Molecular and Electron Microscope Evidence for an Association of Phytoplasma with Citrus Witches Broom Disease.

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ABSTRACT—During April and May 2013 , citrus included sweet orange ***(C. sinensis)*** and mandarin ***(C.reticulate Planco)*** showing typical symptoms of witches broom disease were detected from different fields located at AL-Sharqia, AL-Qalyubia and Ismalia, governorates in Egypt. The detected phytoplasma from diseased samples was transmitted by grafting to healthy citrus plants and by dodder to healthy periwin*kle plants. DNA extracted from symptomatic samples was used as template for amplification of products of 1.8 kb using universal primer* pair P1/P7 and 1.2 kb using primer R16F2n/ R16R2 by direct and nested PCR. Pleomorphic bodies typical to phytoplasma structures were observed in phloem sieve elements in ultrathin sections of infected plants using transmission electron microscope (TEM). Phytoplasma infection resulted in ultrastructure changes especially in sieve elements, as obliteration and necrosis were observed. The ultrastructure changes were expanded to the cell wall, cell membrane and callose deposition on the cell wall was also observed.

keywords— Phytoplasma, Citrus witches'- broom, Grafting, PCR and TEM.

1 INTRODUCTION

The genus citrus L. (Family: Rutaceae, Sub family: Aurantioideae). Citrus fruits are recognized as an important component of the human diet providing a variety of constituents important to human nutrition , including vitamin C (ascorbic acid), folic acid , potassium , flavonoids ,coumarins, pectin and dietary fibers (Dugo and Di Giacomo, 2002). World production of citrus reached more than 129 million tons from cultivated trees in 140 countries around the world. Spain is the leading producing country where as Italy and Egypt rank second and third respectively. Egypt 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 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 witches' broom disease caused by a prokaryotic pathogen phytoplasma (Ghosh et al. 1999 a, b). Phytoplasmas are wall-less prokaryotes in class Mollicutes, unicellular wall-less units bounded by membrane and appear in sieve elements as rounded or ovoid units ranging in diameter from 400 to 900 nm. Phytoplasma are transmitted by grafting, dodder and insect vectors, especially leaf hoppers. (Musetti et al., 2011 and El-Banna et al., 2013). Among the major constraints, Witches' Broom Disease of citrus (WBD) is a very serious disease in most citrus growing regions and dramatically decreases citrus yields (Gunderson and Lee 1996, Firrao et al., 2005). Phytoplasma was identified as the causal agent of witches' broom disease of citrus (Bove, 1986). The WBD Phytoplasma was classified to 16SrIIb (Peanut WB group) and named as "Candidatus Phytoplasma aurantifolia" according to sequences of the 16S rRNA gene (Zreik et al., 1995). The concentration of phytoplasma in woody plants like citrus remains very low and is unevenly distributed within the infected plants (Lee et al., 2000). Until recently identification of phytoplasma induced witches, broom disease in citrus was based on symptomatology, host range, vector specificity and EM studies (Ghosh et al. 1999 a, b). Because phytoplasma is not cultivated in nutrients media, the methods of its detection and diagnosis are restricted. So electron microscopy is used to check the presence of phytoplasma units in phloem tissues of infected plants (El- Banna et al., 2000 and Rocchetta et al., 2007). PCR based methods are also used for detection and identification of phytoplasma (El-Banna et al., 2007; Samuitiene et al., 2007).
The aim of the present investigation is to detect phytoplasma associated with disorders observed on citrus trees in different locations in Egypt, verify the association of the detected phytoplasma with the disease and study the ultrastructural changes in tissues of infected trees.

2MATERIALS AND METHODS

Source of samples

During April and May 2013 , citrus included sweet orange (C. sinensis) and mandarin (C.reticulate Planco) showing typical symptoms of witches broom disease were detected from different fields located at AL-Sharqia, AL-Qalyubia and Ismalia, governorates in Egypt. Samples were taken from both symptomatic and asymptomatic shoots and fruits of orange and mandarin trees.

Pathogenicity test

The pathogenicity of the suspected phytoplasma was verified by grafting and dodder transmission according to Salehi et al. (2005). Sour orange (C. aurantium L.), Volkamer lemon (C. volkameriana Tan. & Pasq.) and rough lemon (C. jambhiri Lush.) were used as rootstock in grafting transmission. Ten replicates were used for each species. Naturally, infected orange and mandarin trees exhibited the symptoms of witches-broom were served as the budwood plant. For negative control

healthy rootstock from the three species were grafted with scions taken from healthy bud wood plants.

Dodder (Cuscuta odorata) seeds were germinated in vitro in petri dishes and were then transferred to the stems of naturally infected (symptomatic) orange and mandarin the tested plants were kept under greenhouse conditions and observed for symptoms appearance, healthy plants used as negative control.

Molecular biology studies

Molecular biology studies were carried out to detect the suspected phytoplasma.

DNA Extraction and PCR Amplification

DNA was extracted from fresh samples of orange, mandarin trees exhibiting witches broom symptoms and periwinkle plants using the modified Dellaporta extraction method (Dellaporta et al., 1983).

The extracted DNA were used as a template for PCR. The primer pair P1 / P7 illustrated in Table (1) (Sinclair et al ., 2000) was used in the amplification of 1.8-kb product of 16SrRNA gene, the spacer region between the 16S and 23SrRNA gene and the start of the 23SrRNA gene regions of the Phytoplasma genomes (Fig.1). To increase the sensitivity of the PCR, the primer pair R16F2n/R16R2 designed to amplify a portion of 16SrRNA gene (1.2 kb) was used as a nested –PCR. (Wang and Hiruki, 2001). 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) and VC 1Kb Plus DNA ladder (Vivantis) were used as PCR Markers.

Table 1. Sequences, size and specificity for primers used for DNA amplification.

Primer	Sequence	Specifity and size of the product
P_1	5-'AAGAGTTTGATCCTGGCTCAGGATT-3'	Universal 1.8kb
P_7	5'-CGTCCTTCATCGGCTCTT-3'	
R16F2	5-'GAAACGACTGCTAAGACTTGG-3'	Nested 1.2 kb
R16R2	5'-TGACGGGCGGTGTGTACAAACCCC-3'	(Aster yellow)

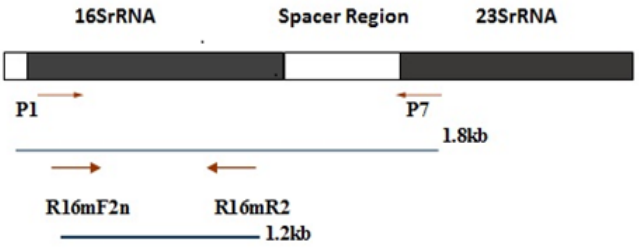

Fig. (1): Illustration of a phytoplasma rRNA operon that shows the 16S and 23S genes and intergenic spacer region. The location of oligonucleotide primers are marked with arrows.

Ultrastructural changes

Transmission electron microscopy was carried out to detect phytoplasma units inside the infected tissues of the different infected citrus samples.

Preparation of plant tissue for examination of electron microscope

Materials prepared for electron microscopy included leaf midribs of orange, mandarin and albedo layer of orange fruit representing both symptomatic and asymptomatic samples. Pieces of about 2 x 2mm taken from samples were cut and transferred to a separate vial to be fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for an hour. After removing the fixative solution, the tissues were washed in sodium phosphate buffer three times for 30 min each .After washing, the buffer was pulled out, and 1% of osmium-tetroxide (OsO4) was added to the tube and allowed for 1.5 h at 4°c. After removing the fixative solution, the samples were dehydrated in an ethanol series of 15% , 30%, 50%, 70%, 80% and 95%, before exposing to 100% for 15 minutes for every step except the step of 100% ethanol, which was repeated twice according to the method described by ***El-Banna et al .(2007).*** Infiltration with Spur's epoxy resin, one large drop into the sample tube every 15 minutes, until at 75% resin overnight with rotating. Samples were put into 100% resin, for at least a day, then samples were placed into flat BEEM capsule molds, hardening the resin was done overnight in an oven at 60°C. Samples were then sectioned (90-100 nm thick) with the ultra –microtome (Leica model EM-UC6) mounted on copper grids (400 mish). Sections were double stained with 2% urany1 acetate and 10% lead citrate, and then allowed to dry well. 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 work was carried in TEM lab, Faculty of Agriculture, Cairo University. Research Park (FARP).

3 RESULTS

Diseases symptoms

Naturally infected orange (C. sinensis) trees showed appearance of small chlorotic rectangular leaves with reduced blade. The infected trees were characterized with multiple sprouting, shortened internodes, leaves were abnormally very small and crowded at the top stem and this was characteristic of witches' broom **(Fig.2 A&B).** Orange fruits were deformed, abnormal in size and color, rough touch, wrinkle and albedo layer is thick in the inner part of the orange fruits. On mandarin (C.reticulate Planco) leaves showed deformation, marginal necrosis and the buds at the top of the plant enlarged in size. Overgrown branches, wrinkle and irregular growth. Apical stems were thick and assumed an upright growth habit. Infected plants appeared bushy because of shortened internodes and small leaves. Leaves drop prematurely and infected branches have distorted twigs characteristic of witches broom symptoms. In advanced stages, infected branches show die back symptoms **(Fig.2 C&D).**

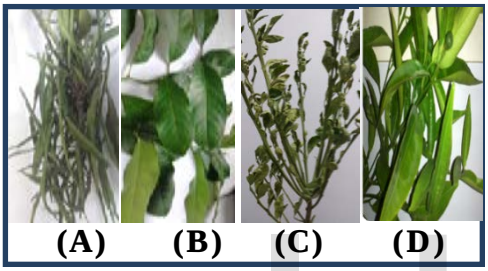

Fig. (2): Symptoms of citrus witches broom and healthy one
(A) Witches broom symptoms on orange leaves
(B) Healthy orange leaves
(C) Witches broom symptoms on mandarin leaves
(D) Healthy mandarin leaves

Pathogenicity test

Grafting transmission

By grafting scions taken from diseased orange and mandarin trees (budwood) into healthy citrus species (rootstock), the same symptoms of witches-broom, yellowing and little leaf were obtained on the newly formed plant parts after 55-65 days from grafting. Percentage of transmission by grafting reached 80% in both mandarin and orange. On the other hand, no symptoms were observed on graft unions with scions taken from healthy plants.

Dodder Transmission

The pathogenicity of the detected Phytoplasma was also checked by dodder transmission. Dodder stolons were applied on naturally infected (symptomatic) mandarin and orange seedlings for 16 days and then applied on healthy citrus and periwinkle plants, the symptoms were detected on the new plant parts after 30 days. The periwinkle plants exhibited yellowing of leaves which were deformed. Typical witches broom symptoms were observed and the growth of the infected plants was generally disturbed and no flowers were formed on the infected plants if compared with healthy ones Percentage of transmission by dodder reached 100%. No symptoms were observed on tested plants in which stolon's of dodder were transferred to them after parasitizing on healthy plants.

Molecular biology studies

With the universal primer pair P1/P7 adopted for detection of the 16SrRNA, 23SrRNA and the spacer region (SR) fragments of approximately 1.8kb was amplified by direct PCR from all total nucleic acid extracted from samples of infected orange, mandarin representing witches-broom symptoms **(Fig.3)** and periwinkle plants exhibiting reduction in size, leaf proliferation of lateral shoots, and stunting symptoms after dodder transmission **(Fig.4)**. No visible bands were detected from the corresponding healthy samples. In the present study nested PCR assays with the primer pair P1/P7 followed by the primer pair R16F2n/R16R2 yielded a strong band of approximately 1.2kb DNA fragment extracted from infected orange, mandarin and periwinkle plants **(Figs.5and6)** . PCR amplified products obtained from healthy plants were used as a controls.

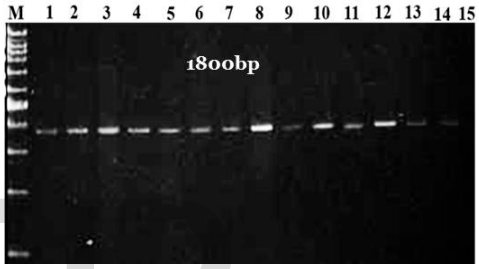

Fig.(3): Gel electrophoresis analysis of the detected phytoplasma in citrus leaves using the universal primers pair P1/ P7 (1800bp). M: DNA marke, VC 1kb, lanes: (1to14): various symptomatic citrus samples, lane15: healthy citrus sample used as negative control.

Fig.(4): Gel electrophoresis analysis of the detected phytoplasma in periwinkle after dodder transmission using the universal primers pair P1/ P7 (1800bp). M: DNA marker, VC 100bp. L1, L2 symptomatic periwinkle samples, L3: healthy periwinkle sample used as negative control.

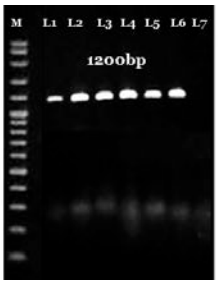

Fig.(5): Gel electrophoresis analysis of the detected phytoplasma in citrus samples using nested PCR with the primers R16F2n/R16R2 (1200bp). M: DNA marker weight VC 100bp, lanes (1to 6): symptomatic citrus samples, lane7: healthy citrus sample as negative control.

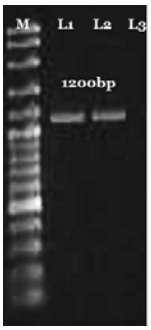

Fig.(6): Gel electrophoresis analysis of the detected phytoplasma in periwinkle after dodder transmission using nested PCR with the primers R16F2n/R16R2 (1200bp). M:DNA marker VC 100bp, L1, L2 symptomatic periwinkle samples, L3: healthy periwinkle sample used as negative control.

Ultrastructural changes

Electron microscopy

Ultrathin sections prepared from mandarin, orange leaf midrib and albedo layer from orange fruit representing witches broom symptoms were investigated through transmission electron microscopy at different magnifications. The examination revealed the presence of numerous phytoplasma units in the sieve elements of the infected orange tissues arranged next to the cell membrane and ready to pass the sieve pores. These units were rounded, elongated or pleomorphic bounded by a unit membrane, lacking cell wall **(Fig.7A)**. Budding of phytoplasma units was also remarkable **(Fig.7B)**. On the other hand, necrotic areas were observed between phloem units, and the cell wall of these areas begins to lose its integrity if compared with those of healthy orange leaf **(Fig.7Cand7D)**.
Yahyai et al. (2014). These observations are confirmed by the findings of Khadhair et al. (1998) who stated that phytoplasma taxonomy can be compared for many characters including the propensity to induce particular symptoms such as phyllody, proliferation, witches-broom or hyperplasia. Amaral-Mello et al. (2006) pointed out that phytoplasmas may alter the balance of hormones in the host plant, eventually inducing distortions of growth. They also stated that phytoplasmas produce certain proteins, e.g. glucanases and hemolysin-like proteins, which can act as virulence factors. In addition, phytoplasmas import numerous metabolites from the host plant, which eventually could change the physiological equilibrium of the host. On the other hand, Pracros et al. (2007) indicated that phytoplasma multiplication in the phloem sieve tubes, results in deregulation of floral meristem gene expression, recorded that expression of genes controlling the maintenance of the shoot apical meristem and the floral organ identify were ap3, ag, Ify and regulated resulting in deformations and distortion of infected plants , as the lateral shoot growth is stimulated by the absence of the apical dominance resulting in witches –broom symptoms .

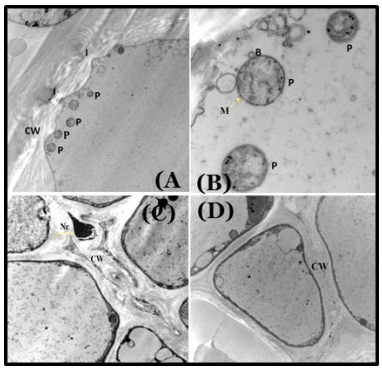

Fig.(7): An electron micrograph of phloem sieve elements in infected orange leaf

(A) Showed numerous phytoplasma (P) units arranged next to the cell membrane and invaginations (I) in cell wall (CW) are obvious (6000X).

(B) Showed phytoplasma units (P) bounded with membrane (M) at high magnification, bud formation (30000 X).

(C) Showed phloem necrosis (Ne) and the cell wall begins to lose its integrity (12000X).

(D) Healthy orange leaf containing no phytoplasma units and normal structure of the cell wall (CW) (10000X).

Regarding the ultrastructural changes occurring in infected mandarin leaves, investigations showed that the phloem parenchyma cell contained also number of phytoplasma units. Phloem abnormality was obvious, in addition to intercellular necrosis of the phloem elements if compared with those of healthy mandarin leaf **(Fig.8A and 8B).** On the other hand, cytoplasm of phloem parenchyma of infected mandarin leaf contains different vacuoles, phytoplasma units and invaginations of cell wall appeared clearly **(Fig.8C)**. Investigation of the ultrathin sections prepared in the albedo layer in infected orange fruit also revealed the presence of different sized phytoplasma units some of which were attached to the cell membrane (Fig.9A). On the other hand, the albedo cells abnormally contained vacuoles. The cell wall invaginations were clear accompanied with callose depositions on its inner side **(Fig. 9B)**.

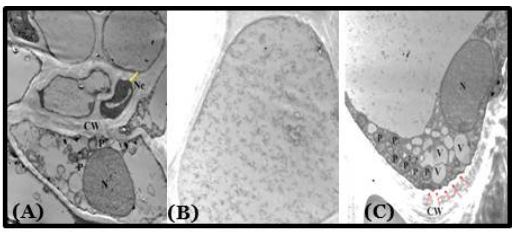

Fig.(8): An electron micrograph of phloem in infected mandarin leaf

(A) Showed phytoplasma units (P), phloem necrosis (Ne) and the cell wall (CW) begins to lose its integrity, nucleus (N) is not affected (8000X).

(B) Sieve elements in healthy mandarin leaf (12000X).

(C) Containing numerous phytoplasma units (P). The cytoplasm contains several vacuoles (V) Invagination and disassociation of the cell wall (arrows) also occurred. The nucleus (N) is not affected (8000X).

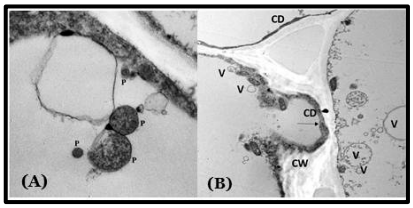

Fig.(9): An electron micrograph of phloem cell in albedo layer of infected orange fruit
(A) Containing phytoplasma units (P) in different sizes (5000X).
(B) Showed phloem cell contain different sizes of vacuoles (V) at the invaginated cell wall (CW) and callose deposition (CD) on the inner side of it (5000X).

4 Discussion

In the present study, phytoplasma was detected and characterized from naturally infected citrus grown in different locations in AL-Sharqia, AL-Qalyubia and Ismalia, governorates in Egypt. The collected samples exhibited typical symptoms of witches-broom, little leaves and yellowing. These symptoms are characteristic to phytoplasma infection and are typical to those described by Bove and Garnier (2000), Lee et al. (2003), Bertaccini and Duduk (2009), Ghosh et al. (2013) and Al-

The pathogenicity of the suspected phytoplasma was verified by grafting and dodder transmission. The obtained results indicated that phytoplasma in orange and mandarin which were grafted by budding to three species of citrus, exhibited the same symptoms of witches broom, little leaves and yellowing after 65 days from grafting. In almost all research work concerning phytoplasma diseases, grafting was the perfect method for experimental transmission with the tested phytoplasma Al –Zadjali et al. (2007), Mikhail et al. (2012) and El-Banna et al. (2013).

Periwinkle plants were also experimentally inoculated with different samples infected with phytoplasma by dodder transmission and kept as a maintaining host. The inoculated periwinkle plants exhibited symptoms of stunting, malformation and yellowing of the newly developed leaves and this agree with Salehi et al. (2000), Chen et al. (2009), Pribylova et al. (2011), Mikhail et al. (2012) and El-Banna et al. (2013). The infected plants did not develop flowers for a long period of time and when formed they were malformed or in very few numbers (small pale flowers) and these findings are more or less similar to those found by Kaminiska et al.(2001) who studied rose phyllody disease and proved its phytoplasma etiology . Periwinkle plants are known to harbor almost all the known phytoplasmas, so it is used as an assay host for phytoplasmas by grafting or dodder transmissions Amaral-Mello et al. (2006), Mikhail et al. (2012) and El-Banna et al. (2013). In the present investigation, the detected phytoplasmas were transmitted into periwinkle plants by dodder and the symptomatic plants were used as a source of phytoplasma studies.

For molecular detection and characterization of phytoplasma in citrus and periwinkle plants, the primer pair p1 p7 which gave products of the expected molecular size 1.8 kb were used. The same primer was used by many investigators for the same purpose Salehi et al. (2005), Arocha et al. (2007) Chen et al. (2009), El-Banna et al. (2013) and Al-Yahyai et al. (2014). The success of PCR in detecting phytoplasma in field-collected samples largely depends on obtaining total nucleic acid preparations of good quality and enriched with phytoplasma DNA, but this has always been difficult Firrao et al. (2007). The amount of phytoplasma DNA is often lower than 1% of total DNA extracted from tissue Bertaccini (2007).

Nested PCR was also used for detection of phytoplasma in the samples collected from the citrus and periwinkle plants. The two universal primer pairs p1 p7 and R16F2n/ R16R2 designed for amplification of phytoplasma 16S rDNA were used to detect phytoplasma with a product approximately 1.2 kb Djavaheri and Rahimian (2004), Alhudaib et al. (2009), Ghosh et al. (2013) and Al-Yahyai et al. (2014).

Examination of ultrathin sections from leaves of diseased orange, mandarin and orange fruit revealed the presence of numerous phytoplasma units in sieve tubes of phloem leaves but not in healthy leaves, the detected units were bounded by one unit membrane and contained multiple DNA segments and ribosomes. Phytoplasmas were particularly abundant in mature sieve tubes. When present in low concentrations, phytoplasmas were generally restricted to the periphery of sieve tubes. The presence of phytoplasma units in phloem tissues confirmed the biological studies of grafting and dodder transmission and its responsibility for witches broom disease Ghosh et al. (1999a), Ghosh and Shyam (2002), Omar et al. (2008), Musetti et al. (2011) and Mikhail et al. (2012).

The number of phytoplasma units was not similar in the different inspected tissues and this was correlated with the severity of symptoms .This observation was also mentioned by Kesumawati et al. (2006). On the other hand, Kaminiska et al. (2001) stated that there was no correlation between the number of the detected phytoplasma units and the severity of rose phyllody symptoms. Montano et al. (2010) and Li et al. (2012) remarked that the titer of phytoplasma was high in leaf phloem tissues which are source of nutrients. The number of phytoplasma units was high (75 units/cell) in phloem parenchyma cells, budding and binary fission of phytoplasma were also observed as a developmental stages of its growth as stated by El-Banna and El-Deeb (2007). The anatomical changes observed during the present investigation were indicated through visualizations of ultrathin sections. The most remarkable was necrotic areas between phloem units, callose deposition and cell wall thickening which resulted in almost all cases in reduced lumen of sieve tubes. Kaminiska et al. (2001) pointed out to the cell wall thickening of sieve elements as a result of infection with phytoplasma. The presence of vacuoles in phloem tissues containing phytoplasma units was reported also by Singh et al. (2011), who mentioned that it might be attributed to autophagic activity. Phytoplasma units are clearly

distinguishable from the autophagic vesicles by the presence of ribosomes and pieces of nucleic acid. The phytoplasma units were observed adjacent to the cell membrane as it's well-known that they utilize the phospholipids sharing in membrane structure.

REFERENCES

[1.] Alhudaib, K.; Arocha, Y.; Wilson, M.and Jones, P. (2009). Molecular identification potential vectors and alternative host of the phytoplasma associated with a lime decline disease in Saudi Arabia. Crop Prot., 28: 13–18

[2.] Al-Yahyai, R.; Al-Subhi, A.; Al-Sabahi, J.; Al-Said, F.; Al-Wahaibi, K. and Al- Sadi, A. (2014). Chemical composition of acid lime leaves infected with Candidatus Phytoplasma aurantifolia. J. Agric. Sci., 5 (1): 66-70.

[3.] Al-zadjali, A.D.; Natsuaki, T and Okuda, S. (2007). Detection, identification and molecular characterization of a Phytoplasma associated with Arabian Jasmine (Jasminum sambac L.) Witches-Broom in Oman. J. Phytopathol ., 155:211-219.

[4.] Amaral-Mello, A.P.O.; Bedendo, I.P.; Kitajima, E.W.; Ribeiro, L.F.and Kobori. (2006). Rose phyllody associated with a Phytoplasma belonging to group 16SrIII in Brazil. Inter. J.Pest Manag., 52(3):233-237.

[5.] Arocha, Y. A.; Bekele, B. B.; Tadesse, D. C.and Jones, P. D. (2007). First report of a 16SrII group phytoplasma associated with die-back diseases of papaya and citrus in Ethiopia. J.Plant Pathol.,Plant Pathology, 56: (6) 1039-1039.

[6.] Bertaccini, A. (2007). Phytoplasmas: diversity, taxonomy, and epidemiology. Frontieres in Bioscience, 12: 673-689.

[7.] Bertaccini, A. and Duduk, B. (2009). Phytoplasma and phytoplasma diseases: a review of recent research. Phytopathology, 48:355–378.

[8.] Bové, J.M. (1986). Witches' broom of lime. Bull. FAO Plant Protec.., 34: 217-218.

[9.] Bove, J.M. and Garnier, M. (2000). Witches' broom disease of lime. Arab. J. Plant Protec., 18: 148- 152.

[10.] Chen, J.; Pu, X.; Deng, X.; Liu, S.; Li, H. and Civerolo, E. (2009). Phytoplasma related to 'Candidatus Phytoplasma asteris' detected in citrus showing Huanglonbing (Yellow Shoot Disease) symptoms in Guangdong, P. R. China. Phytopathology, 99:236–242.

[11.] Dellaporta, S. L.; Wood, J. and Hicks, J. B. (1983). A plant DNA mini preparation: version II. Plant. Mol Biol. Rep., 1: 19-21.

[12.] Djavaheri, M.and Rahimian, H. (2004). Witches'-broom of bakraee (Citrus reticulata hybrid) in Iran. J. Plant Dis., 88(6):683.

[13.] Dugo, G.and Di Giacomo, A. (2002). Citrus: the genus Citrus, medicinal and aromatic plants – industrial profiles. Taylor &Francis group, London, pp 76-77.

[14.] El-Banna, Om-Hashem M.; Abada, K.A. and Mostafa, M.A. (2000). Anew disease of strawberry in Egypt caused by Spiroplasma citri.Proc.9th Cong.of the Egypt. Phytopathology, pp.1-17.

[15.] El-Banna, Om- Hashem M. and El-Deeb, S. H. (2007). Phytoplasma associated with mango malformation disease in Egypt. J. Phytopathol., 35(2):141-153.

[16.] El-Banna, Om-Hashem M.; Mikhail, M.S.; Farag, Azza.G. and Mohamed, A.M.S. (2007). Detection of phytoplasma in tomato and pepper plants by electron microscope and molecular biology based methods. Egyp. J. Virol., 4:93-111.

[17.] El-Banna, Om-Hashem M.; Mikhail, M.S. ; El-Attar, A.K. and Algamali, A.A.R. (2013).Molecular and Electron Microscope Evidence for an Association of Phytoplasma with Sesame Phyllody in Egypt .J. Phytopathol., 41(2):1-14.

[18.] F.A.O (2013). Citrus Statistics 2013. Food and agriculture organization of the United Nations.

[19.] Firrao, G.; Gibb, K. and Streten, C. (2005). Short taxonomic guide to the genus "Candidatus Phytoplasma". J. Plant Pathol., 87: 249-263.

[20.] Firrao, G.; Garcia-Chapa, M. and Marzachi, C. (2007). Phytoplasma genetics, diagnosis and relationships with the plant and insect host. Front Biosci., 12:1352–1375.

[21.] Ghosh, D.K.; Das, A.K.; Singh, S.; Singh, S.J. and Ahlawat, Y.S. (1999a). Occurrence of witches' broom – a new Phytoplasmal disease of Acid lime (Citrus aurantifolia) in India. Plant Dis., 83:302.

[22.] Ghosh, D.K.; Das, A.K.; Singh, S.; Singh, S.J. and Ahlawat, Y.S. (1999b). Association of phytoplasma with witches' broom, a new disease of acid limes. Curr .Sci., 77:174–177.

[23.] Ghosh, D. K.and Shyam, S. (2002). Phytoplasmal infection in Nagpur mandarin orchards in central India. J. Phytopathol., 55(1):87-89.

[24.] Ghosh, D.; Bhose, S.; Manimekalai, R.and Gowda, S. (2013). Molecular detection of Candidatus Phytoplasma spp. causing witches' broom disease of acid lime (Citrus aurantifolia) in India. J. Plant Biochem. Biotechnol., 22(3): 343-347

[25.] Gunderson, D.E. and Lee, I.M. (1996). Ultrasensitive detection of Phytoplasmas by nested-PCR assays using two universal primer pairs. J. Phytopathol .Mediterr., 35: 114-151.

[26.] Kaminiska, M.; Sliwa, H.; Rudzinska L. and Angwald, A. (2001). The association of Phytoplasma with stunting, leaf necrosis and witches-broom symptoms in agnolia plants .J.Phytopathol., 149:719-724.

[27.] Kesumawati, E.; Kimata T.; Uemachi, T.; Hosokawa, M. and Yazawa, S. (2006). Correlation of Phytoplasma concentration in Hydrangea macrophylla with green –flowering stability. Scientia Hortic., 108: 74-78.

[28.] Khadhair, A.H.; Kawchuk, L. M.; Taillon, R.C. and Botar, G. (1998). Detection and molecular characterization of aster yellows phytoplasma in Parsley. J. Plant Pathol ., 20:55-61.

[29.] Lee, I.M.; Devis, R.E.and Gundersen-Rindal, D.E. (2000). Phytoplasma: phytopathogenic mollicutes. Ann. Rev. Microbiol., 56:1593–1597.

[30.] Lee, I.M.; Martini, M.; Bottner, K.D.; Dane, R.A.; Black, M.C. and Troxclair, N. (2003). Ecological implications from a molecular analysis of Phytoplasmas involved in an aster yellows epidemic in various crops in Texas. Phytopathology, 93:1368-1377.

[31.] Li, Z.N.; Zhang, L.and Wu, Y.F. (2012). Anew Phytoplasma associated with witches-broom on Japanese maple in China. Forest Pathology, 42(5):371-376.

[32.] Mikhail, M.S.; El-Banna, Om-Hashem M.; Khalifa, Elham A. and Mohammed, A.M.S. (2012). Detection and control of rose

phytoplasma phyllody disease .Egypt .J.Phytopathol., 40:87-100.

[33.] Montano, H.G.; Davis, R.E.; Dally, E.L.; Hogenhout, S.;Pimentel,J.P. and Brioso,P.S.T. (2010). Candidatus Phytoplasma brasiliense a new Phytoplasma taxon associated with hibiscus witches broom disease. Int. J. Syst. Evolut. Microbiol., 51:1109-1118.

[34.] Musetti, R.; Grisan, S.; Polizzotto, R.; Martini, M.; Paduano, C. and Osler, R. (2011). Interactions between 'Candidatus Phytoplasma mali' and the apple endophyte Epicoccum nigrum in Catharanthus roseus Plants. J. Appl. Microbiol., 110(3):746-756.

[35.] Omar, A. F.; Emeran, A. A.and Abass, J. M. (2008). Detection of phytoplasma associated with periwinkle virescence in Egypt. J. Plant Pathol., 7(1):92-97.

[36.] Pracros, P.; Joel, R.; Sandrine, E.; Armand, M. and Michel, H. (2007). Tomato flower abnormalities induced by stolbur Phytoplasma infection are associated with changes of expression of floral development genes. Mol. Pl. Mic. Int ., 19: 62-68.

[37.] Pribylova, J.; Petrzik, K.and Spak, J. (2011). Association of aster yellows subgroup 16Sri-C phytoplasmas with a disease of Ribes rubrum. Bull. Insectol., 64: 65-66.

[38.] Rocchetta, I.; Leonard, P.I.and Filho, G.M.A. (2007). Ultrastructure and x-ray microanalysis of Euglena gracilis (Euglenophyta) under chromium stress. Phycologia, 46:300-306.

[39.] Salehi, M.; Izadpanah, K.and Taghizadeh, M. (2000). Herbaceous host range of lime witches' broom phytoplasma in Iran. J. Plant Pathol., 36(3): 343-353.

[40.] Salehi, M.; Nejat, N.; Tavakoli, A. R.and Izadpanah, K. (2005). Reaction of citrus cultivars to Candidatus phytoplasma aurantifolia in Iran. J. Plant Pathol., 41(3): 147-149.

[41.] Samuitiene, M.; Jomantene, R.; Valiuans, D; Navalinskiene, M. and Davis, R.E. (2007). Phytoplasma strains detected in ornamental plants in Lithuania. Bull. Insectol., 60(2):137-138.

[42.] Sinclair, W.A.; Griffiths, H.M. and Davis, R.E. (2000). Ash yellows and llilac witches-broom: phytoplasma diseases of concern in forestry and horticulture. J.Plant Dis., 80:468-475.

[43.] Singh, M.; Chaturvedi, Y.; Tewari, A.K.; Govind, P.R.; Sunil, K.S.; Shri, K. and Khan, M.S. (2011). Diversity among phytoplasmas infecting ornamental plants grown in India. Bull. Insectol., 64:569-570

[44.] Wang, and Hiruki, H. (2001). Use of Heteroduplex Mobility Assay for identification and differentiation of phytoplasma in the aster yellows group and the clover proliferation group, Phytophathology, 91:246-252.

[45.] Zreik, L.; Carle, P.; Bove, J.M. and Garnier, M. (1995). Characterization of the mycoplasma-like organism associated with witches' broom disease of lime and proposition of a Candidatus taxon for the organism "Candidatus Phytoplasma aurantifolia". Int. J. Syst. Bacteriol., 45: 449-453.