healthy plant; 2(B) lopsided, with immature acorn-shaped infected fruit; 2(C) rough touch and wrinkle infected fruit and 2(D) lopsided half fruit with thick albedo layer and stylar-end breakdown.

Culturing and Isolation of *Spiroplasma citri* on C-3G Medium

The causal agent of stubborn disease was isolated from young leaves of common citrus showing typical symptoms of citrus stubborn disease. The organism was cultured in the C-3G medium and incubated at 32°C. Changing in the color of the inoculated liquid media was observed. The colors gradually changed from red to yellow indicate to the presence of *Spiroplasma* in the cultured samples **(Fig 3A)**. Changing in the color was obtained when the inoculation was done on solid C- 3G media containing 0.8% agar **(Fig. 3B)**. Typically fried-egg shape colonies was developed **(Fig.3C)** and examined under x200 light microscope. The development of the typically fried-egg colonies confirmed the presence of *S. citri*, the isolated pathogen from the stubborn disease infected plants.

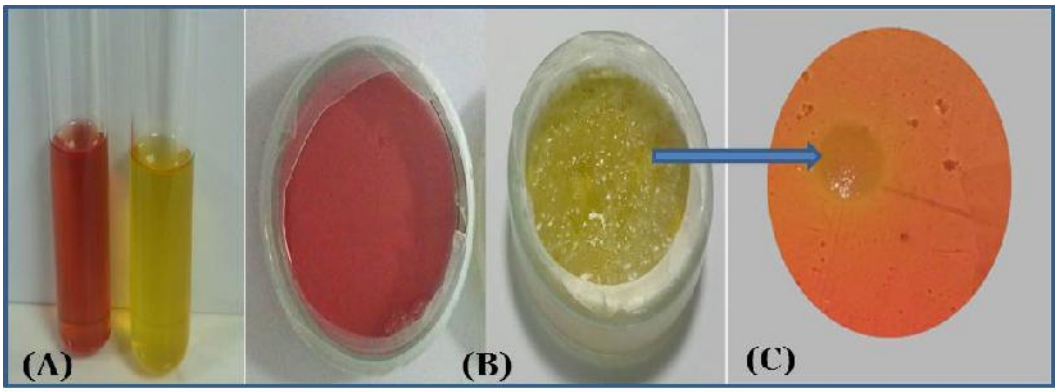

Fig. (3): Changing in the colour of liquid and solid media due to the *S. citri* infection, 3(A) Change in the color of the culture liquid media from red to yellow indicated the presence of *S. citri* growth in the media; 3(B) Change in the color of the culture solid media from red to yellow;3(C) Typically Fried – egg shape colonies of *S. citri* on solid C-3G media (x 200).

Molecular biology studies

The polymerase chain reaction was used as a detection method to detect the naturally infected citrus plants with *S. citri* collected from different Governorates in Egypt. The extracted DNA was amplified by PCR with spirallin specific primer as described in **(Table 1)**. The amplicons of the expected size of 675bp were produced using Spiralin primers **(Fig. 4)**. No visible bands were detected from the corresponding healthy samples. PCR amplified products obtained from healthy plants were used as a controls.

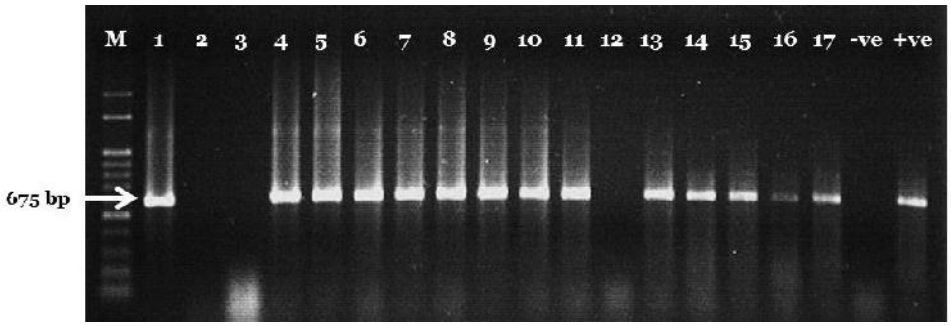

Fig (4): Agrose gel electrophoresis analysis of PCR amplified citrus samples showing stubborn symptoms using primer pair (Spiralin- f / Spiralin- r) to amplify the adhesion gene at 675 bp fragment. M= VC100 pbplus DNA marker, Lanes (1-17) = various symptomatic citrus samples, Lane 18 = negative control (healthy citrus sample), Lane 19= positive control (infected citrus sample).

Spiralin gene Sequence analysis:

Nucleotide sequencing of the PCR amplified product for spiralin gene was cloned, sequenced to determine if this PCR fragments was from the *Spiroplasma citri* gene or not and to compare the sequences from this isolate with those of other sequences of spiralin gene of *Spiroplasma citri* available in Gene Bank. The predicted spiralin gene is 675 nucleotides in size, starting from ATG start codon (methionine), as obtained by comparison with other spiralin sequences and ending with a TGA stop codon from which the 3`NCR (non-coding region) ends. Multiple sequence alignment of the nucleotide sequence of spiralin gene of *Spiroplasma citri* [Egypt] with the corresponding sequence of 16 different *Spiroplasma citri* isolates available in GeneBank were analyzed using DNAMAN software. Phylogenetic tree was constructed based on alignment and displayed as rectangle tree **(Fig. 5)**. A comparative analysis of the spiralin gene sequences of 16 isolates of *S. citri* available in Gene Bank revealed that spiralin gene (Egypt) is closely related to *Spiroplasma citri* with percentage of similarity reach to 100%.The highest degree of similarity with spiralin gene of *S. citri* of Egypt isolate was found with Qualubia isolate (AM157771) from Egypt. The sequence result also observed the high similarity between spiralin gene of *S. citri* of Egypt isolate and the Fewa isolate from Egypt (AM157770), SPF1 isolate from Iran (KT834818) and israel/asp1 isolate from France (U13996) in addition to the isolates described in Table (2). The previous results from the sequence analysis observed that the isolate under study is belonging to *Spiroplasma citri* (Egypt strain).

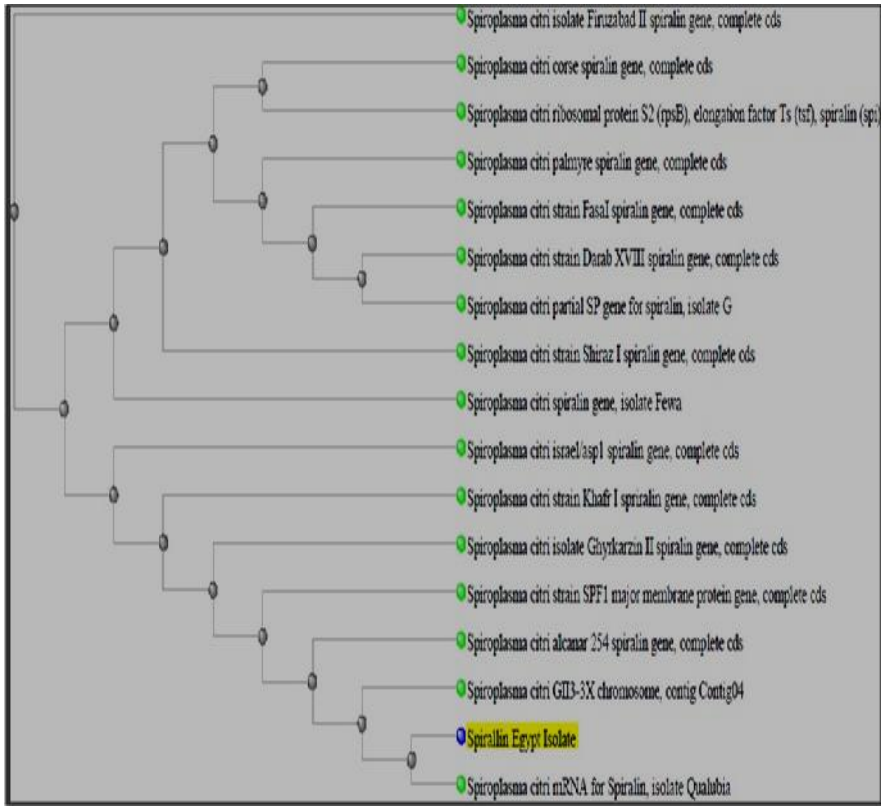

Fig (5): Dendrogram showing phylogenetic tree obtained from the alignment of nucleotide sequence analysis of spiralin gene of Egyptian isolate and 16 sequence for spiralin gene collected for Gene bank.

Table (2): Spiralin gene sequence accession number for *S. citri* isolates collected from Gene bank, used for nucleotide sequence comparison.

Accession number	Isolate	Host	References	Country	Identity
AM157771	Qualubia	*Citrus sinensis*	Omar, Unpublished data	Egypt	100%
AM157770	Fewa	*Citrus sinensis*	Omar, Unpublished data	Egypt	99%
KT834818	SPF1	Citrus	Fallah *et al*., Unpublished data	Iran	99%
KP148816	Ghyrkarzin II	Periwinkle	Zarei *et al*., Unpublished data	Iran	99%
JN974243	Khafr I	Safflower	Khanchezar *et al.*,Unpublished data	Iran	99%
U13996	israel/asp1	*Citrus sinensis*	Foissac*et al*., (1996)	France	99%
U13994	alcanar 254	*Citrus sinensis*	Foissac *et al*., (1996)	France	99%
AM285305	GII3-3X	*Circuliferhaematoceps*	Carle *et al*., (2010)	France	99%
JN860712	Shiraz I	sweet orange	Khanchezar *et al*., (2014)	Iran	98%
U13995	corse	*Circuliferhaematoceps*	Foissac*et al*., (1996)	France	98%
AF012877	R8A2	Citrus	Le Dantec*et al*., (1998)	France	98%
KP148818	Firuzabad II	periwinkle	Zarei *et al.*,Unpublished data	Iran	96%
U13997	palmyre	*Circuliferhaematoceps*	Foissac *et al*., (1996)	France	96%
FJ755921	FasaI	*Circuliferhaematoceps*	Khanchezar *et al.*,Unpublished data	Iran	94%
KP666139	Darab XVIII	Semsem	Khanchezar *et al*., (2014)	Iran	94%
LN713947	G	Citrus columella	Drais *et al*., Unpublished data	Algeria	94%

4. Discussion

In the present study, *Spiroplasma citri* was detected and characterized from naturally infected citrus grown in different locations in Ismailia, Al-Qalyubia and Kafr El- Shakh governorates in Egypt. The collected samples exhibited typical symptoms of stubborn disease which are characteristic to *Spiroplasma citri*. The symptoms of collected samples showed of small, upright with chlorotic mottle near the margins. The trees appeared stunted, compressed with multiply axillary buds and off seasonal blooming compared to the healthy. Fewer, smaller fruits were produced with malformed and lopsided shape. Fruits were paler in color and dropped in mature. These symptoms are typical to those described by **(Om-Hashem, 1995 and Shi *et al*., 2014).**

Symptoms related to tree size and fruit yield are likely related to the fact that *S. citri*, a phloem resident, requires carbohydrates and sterols from its plant host **(Andre *et al*., 2005; Chang, 1989)**. While living in plants, *Spiroplasma* competes with their hosts for these energy sources, causing depletion of some sugars and hormones and accumulation of others.

The resulting imbalance affects the normal metabolism of the citrus plant, causing stunting, leaf mottling, production of smaller and fewer fruit and off-season blooming **(Gaurivaud *et al*., 2000a)**.

The causal agent was isolated from young leaves of suspected plants. Results of various tests conducted led to the identification and cultivation of the causal organism, *Spiroplasma citri* (Mollicutes, Spiroplasmateaceae) for example, change the color of cultivated medium from red to yellow indicated the presence of *Spiroplasma* in the cultivated medium. The change in color was attributed to the increase in acid production resulted from the culture growth. Reduction in the media pH caused the pH indicator, the phenol red to change to yellow. Similar results were previously demonstrated when *Spiroplasma* were cultured similar medium **(Lee and Davis, 1984, and Omar; 1999).**

This presence of *S. citr*i was further confirmed by the appearance of typical fried-egg shape colonies on C-3G medium containing 0.8%. Nobel agar inoculated with the isolates. The colonies were very small in size, appeared as pinheads and clear in color. These colonies were circular in form with a dark center surrounded by a lighter color, when examined under light microscopy. The observations were in line with those obtained by several workers (**Saglio *et al*., 1973, and Sidaros, 2000**). The fried egg colonies developed because the helical mollicute, *S. citri* when growing on low-agar medium, would form fuzzy colonies with occasional surrounding satellite colonies due to the ability of the *Spiroplasmal* cells to move and penetrate through the agar matrix **(Jacob *et al.*, 1997)**.

For molecular detection and characterization of *Spiroplasma* in citrus plants, the primer pair Spiralin- f / Spiralin- r which gave products of the expected molecular size 675 bp were used. The same primer was used by many investigators for the same purpose **(Foissac *et al*., 1996; Mannaa *et al*., 2013)**. Previously, (**Duret *et al*., 2003)** reported that spiralin,is the major protein of *S. citri* membrane. (**Bove´ *et al*., 1993)**, reported that spiralin is not essential for pathogenicity of *S. citri* but showed that this gene is needed for efficient transmissibility by the insect vectors. Indeed, spiralin is acting as a lectin that is able to bind insect vector glycoproteins **(Killiny *et al.*, 2005).**

Spiralin gene for *S. citri* of Egyptian isolate was sequenced and analysed. Almost similar gene was reported with the 16 other strains of s. citri, such as the R8 A2HP, C189, RBA2B Corse. Israel, Asp1, Alcanar and 78 **(Saillard *et al*., 1993)**.The similarity value between sequences of spiralin gene for *S. citri* of Egyptian isolate was 100% with Qualubia isolate. The isolate also has more than 99.59% similarity to the sequences of the Fewa isolate and the Israel isolate, and more than 96 % to the Palmyreisolate. The relatively lower value of similarity for FasaI strain (94%) as compared to the other strains 99% was not fully understood.

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