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RESEARCH

Biological and Molecular Characterizations for Identification of a Phytoplasma Associated With Lemon Witches'-Broom in Egypt

Rasha M. Mahrous[1,4], Ahmed K. El-Attar[2], Ahlam A. AlWatban[1], Sohair I. EL-Afifi[3], Nagwa M. Aref[1,3]

ABSTRACT

Background: Phytoplasma Associated with Witches' broom disease (WB) is a very serious disease of citrus with a major limiting factor for lime production. Symptoms of Witches' broom were observed on limes in AL-Qalyubia governorate, Egypt. This study identified and classified the isolated WB phytoplasma.

Method: Phytoplasma was isolated from lime trees exhibited Witches' broom Symptoms in AL-Qalyubia governorate, Egypt by grafting on indicator (*Vollka marina*) plants. The Phytoplasma was transmitted from naturally infected Lemon (*Citrus limon*) to healthy periwinkle (*Catharanthus roseus*) by dodder (*Cuscuta reflexa Roxb*) and Lemon (Vollka marina) plants by eye bud grafting. Cytopathological detection referred to Diene's stain was used to differentiate the phloem tissues of leaf petiole sections from infected trees. The phytoplasma was detected in the sieve tubes and parenchyma cells of leaf midribs by Transmission Electron Microscopy (TEM). Phytoplasma was molecularly detected in symptomatic samples using the specific primers of their 16S-23S rRNA gene by PCR.

Results: The Phytoplasma was isolated on indicator (*Vollka marina)* plants. It was transmitted on Lemon (*Citrus limon*) by grafting and on periwinkle by dodder. It was detected in the sieve tubes and parenchyma cells of leaf midribs tissues using Diene's stain and TEM. Infection of Lemon trees with Phytoplasma associated witches' Broom was detected by PCR amplification of the intergenic spacer region (SR) using a witches' broom-specific PCR primers (SR1 and SR2). Molecular characterization of the isolated Phytoplasma was performed through the successful cloning and sequencing of the PCR fragment that amplified from 16S rRNA gene. The nucleotide sequences were published to the GenBank as Lemon Witches' broom-Egyptian strain (LWB-Eg strain) with accession number "KJ948653". Phylogenetic analysis showed a high similarity (99%) with Oman isolate.

Conclusion: Phytoplasma strain of LWB-Eg *Candidatus Phytoplasma* that reported as a natural causal agent from Lime WB strain in Egypt was classified as 16SrII subgroup.

Keywords

Witches' broom, Phytoplasma, Electron microscopy, PCR, spacer region (SR), universal primers.

BACKGROUND

Citrus fruits have long been valued as part of a nutritious and tasty diet rich in vitamins, minerals and dietary fiber (non-starch polysaccharide) that are essential for normal growth (Dugo and Di Giacomo, 2002; EL-Banna *et al.*, 2015). However, it is appreciated that these and other biologically active, non-nutrient compounds found in citrus and other plants (phytochemicals) can also help to reduce the risk of many chronic diseases (Christine and William, 1999). In the Middle East region, the lemon considered as one of the most economically important crops according to the proper climate.

Witches' broom disease is a very serious disease of acid limes. The first report of the disease was-in Oman by Walker and Bridge in 1978 as "unknown origin disease" and then described by Bove in 1986 (AL-Zadjali *et al.*, 2014). Witches'- broom was detected in Egypt on different crops such as Hibiscus (Mokbel *et al.*, 2013), Faba Bean (Hamed *et al.*, 2014), Tomatos

Correspondence: r.mahrous@seu.edu
Full list of author information is available at the end of the article

(Ahmed *et al.,* 2014) and citrus - included sweet orange (*C. sinensis*) and mandarin (*C.reticulate Planco*) (EL-Banna *et al.,* 2015).

Witches' broom disease of lemon (WBDL) kills lemon trees in less than 3 years and has become a major limiting factor for lime production. The disease is caused by a phytoplasma that is designated as *Candidatus Phytoplasma aurantifolia* (Agrios, 1997 and Chung *et al., 2*006). Affected trees show witches' brooms, that are characterized by their compactness and their very small, pale green leaves. It shows many secondary thin shoots, developed from axillaries buds that normally stay dormant, with shortened internodes. The advanced stage of the disease has dry leaves, many witches' brooms. In four or five years the tree collapses. No flowers or fruits are produced on witches' brooms and the ones produced on normal shoots are reduced in size (Bulletin OEPP/EPPO, 2013).

Phytoplasmas were described, identified and differentiated mainly on the basis of their biological properties such as the symptoms they induce, on the host plant affected, as well as the methods of transmission by grafting and dodder transmission (Suzuki*et al.,* 2006 and EL-Banna *et al.,* 2007). Dienes' stain was used to detect plant diseases induced by Phytoplasma like organisms (Deeley *et al.,* 1979). Histological study was performed on the different tissues that were affected (EL-Banna *et al.,* 2015). Transmission electron microscopy was carried out for characteristic phytoplasma symptoms by many investigators (EL-Banna *et al.,* 2007; Rita and Favali, 2003).

The application of nested-PCR assays using R16F2n/R2 and P1/P7 primers as well as the use of conserved sequences and sequencing the new emerged strains allow to broadly detect and classify the evolution and virulent phytoplasma strains in field(Duduk and Bertaccini, 2011 and Madhupriya *et al.,* 2017).The primer pair SR1/SR2 was used to amplify a DNA fragments of approximately 241bp and 325bp from Tomato Big Bud (TBB) and Pepper Witches' broom (PeWB) phytoplasmas respectively (EL-Banna *et al.,* 2007). Molecular characterization and strain classification of different hytoplasmas were performed based on 16S rRNA gene and 16S-23S intergenic spacer region (SR) sequences (Davis *et al.,* 2017).

The present study aimed to biological, molecular and phytopathological characterization of the potential transmitted associated causal agent of witches' broom disease in lemon reported in Egypt obtained from the open cultivated fields of Citrus trees in Giza and AL-Qalyubia. Molecular classification was performed using cloning, DNA sequencing and phylogenetic analysis of the spacer region between 16S and 23S rRNA fragment of the isolated phytoplasma genome.

MATERIALS AND METHODS

Source of samples

Leaves from different lemon trees (*Citrus lemon*) showed symptoms of Witches' broom disease were collected from Horticulture Research Institute, Agricultural Research Center (ARC) at Giza governorate and a limit the farm belong to AL-Qalyubia governorate in Egypt.

Dodder transmission:

The infected lemon leaves were used to transmit the phytoplasma onto healthy periwinkle (*Catharanthus roseus*) plants through dodders (*Cuscuta reflexa Roxb*) according to the method of (Moustafa TA; 1990)

Grafting: Indicator hosts, healthy Lemon (*Vollka marina*) and periwinkle (*Catharanthus roseus*) as root stocks were used in grafting transmission. Eye bud sections from symptomatic trees were grafted using T- Grafting (Roistacher C. 1991 and Mahrous RM., 2005).

Dienes' stain:

Dinenes' stain was used as a characteristic tool for the detection of the phytoplasma in infected plants. Hand sections of healthy, treated and infected stems and leaves were prepared. The sections were transferred to the stain for 5 min, then washed in d.H_2O, then examined immediately by light microscope (Deeley *et al.,* 1979)

Transmission Electron Microscopy (TEM):

Transmission electron microscopy was carried out to reveal the Witches' broom phytoplasma units inside the infected leaf tissues of lemon (*Vollka marina*) according to (Rita and Favali, 2003). Tissues were cut into small pieces about 1-2 mm and fixed in 0.1 M potassium phosphate buffer pH 7.3 with 5% glutaraldehyde and 4% sucrose for 20 min at room temperature under mild vacuum then rinsed in 0.1 M Na-Cacodylate buffer, pH 7.2, for 45 minutes, with buffer changes at 15 and 30 minutes. Further fixation in 1% Osmium tetroxide in Na-Cacodylate buffer, under intermittent vacuum, took place for 1.5 hours. Samples were then rinsed again in the Na-Cacodylate buffer. Samples were dehydrated through an ethanol series in buffer: 35% - 50% - 70% - 80% - 95% - 100% for 60 minutes each. Then Infiltrate with resin as follows: propylene: resin 2:1 /1 hr, propylene: resin 1:1 /1 hr, propylene: resin 1:2/ 1 hr, Pure resin 1 hr, Pure resin overnight, pure resin with accelerator 2hr, embedded in Durcupan (Frfinova *et al.,* 1996). Semi-thin sections were prepared on glass slides through cutting at (200 nm) using Leica model EM-UC6 ultra microtome. Sections were stained with Toluidine blue for 5 min and examined by light microscope model M-200M. Ultra-thin sections (70 nm) were cut using ultra microtome, mounted on copper grids (400 mesh). Sections were stained with double stain Uranyl acetate 2% for 10 min followed by Lead citrate for 5 min and examined by transmission electron microscope JEOL (JEM-1400) at the candidate magnification. Images captured by CCD camera model AMT. (ZeissEM9.60KVFacarlZeiss.Oberkochen).

Detection by PCR:

Total DNA was extracted from both symptomatic and asymptomatic lemon leaves using CTAB (cetyl tri-methyl ammonium bromide) according to (Ahrens and Müller, 1992). The Phytoplasmas' DNA was detected in symptomatic lemon samples through the specific amplification of the spacer region between the 16S and 23S rRNA gene, and the start of the 23S rRNA gene regions of the phytoplasma genome using the universal phytoplasma primer pair P1/P7 (Schneider *et al.,* 1995) in direct PCR followed by primer pair R16F2n/R16R2 in nested PCR (Sinclair *et al.,* 2000). The PCR mixture contained 25 pmol of each primer; 20 ng of template DNA; 200 µM of each dNTP; 1x polymerase reaction buffer; 2.5 mM MgCl2; 1.25 U of dream-*Taq* polymerase (Fermentase - USA) and sterile water to a final volume of 25 µl. Amplification was started with denaturation at 94°C for 3 min followed by 35 cycles starting with denaturation at 94°C for 1min, annealing for 2 min at 55°C and primer extension for 3 min at 72°C with a final extension step at 72°C for 10 min. One microliter of (1:10) diluted PCR product from the first amplification was used as template in the second round, nested, PCR with the same PCR parameters and reaction conditions.

The detection of *phytoplasma* associated with witches' broom using primer pair R16F2n/R16R2 was confirmed through direct PCR using witches' broom-specific primers SR1: 5'-AGG CGG ATC CTT GGG GTT AAG TCG TAA-3 and SR2:5'- AGG CGA ATT CCG TCC TTC ATC GGC TCT-3' representing the phytoplasma-specific 16S/23S rRNA (rDNA) intergenic spacer region (SR) (Liefting *et al.,* 1996). Amplification was started with a denaturation step at 94°C for 2 min followed by 5 cycles at 94°C for 15s, 45°C for 15s, and 72°C for 30s followed by 25 cycles of 15s at 94°C, 15s at 55°C and 30s at 72°C, with a final extension of 10min at 72°C. The PCR products were stained with gel star (Lonza, USA) and analyzed by

electrophoresis in 1.0% agarose gel and visualized by UV illumination using Gel Documentation System (Gel Doc 2000, Bio-Rad, USA).

Molecular cloning and DNA sequencing

The fragment that amplified in the nested-PCR was purified and cloned directly into pCR4-TOPO cloning vector (Invitrogen, *Cat.*: K4530-20). The plasmid was introduced into DH5α *E.coli* cells using heat chock (Ausubel*et al.*, 1999). The recombinant plasmid was amplified and isolated from selected white colonies, and purified using the QIAprep Spin Miniprep Kit (Qiagen, Germany). The obtained recombinant plasmid DNA was analyzed with restriction digestions using *EcoR*I enzyme and verified by automated DNA sequencing**.** The universal M13 (-20) forward and M13 reverse primers were used for DNA sequencing. The sequence analysis as well as the construction of phylogenetic tree was performed using DNAMAN software (Lynnon BioSoft. Quebec, Canada). The nucleotide sequences were assembled and our findings were deposited in GenBank at National Center for Biotechnology Information (NCBI, Bethesda, USA). Blast search was performed at NCBI using the Standard Nucleotide BLAST to search for sequence similarity. To perform the phylogenetic analysis using DNAMAN Software; the nucleotide sequences were aligned with selected partial sequences of the 16S ribosomal gene from representative strains of '*Candidatus* Phytoplasma' available in GenBank and the phylogenetic tree was constructed based on that the horizontal distances in the tree indicate degree of relatedness between strains. The Lime WB phytoplasma-related strains retrieved from GenBank and used for phylogenetic analysis were reported in Table 1.

RESULTS

Histological characters of the WB Phytoplasma isolate

Symptomatology and transmission

Typical Witches' broom symptoms were observed on lemon included proliferation of shoots, yellowing of leaves and shortened internodes (Figure 1). Dodder plant showed a high ability to transmit the causal agent from infected lemon and periwinkle to healthy ones and the symptoms were appeared 2-3 weeks post transmission (Figure2).

T- Grafting was used to transmit the phytoplasma from the naturally infected citrus plants to healthy ones and periwinkle seedlings well. Typical symptoms were observed 2-4 months after grafting (Figure2).

Fig. 1: Photo plate showing Witches' Broom Disease (LWBD) Symptoms of the infected lemon plants, leaves are abnormally very small, excessive proliferation of shoots.

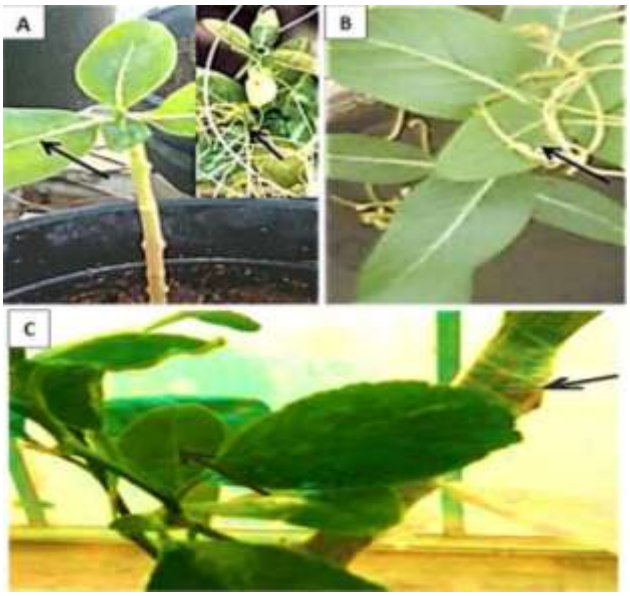


Fig. 2: Photo plate showing transmission of witches' Broom symptoms from infected lemon by plant dodder on periwinkle (A); healthy periwinkle (B), and grafted lemon **(C).**

Histological changes

Light Microscopy was used for internal investigation of naturally infected symptomatic plants. The symptomatic plant samples for lemon showed blue stained phloem but not the non-symptomatic ones (Figure3A and B). Ultra-thin sections of symptomatic tissues showed the symmetrical tri-unit laminar membranes (TUM) (Figure 4A), characteristic numerous pleomorphic bodies resembling budding structures of phytoplasma such as, budding forms, small and/or large vesicles bodies (Figure 4C, D). The size of spherical phytoplasma cells ranged from 200 - 400 nm in diameter. No phytoplasma or virus 'particles-were detected in non-symptomatic samples.

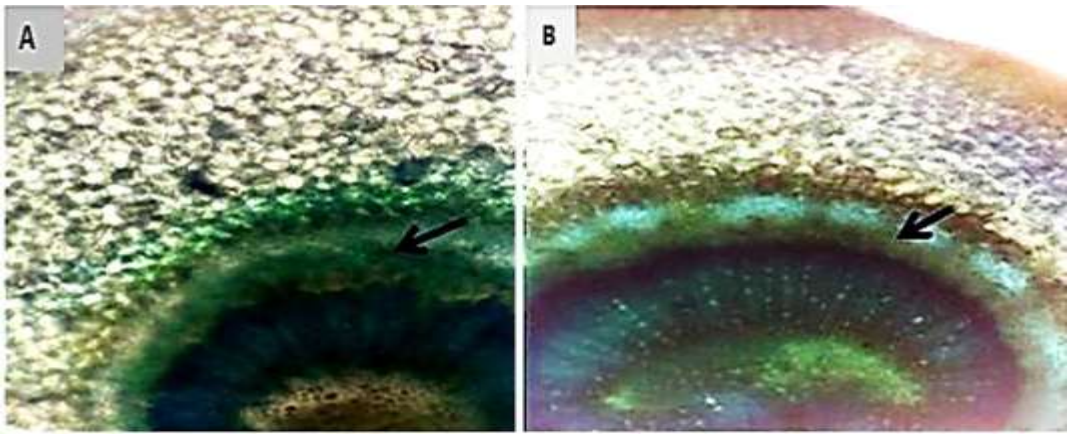


Fig 3: A: Cross sections of uninfected stem of healthy citrus plant B: Cross sections of stem infected by WBDL showing phloem tissues of citrus stained with dark blue at magnification: 400X.

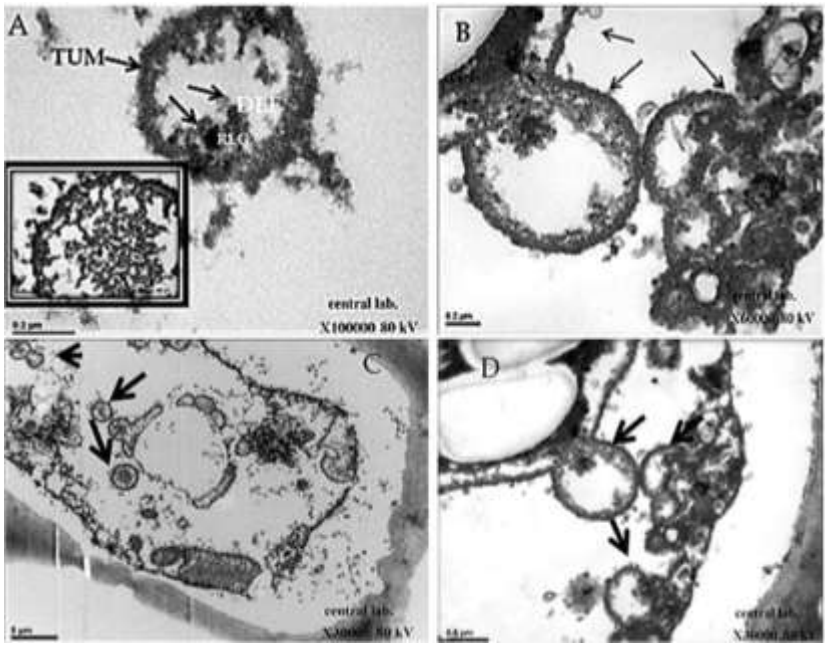
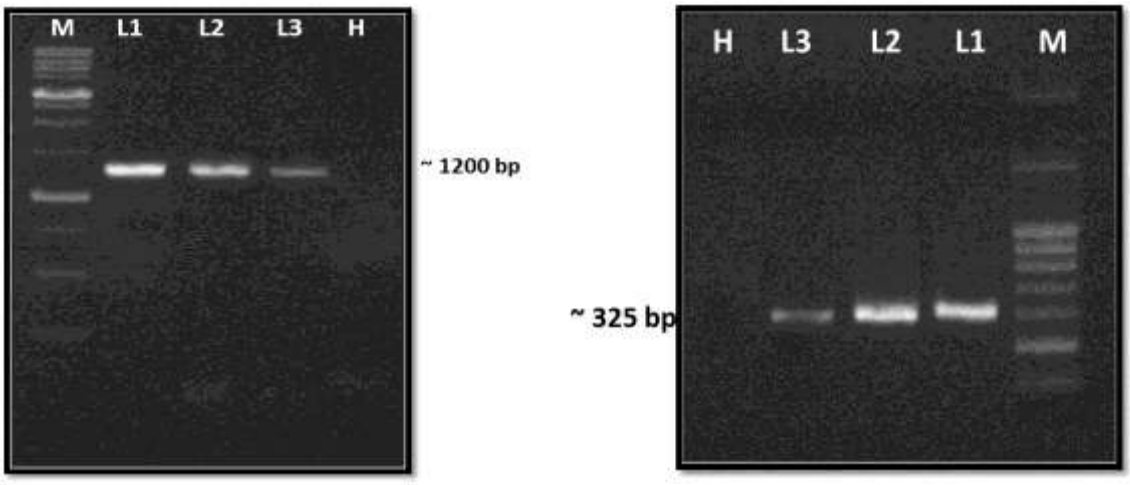


Fig. 4: Cross sections of infected lemon with witches' broom *phytoplasma* cells.A: pleomorphic bodies with tri-unit membrane (TUM), Ribosome–like granules (RLG) and DNA-like fibrils (DLF), bar =0.2 microns. B,Cand D showing *phytoplasma* pleomorphic units scattering inside the phloem elements,budding forms (arrows),bar=0.2,5,0.5 microns respectively.

Molecular identification of phytoplasma:

Total DNA isolated from infected lemon plants was used for the PCR detection of phytoplasma. All symptomatic samples infected with phytoplasma showed individually clear band at ~1200 bp as a result for the nested PCR amplification utilizing the universal phytoplasma-specific primer R16F2n/R16R2 (Figure 5A). As well as electrophoresis analysis of the PCR product showed a single amplified fragment of ~325 bp while no fragments were amplified from the DNA extracted from symptomless or healthy plants (Figure. 5B).



[A] **[B]**

Fig.5: Gel electrophoresis for the detection of the phytoplasma in lemon symptomatic leaves using universal phytoplasma-specific PCR primers R16F2n/R16R2 [A] and witches' broom-specific PCR SR1/SR2 [B]. L1, L2 and L3 are infected samples showed WB symptoms. H: Healthy Plant Control. M: 1 Kb DNA Ladder in A and 100 bp DNA Ladder in B.

Sequence analysis, Phylogenetic estimation and identification of phytoplasma

To identify the phytoplasma strain, the amplified 1247 bp PCR fragment of 16S rRNA gene was cloned successfully into the TOPO cloning vector and directly sequenced. The nucleotide sequence was published to the GenBank with accession number "**KJ948653**" as **LWB-Eg** strain.

Multiple sequence alignment and phylogenetic analysis was performed to compare the Egyptian strain (LWB-Eg) with the corresponding sequences of the other phytoplasma strains on GenBank (Figure 6). Phylogenetic tree showed that, LWB-Eg was 99% similar to Lime WB (strain LWB, EF186828, **16SrII**) from Oman. The identity was about 98% with Soybean WB (Strain Moz-13, HQ840717, 16SrII) from Mozambique, Cactus WB (Strain YN02, EU099547) from China, Alfalfa WB (DQ233655 and DQ233656) from Iran and Faba bean phyllody (FBP, EF193354) from Sudan. There was about 97.5% identity with several strains all in subgroup 16SrII like Jasmine WB (Strain JasWB, AB257290) from Oman, Echinacea (Strain EWB2, JF340077) from Australia, Alfalfa WB (strain AlfWB, AB259169) from Oman and Peanut WB (strain PnWB, L33765) from Taiwan. There was higher than 95% but less than 96% identity with Hibiscus WB (strain HibWB26, AF147708) from Brazil, Guazuma WB (strain GWB 10, HQ258883) from Costa Rica and Hibiscus W.B (strain WBAZ3, KF716175) from Egypt. The identity between the Egyptian isolate (LWB-Eg) and the other phytoplasma strains was presented in Table 1.

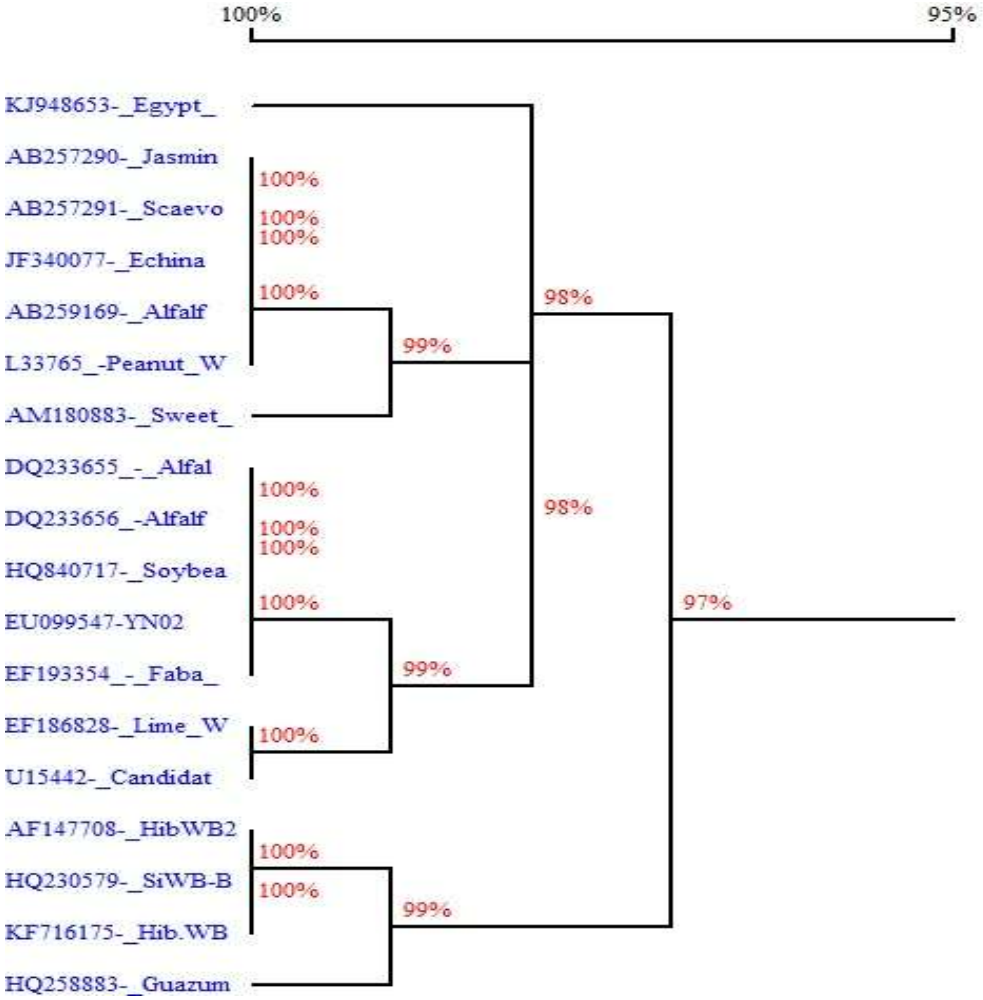


Fig. 6: Sequence analysis and the phylogenetic tree for our isolate LWB-Eg (KJ948653) showed range of 95-99% identity with the sequence of different *phytoplasma* strains in the GenBank.

Table 1: Sequences of Lime WB *phytoplasma*-related strains retrieved from GenBank and used for phylogenetic analysis of the 16S rDNA gene

Accession No.	Phytoplasma strain	Abbreviation	Origin	16SrRNA group	Identity (%)
AB257290	Jasmine WB	JasWB	Oman	**16SrII**	**97.55**
AB257291	Scaevola WB	ScaT	Oman	U.C	97.51
JF340077	Echinacea	EWB2	Australia	**16SrII**	**97.51**
AB259169	Alfalfa WB	AlfWB	Oman	**16SrII-D**	**97.55**
L33765	Peanut WB	PnWB	Taiwan	**16SrII-A**	**97.50**
AM180883	Sweet potato little leaf	Aus142A	Australia	**16SrII-D**	**97.35**
DQ233655	Alfalfa WB	Fars	Iran	**16SrII**	**98.16**
DQ233656	Alfalfa WB	Yazd	Iran	**16SrII**	**98.16**
HQ840717	Soybean WB	Moz-13	Mozambique	**16SrII**	**98.24**
EU099547	Cactus WB	YN02	China	**16SrII**	**98.16**
EF193354	Faba bean phyllody	FBP	Sudan	**16SrII**	**98.07**
EF186828	Lime WB	LWB	Oman	**16SrII**	**99.00**
U15442-	Ca. P. aurantifolia	WBDL	Oman	**16SrII-B**	**98.70**
AF147708	Hibiscus WB	HibWB26	Brazil	16SrXV	95.91
HQ230579	Sida rhombifolia	SiWB-Br1	Brazil	U.C.	95.81
HQ258883	Guazuma WB	GWB 10	Costa Rica	16SrXV	95.83
KF716175	Hibiscus W.B	WBAZ3	**Egypt**	16SrXV	95.99

U.C.: Unclassified phytoplasma;Ca. P.: Candidatus Phytoplasma

DISCUSSION

In spite of being several diseases associated with phytoplasma had been reported in Egypt no reports have been mentioned before about characterization and classification of lemon Witches' broom agents from the open field. In this study, Witches' broom disease of lemon was found as a natural infection in lemon grown in Giza and AL-Qalyubiagovernorates. The causal agent of Witches' broom in lemon was characterized using both biological and molecular tools. All findings were in accordance with the description of phytoplasma associated Witches' broom disease of lemon that described by Agrios *et al.,* 1997; Chung *et al.,* 2006 and Mardi *et al.,* 2011. The mode of transmission for the phytoplasma associated Witches' broom was proved to be induced by dodder from infected lemon or periwinkle plants to healthy ones. The same result was obtained by Ghosh *et al.,* 1999; Mahrous 2012 and EL-Banna *et al.,* 2015. The conformation of successfully transmitted phytoplasma from infected bud sticks grafted on lemon seedlings grown in an insect-free greenhouse was also obvious as shown in Figure1A. The grafted plants

were regularly observed for symptom development in agree with another studies presented by Ghosh *et al.,* 1999 and EL-Banna *et al*., 2007.

Dienes' stain was successfully used as a deferential tool for detection of phytoplasma locations in the phloem tissues Gamal *et al.,* 1983 andSidaros *et al.,* 2000. The presence of phytoplasma in the phloem sieve tubes was confirmed by EM from lemon leaf midribs that revealed considerable number of polymorphic bodies of phytoplasma. This result is the same as with those obtained by Frfinova *et al.,* 1996 and EL-Banna *et al.,* 2015.

Molecular diagnosis through the PCR analysis for the symptomatic samples confirmed the infection of Lemon plants with phytoplasma associated with witches' broom in Egypt. The DNA extracted from the Lemon plants, those showed witches' broom symptoms, was used as template for nested PCR utilizing universal phytoplasma-specific PCR primers and witches' broom-specific PCR primers respectively. The results of the nested PCR using the universal phytoplasma-specific primers (P1/P7 followed by R16F2n/R16R2) showed a clear band at the specific size 1200 bp while the direct PCR using the witches' broom-specific PCR primers (SR1 and SR2) showed a clear band at ~325 bp that agrees with the results obtained by Al-zadjali *et al.,* 2014 and Hamed *et al.,* 2014. Those PCR results clearly demonstrated and confirmed the natural infection of Lemon with phytoplasma associated with Witches' broom which agree with the data obtained by EL-Banna *et al.,* 2015. PCR results for Lemon plants those were experimentally infected with phytoplasma confirmed the successful transmission of the Phytoplasma Associated with Witches' broom into the healthy Lemon plants using Dodders.

The identification and molecular classification of the phytoplasma strain studied in this research was based on 16S ribosomal gene sequence and performed through the sequence analysis and construction of a phylogenetic tree for different groups of well defied phytoplasma strains. The results of the *Eco*RI digestion analysis (data not shown) as well as the nucleotide sequencing successfully characterized this phytoplasma strain. The nucleotide sequence obtained from the 1246 bp PCR product associated with infected lemon was submitted to the GenBank with the name Lime witches' broom in Egypt, abbreviated as LWB-Eg and accessioned by GenBank accession number "KF716175" as the first classification of phytoplasma associated witches' broom affecting lime in Egypt.

Sequence of LWB-Eg was submitted to BLAST analysis and showed a 99 % identity with reference strain of Lime witches' broom from Oman, belonging to 16SrII-group. The data of the phylogenetic analysis and homology with the sequence of the 16S ribosomal RNA (rRNA) gene that showed in Table 1 confirmed the high similarity with several phytoplasma strains; Soybean WB (Strain Moz-13, HQ840717, 16SrII), Cactus WB (Strain YN02, EU099547), *Candidatus Phytoplasma aurantifolia* (WBDL, U15442), Alfalfa WB (DQ233655 and DQ233656) and Faba bean phyllody (FBP, EF193354) all in group 16SrII. These data indicated that; the phytoplasma strain studied in this research (LWB-Eg) clustered in the **16SrII** group.

CONCLUSION

The results presented here will inform future population level analyses of Witches' broom disease (WB) associated with citrus to investigate if these, or a subset of these genes, can define this economically important pathotype and be used as identified or classified targets.

Results in this research suggest that, LWB-Eg represent a limited emerging phytoplasma recorded in Egypt. Strikingly, its sequence type share an epidemiological profile very similar (99%) to Lime WB strain from Oman (EF186828) which classified as **16SrII** group. The pathogenic variants phylogroup is 16SrII but less likely amongst other phylogroup 16SrXV.

Availability of supporting data

This research utilized publicly available phytoplasma genome sequence data for which there are no ethical issues. The data sets supporting the results of this article are included within the article. The studied nucleotide sequences were published to the GenBank with accession number "KJ948653"and named as LWB-Eg strainthat is available online.

Competing interests

The authors declare that they have no competing interests

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AUTHOR DETAILS

[1]Department of Botany and Microbiology, College of Science, King Saud University, Riyadh-11495, Kingdom of Saudi Arabia.
[2]Virus and Phytoplasma Research Department, Plant Pathology Research Institute, Agricultural Research Center (ARC), Giza, Egypt.
[3]Agricultural Microbiology Department, Faculty of Agriculture, Ain Shams University, Hadayek Shobra, 1241, Cairo, Egypt.
[4]Public Health department, College of Health Sciences , Saudi electronic university - Riyadh - 11673,Kingdom of Saudi Arabia.

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