Phytoplasma Disease of Jasminum sambac L. in Egypt: Molecular Identification, Host Responses, and Pathobiological Implications

Maha A. El-Abhar

elabharmaha@yahoo.com

Plant pathology research institute, ARC, Egypt https://orcid.org/0000-0002-6118-4769

Dr/ Sherin Nahas

Plant pathology research institute, ARC, Egypt https://orcid.org/0000-0002-7702-0857

Farrag A.A.

Plant pathology research institute, ARC, Egypt https://orcid.org/0000-0002-2306-474X

Ahmed A. Kheder

Plant pathology research institute, ARC, Egypt https://orcid.org/0000-0002-1647-5954

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Phytoplasma Disease of Jasminum sambac L. in Egypt: Molecular Identification, Host Responses, and Pathobiological Implications

Maha A. El-Abhar[1], Shereen E.M. El-Nahas[2], Farrag, A.A.[1] and Ahmed A. Kheder[1]

[1,] Virus and phytoplasma Res. Dep., Plant Path. Res. Inst., ARC, Giza, Egypt

[2] Integrated Pest Control Res. Dep., Plant Path.Res. Ins., ARC, Giza, Egypt.

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Abstract:

In September 2023, yellow blotches, slight leaf malformation, stunting, flower color alteration and thickened bark above the phloem interruption point symptoms, as a virus or phytoplasma-like infection, were observed on *Jasminum sambac* L. plants in Ismailia and Giza governorates, Egypt. Collected samples were tested by direct ELISA, using the available antisera specific for AMV, PVY, ToRSV, PVX, PLRV, TMV, CMV, ToMV, and TYLCV; however, all tests were fruitless (negative). To diagnose these symptoms as phytoplasma-associated, Dienes' stain was used, followed by examination under incident light microscopy of the *Jasminum sambac* L. plants. Blue-stained areas were clearly obcerved in the infected phloem tissues, indicating the presence of phytoplasma while we did not notice those blue areas in the healthy phloem (control). Furthermore polymerase chain reaction (PCR) was used successfully with positive results. The amplified products of nested PCR were purified and then sequenced. Sequencing and phylogenetic analysis were performed to identify the detected phytoplasmas. The partial sequence was conducted by the NCBI GenBank database with accession number PP970365, and its definition was *'Jasminum sambac* L.' phytoplasma clone PVIDL 16S ribosomal RNA gene, partial sequence with Taxonomy ID: 2030933. Phylogenetic analysis showed a high similarity (97%) with *Candidatus Phytoplasma* sp.16Sr-IID isolates. Phytoplasma affects plants through different mechanisms, and so, the extent of the effect of phytoplasma on individual *Jasminum sambac* L., plants was studied in terms of anatomical and biochemical studies to demonstration the relationship between plant defense and effect of phytoplasma on histological and cytological changes, and subsequently, biochemical changes. This study aims to provide the first documentation of the morphological symptoms of phytoplasma infection of Arabian jasmine *(Jasminum sambac* L.) by the plesiidae insect. This plant is cultivated in Egypt for ornamental, aromatic, and medicinal purposes. The study also aims to achieve molecular confirmation of phytoplasma infection using polymerase chain reaction (PCR), deposit the extracted sequence in GenBank, and characterize the phytoplasma strain and its evolutionary relationships. Furthermore, it seeks to determine the plant's defense mechanisms and

resistance, the effects of the disease on the plant, and its pathological indicators, thus contributing to the integrated management of this disease in ornamental plants in general and jasmine in particular.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author Contributions
M.A. El-Abhar conceived and designed the study, conducted the experiments, performed the electron microscopy and biochemical analyses, analyzed and interpreted the data, and prepared the manuscript. S.E.M. El-Nahas participated in the chemical analyses and manuscript revision. A.A. Kheder and A.A. Farrag contributed to the molecular characterization of the phytoplasma. All authors read and approved the final version of the manuscript.

Correspondence: Maha A. El-Abhar
maryam.maher73@gmail.com
https://orcid.org/0000-0002-6118-4769
Shereen E.M. El-Nahas
Shereen.nahas@gmail.com
https://orcid.org/0000-0002-7702-0857
Farrag, A.A.
PL_VIROLOGY@yahoo.com
https://orcid.org/0000-0002-2306-474X
Ahmed A. Kheder1
Ahmedkheder@hotmail.com
https://orcid.org/0000-0002-1647-5954

Introduction:

Jasminum sambac L. is a tiny shrub growing up to 0.5 to3 m in height of ornamental plant belongs to the genus Jasminum of Oleaceae family. The species is highly variable; there are several varieties of jasmine plant relying on the flower size and the number of petals per flower. possibly a result of spontaneous mutation, natural hybridization, and autopolyploidy. The flowers may be used as a fragrant ingredient in jasmine tea, and it's are not only essential perfumes, but also have been highly appreciated for great medicinal value for treating as severe depression, respiratory tracts, for muscle pain and for toning the skin, this oil is expensive (Klaus and Joachim 2004; Skaria 2007; Lokhande *et al.,* 2015; Patil *et al.,* 2018). Jasmine sp. are infected with many diseases including phytoplasma that are causing a wide range of diseases include discoloration of leaves, leaf curling, virescence, phyllody, witches' broom, stunted growth, mild yellowing, and general decline to death

(Dijkstra and Lee, 1972.; Lee *et al.,* 2004; Bhat *et al.,* 2006 and Al-Kuwaiti *et al.,* 2018; Hemmati *et al*., 2021). Phytoplasma are found on a wide range of economically important plant species, causing damage globally that affects economic, social, and environmental inclusion. (Chaturvedi *et al.,* 2010; Bertacini and Dudok, 2009; Bertacini *et al.,* 2014). In the Middle East (a cross-border region including West Asia, Iran, Egypt, and Turkey) agriculture plays a vital economic role that affect by phytoplasma, which have been reported as a concern in the Middle East. And to date, there are fourteen 16Sr groups that have been identified from phytoplasma in association with more than 164 plant species (Al- and Mardi *et al.,* 2011; Al-Harthi *el al.,* 2013; Kolmer *et al*., 2020). In Egypt many phytoplasma groups/subgroups have increased reports during the last two decades and are registered on the gene bank website NCBI; from 2009 to 2023 it has recorded approximately 38,838 isolates on different plants.

Among the methods for detecting phytoplasma are (1) biological (by symptoms), which is the first indicator of the presence of the disease; (2) light microscopy using stains such as dienes' stain and aniline stain; (3) electron microscopy, whether semi-thin section or ultra-thin section; (4) serological examination methods that produce monoclonal or polyclonal to identify phytoplasma, which are of a high degree of accuracy and specialization, but requires high concentrations of phytoplasma in the plant; and (5) using molecular biological methods, especially PCR. (Bai *et al.,* 2009, 2022). Phytoplasmas are transmitted experimentally by vegetative propagation; grafting (Caglayan *et al.*, 2019), and they are spread mainly by insects vectors, primarily leafhoppers, planthoppers, and psyllids (Salehi *et al.,* 2005; Weintraub and Beanland, 2006; El-Banna *et al.,* 2007; El-Banna *et al.,* 2015). Using genetic sequences and phylogenetic trees as well as modern detection methods are considered essential for a clear vision of determining the diversity, classification, and evolution of strains and the extent of their effective role in understanding the genetic relationships with each of the pathogenicity traits, the host and the vector, and thus understanding the biology of phytoplasma and developing integrated tool strategies to fight it. Climate change has an impact on the life cycle of insects. Climate change has an impact on the life cycle of insects, host susceptibility, and also the ability of phytoplasmas to infect, as they may become more active and input to new areas, thus expanding their geographical range distribution. Subsequently, the detection of phytoplasmas and the study of their relationship with plants and surrounding environmental factors are essential for the integrated management of phytoplasma diseases, which often aims to prevent them. Based on Dermastia (2019), phytoplasma affects the hormonal balance of the plant, chemical studies, whether on the effect of the phytoplasma on the plant or on the plant's reaction as resistance to the disease, provide information that contributes to control using substances that affect the hormonal balance in

the plant or increase the plant's resistance to the disease, and therefore are an effective tool in developing more plant varieties by understanding resistance mechanisms within the plant.

This study aims to assess the extent of damage and changes caused by infection in terms of biochemical changes using high-performance liquid chromatography (HPLC), anatomical changes using electron microscopy, and molecular identification of infection of the jasmine plant (*Jasminum sambac* L.) as an ornamental, aromatic, and medicinal plant in Egypt.

Materials and Methodes:

Source of the phytoplasma isolate:

Leaves samples were collected on the basis of visual like symptoms of yellowing, blotches, malformation and little leaf, and flower color refraction from (*Jasminum sambac* L.) plants cultivated in Ismaelia and Giza Governorate, Egypt during the growing 2023; note, during the search, the presence of signs and symptoms of insect infestation

Identification and characterization

In the current study, several diagnostic and characterization methods were successfully applied to verify virus- or phytoplasma-like symptoms in the tissues of infected plants including the following:

a- Serological test

Collected samples were tested by double antibody sandwich- enzyme linked immunosorbent assay (DAS-ELISA) to detect and identify AMV, PVY, ToRSV, PVX, PLRV, TMV, CMV, ToMV, and TYLCV as described by Clark and Adams (1977). ELISA kit was supplied by LOEWE (Biochemica Gmbh, D-82054 Sauerlach, Muhlweg 2a, Germany) at Phytoplasma Research Department, Plant Pathology. Research Institute, ARC.

b- Light microscope detection

At this stage, phytoplasma are visually detected by a light microscope through three methods of detecting tissue infected with phytoplasma.

a) Free hand cross sections were taken of the midribs of petioles leaves from infected plants and dienes' stained that were prepared as described by Hayflick (1965), Deeley *et al.,* (1979) and Musetti (2013).
b) According to Mazia *et al.,* (1953) epidermal strips of infected leaves were removed from the underside of infected leaves using forceps. The strips were dipped for 5 minutes in 5% Trediton X-100 solution. After that, they were stained with Dienes' stain for 15min. The treated strips were placed in 0.5% acetic acid for 15min., followed by washing in tap water for 15min.
c) Scraping the underside of the leaf with a sharp scalpel, followed by dienes stained

Any of the previous three methods examined immediately by light microscope (OptiKa B-353, Italy) with a camera (ATPEK) attached (10 X) at Phytoplasma Research Department, Plant Pathology. Research Institute, ARC.

c- Molecular characterization

DNA isolation and PCR-based assay:

Total DNA was extracted from naturally infected and healthy *Jasminum sambac* L. plants collected from Giza and Ismailia governorates, using the Dellaporta extraction method (Dellaporta *et al.,* 1983). About 1g of leaf sample was homogenized and grind into a fine powder using a mortar and pestle 500µl of Dellaporta buffer (100 mM Tris-HCl, 20 mM EDTA, 1.4 M NaCl, pH 8.0), DNA was precipitated by adding an equal volume (v:v) absolute ethanol then collected by centrifugation, washed twice in 70% ethanol, air dried and resuspended in 35µl TE buffer (10mM Tris, 1mM EDTA, pH 8.0). For the first PCR reaction, direct PCR assays were performed with primer pair P1/P7 as described by Schneider *et al.,* 1995. DNA concentration was measured using Nanodrop one C, two µl of extracted DNA was used in a 25µl total PCR reaction mixture containing 12.5µl ama***R*** OnePCR™ ready-to-use PCR reaction mixture (GeneDireX, Inc.); 10 pmol of each primer. PCR product was used as a template for nested PCR with R16F2n/R16R2 (Deng and Hiruki, 1991). DNA amplification was started with 94°C for 3 min for the denaturation step, followed by 35 cycles consisting of 94°C for 3 seconds for denaturation, followed by annealing at 53°C for 1min, extension 72°C for 2min, and a final step for 1 cycle of extension at 72°C for 7min. Total DNA from infected Catharanthus roseus plants (Acc. No. OP730893.1) was used as a positive control as well as DNA from an asymptomatic *Jasminum sambac* L. plant was used as a negative control. The PCR amplicons were stained with EZ view nucleic acid stain (Biomatic, Inc., USA) and analyzed by electrophoresis in a 1.5% agarose gel, then visualized using a UV transilluminator. The PCR fragment size standard was compared with a 100bp DNA ladder (BioMatic, USA).

Table (1). Primers used in PCR reactions to identify phytoplasma on *jasminum sambac* L. plants

Primer			
P1 (forward)	AAGAGTTTGATCCTGGCTCAGGATT	1800 bp	Deng and Hiruki,1991)
P7 (reverse)	CGTCCTTCATCGGCTCTT		
R16F2n (forward)	GAAACGACTGCTAAGACTGG	1200 bp	Schneider *et al.,* (1995)
R16R2 (reverse)	TGACGGGCGGTGTGACAAACCCCG		

Nucleotide sequencing and sequence analysis

The amplicon of the spacer region R16F2n/R16R2 of the phytoplasma genome was purified using the QIAquick PCR Purification Kit, and was directly sequenced in both directions using an ABI 3730XL automated DNA sequencer. Obtained sequences were assembled, and phylogenetic and molecular evolutionary analyses were conducted using MEGA version 11 (Tamura *et al.,* 2021). The nucleotide sequence was compared with thirty-two other phytoplasma isolates from different groups and geographical regions that were deposited in the GenBank.

Electron microscope

Everything that happened at this stage, whether phytoplasma particles, semi-thin or ultra-thin sections, took place at the Agricultural Research Institute (FARP), TEM Laboratory, Faculty of Agriculture, Cairo University Research Institute (CURP).

Phytoplasma particles

Phytoplasma particles were shown in crude sap extracted from *Jasminum sambac* L. infected leaves according to Hunter (1993) method using a transmission electron microscope (JEOL, Tokyo, Japan) at 80 kv., Images were captured by a side-mounted CCD digital camera (AMT Optronics, 1632 x 1632 pixel format).

Histological and cytological changes

Transmission electron microscopy (TEM) was used to examine the ultrastructural effects of phytoplasma infection on Jasminum sambac leaves. Semi-thin and ultra-thin sections of petiole tissues were prepared according to the methods described by Hanker and Giamara (1993) and Bozzola and Russell (1999). Semi-thin sections (0.5–1 µm thick) were prepared using glass knives with a Leica Ultracut UCT ultramicrotome and stained with 1% toluidine blue in 1% borax for light microscopy examination. Ultra-thin sections (75 - 90 nm thick) were cut using ultra microtome, mounted on copper grids (400 mesh). Sections were stained with double stain uranyl acetate and Lead citrate, and allowed to air dry as described by El-Banna *et al.,* (2007); Rocchetta *et al.,* (2007). Finally, the sections were examined using a JEOL (JEM-1400) transmission electron microscope. Images were captured by a side-mounted CCD digital camera (AMT Optronics camera, 1632x 1632 pixel format).

E- Biochemical analysis: Its aim is to assessment plant defense mechanism and investigate the biochemical effect associated with phytoplasma infection. This point was carried out in the Central Laboratory, Agricultural Research Center in conjunction with the Mycology Center at Al-Azhar University

HPLC conditions for phytohormons separation: Samples of frozen leaves from both infected and healthy (*Jasminum sambac* L.) were cut into pieces and mixed thoroughly, with each sample individually according to (Qi, *et al.,* 2021). Phytohormons separation were quantitatively analyzed using a reverse-phase HPLC system with a UV system. The HPLC analysis was conducted using an E-Chrom Tech Model LC-1620A high-performance liquid chromatography (HPLC) system, equipped with a UV-Vis detector. Standard solutions were prepared for the investigated phytohormones, namely: absecic acid, salysylic acid, jasmonic acid and auxin (Sigma-Aldrich,

Merck, Darmstadt, Germany). (Xie and Zhang, 2001; Wu and Hu, 2009). But for the auxin (indole 3-acetic acid), HPLC separation conditions are according to Dumale (2018).

Estimation of enzyme activities: Peroxidase (POD) activity was measured by methods' Singh *et al.,* (2013), and the absorbance of yellow product was recorded at 470 nm.. **Polyphenol oxidase (PPO)** enzyme activity was measured according to Goel and Paul (2017) and absorbance was recorded at 420 nm.. **Superoxide dismutase (SOD)** activity was assayed based on its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) by Kumar (2012). A control reaction was prepared without crude extract. The SOD reaction was carried out by exposing the reaction mixture to white light for 15 min at room temperature. After 15 min incubation, absorbance was recorded at 560 nm. One unit (U) of SOD activity was defined as the amount of enzyme causing 50% inhibition of photochemical reduction of NBT. using a UV-visible spectrophotometer (Milton Roy, Spectronic 1201) to measurement for each PPO, POD and SOD. A standard curve was plotted with the absorbance data in experimental plant samples.

Secondary Metabolite Analysis

Determination of chlorophyll: leaf chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid content were determined using the ARNON method, as described by Gu et al. (2016). A UV-Vis spectrophotometer was used to measure the absorbance of chlorophyll extract at 645 nm, 663 nm, and 470 nm.

Total flavonoid content: the method outlined by Zhishen *et al.,* (1999). The reaction was measured at 510 nm using a spectrophotometer (Spectronic 106). The results were expressed as µg quercetin/g dry weight (DW). Determination of anthocyanin: According to the method described by Fuleki and Francis (1968), anthocyanin pigments of sample extraction from flowers in each infected and healthy plant were performed. The analysis was performed in three replicates for each treatment.

Extraction of essential oils

Essential oil was extracted using a Soxhlet apparatus, utilizing n-hexane as the organic solvent according to Mahmood *et al.,* (2017). 10 grams of jasmine petals were tightly packed in Whatman filter paper and placed within the Soxhlet apparatus. n-Hexane was added to the flask and heated to a temperature between 55 and 60°C. The vaporized solvent traveled through the thimbles, extracting the oil, and was then condensed. The residue was weighed for % yield calculation.

Results and Disscusion:

Phytoplasma identification

Visualization of symptoms by gross anatomy

Any plant pathogens alter normal plant plant developmental processes, resulting in abnormal morphology in infected host plants. In the present study, Phytoplasma was isolated from naturally

infected jasmine (*Jasminum sambac* L.) plants collected from Ismaillia and Giza governorate, Egypt. Visual symptoms as yellow blotches, little malformation leaves, stunting vein necrosis, flower discoloration and thickening bark above the phloem interruption point symptoms as (Fig. 1).

Fig. (1): Naturally (*jasminum sambac* L.) plants show healthy (a) and yellow with blotches, malformation leaf, stunt, vein necrosis, refraction flower color and thick bark above the phloem interruption point symptoms as Phytoplasma-like infection (b)

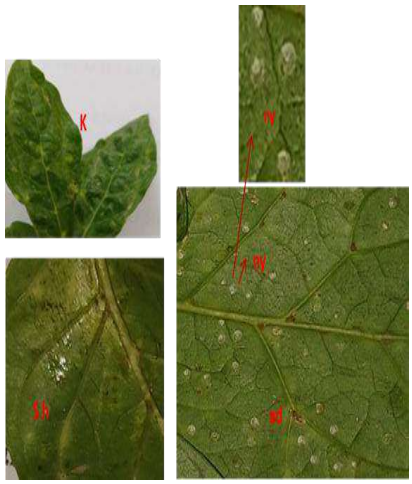

Fig. (2): Symptoms of infestation by the psyllid insect include the nymphs are found on top of the leaves and scale-like flat nymphs (ny), leaf crinkle (c), and sticky honeydew (sh)., while the adults (ad) are orange to brown in color and have wings

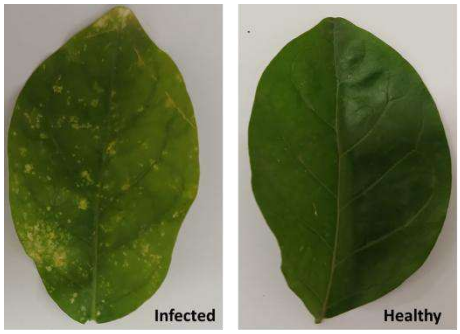

Fig. (3): Comparison between healthy and infected leaf of *jasminum sambac* L with phytoplasma

Phytoplasmas are a large group of plant pathogenic wall-less, non-helical, bacteria associated with plant diseases, collectively known as yellows diseases and others in more than a thousand plant species worldwide (Bertaccini *et al*., 2014). *Jasminum sambac* L. infected with phytoplasma illustrated in more papers such as Al-Zadjali *et al*., (2007); Rao and Khurana (2015); Gopala and Rao (2018); Hemmati *et al*., (2021); Ahmed and Khan (2021). The appearance of these visible symptoms indicates a severe disturbance. It can be attributed to several causes, including disorders in the balance of growth regulators. This is phenomenon considered one of the primary reasons for categorizing phytoplasmas as "genetic diseases," which leads to deformities in various plant organs. On the other hand, gradual partial or complete blockage of phloem flow leads to the accumulation of dissolved organic materials (such as amino acids and sugars), which causes a series of symptoms,

also affecting the roots **(**Arashida, 2008; and Pagliari, 2017**).** Bare bark necrosis or vein necrosis in leaf veins were also observed (Rita and Laura 1875). Additionally, through plant examination, the signs and symptoms of insect infestation by psyllid insects include nymphs, insect eggs, leaf curling, and sticky honeydew. The nymphs are found on the upper side of the leaves and are flat, resembling scales, while the adults are orange to brown and have winged (Figure 2), as mentioned in Tedeschi *et al.,* (2006), Gonella *et al.,* (2008), Marcone (2014), Caglayan *et al.,* (2019), Asudi *et al.,* (2021), Huang *et al.,* (2021), and Wang *et al.,* (2024) who reported that phytoplasmas are mainly transmitted by insects of the families Cicadellidae (leafhoppers), Fulgoridae (planthoppers), and Psyllidae (jumping plant lice), and vegetative propagation techniques. Figure (3) showed comparison between healthy and infected leaf of *jasminum sambac* L.

Serological test

Double antibody sandwich- Enzyme linked Immunosorbent assay (DAS-ELISA) was used to detect and identify AMV, PVY, ToRSV, PVX, PLRV, TMV, CMV, ToMV, and TYLCV as described by Clark and Adams (1977) to all Collected samples were tested by ELISA kit was supplied by LOEWE (Biochemica Gmbh, D-82054 Sauerlach, Muhlweg 2a, Germany). However, all viruses tests were fruitless (negative). Thus diagnose these symptoms as phytoplasma infection via Dienes' stain under light microscopy.

Light microscope (LM)

Dienes' stain is considered one of the earliest diagnostic methods used for preliminary detection of phytoplasmas, and it is also applied to investigate their distribution pattern within the phloem tissues of infected plants (Deeley *et al.,* 1979). This stain revealed irregular blue patches resulting from intense staining of phloem cells surrounding the affected regions in cross sections, which is regarded as an indication of phytoplasma DNA accumulation. These changes can be observed under a light microscope (LM) within the phloem tissues, particularly in sieve elements and companion cells. This was clearly documented in Figure (2a–d) using three different sample preparation methods: hand-prepared cross sections (Fig. 2a), epidermal strip (Fig. 2b), and scraping with a scalpel (Fig. 2c) by illustrative enlargement using Dienes' stain. Meanwhile, figure (2d) represented Mazia *et al.,* (1953) method but used with scraped underside surface instead of epidermal strips infected petiole leaf tissue after pressed between two glass. This showed that the Mercuric Bromophenol Blue (MBB) stain gave intense staining of proteins associated with the presence of phytoplasm, relying on it being an acidic stain that reacts with the active protein groups (Bhandari, 1997), but it is not specific and not confirmatory for diagnosis like Dean's stain as noticed in Deeley *et al.,* (1991) who observed that Diene's stain was specific for phytoplasma diseases and gave no reaction in healthy tissues and in samples infected by other pathogens whereas a quick and easy preliminary test for rapid assessment

of plant samples, this result was confirmed by many sources (Das and Mitra 2004; Ammar *et al.,* 2005; Fadhil, 1997 and Moslemkhani, *et al.,* 2014).

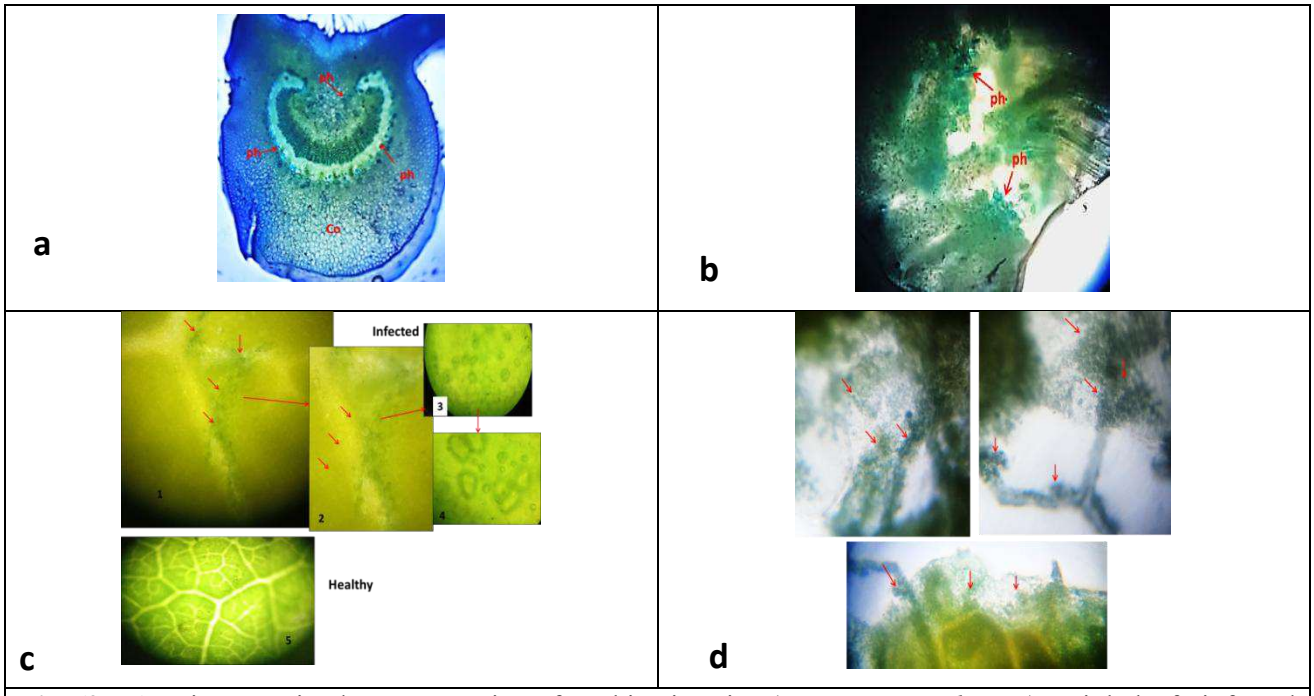

Fig. (2a-d): Diene's stained a cross-section of Arabian jasmine (*Jasminum sambac* L.) petiole leaf infected with phytoplasma (a), and crushed epidermal strips (b), scraped to reveal infected vein tissue (scraping method) (c) with various magnifications from 1to4 of infected and healthy 5. Mazia *et al.,* (1953) method but used underside surface crushed instead of epidermal strips infected petiole leaf tissue (d)

The aim of this point, diagnosis, localization, and identification of phytoplasmas in the infected tissues using light microscopy (LM) techniques that effective only for detecting cells containing high concentrations of the pathogen. Dienes' stain is important as a semi-specific diagnostic stain for phytoplasma. To confirm the identification and achieve more accurate diagnosis, molecular biology techniques were used.

Molecular identification

DNA isolation and PCR-based assay:

Total DNA was successfully isolated from naturally infected and healthy *Jasminum sambac* L. plants collected from Giza and Ismailia governorates, and was used for direct PCR using universal primer pairs. Results showed that all symptomatic samples generated a product about 1200bp. whereas no visible bands were amplified from healthy samples (Fig. 3).

Nucleotide sequencing and sequence analysis

Without molecular sequencing the identity of the infection can't be determined. Therefore, the nucleotide sequence of the nested PCR-amplified product of the 16S rDNA for the Egyptian Jasminum sambac L. isolate (PVIDL) was successfully determined (deposited in the GenBank

database under accession number PP970365.1). Sequence alignment and phylogenetic analysis were conducted to compare with other corresponding sequences in the GenBank database (Fig. 4).

Fig. (3): Agarose gel electrophoresis of PCR amplification products obtained by using nested primer pair R16F2n/R16R2 of phytoplasma 16S rDNA gene detection from infected (*jasminum sambac* L.) samples showing positive results of phytoplasma. M: marker weight marker (100bp ladder). Lanes (L1 to L5 and L7): DNA isolated from infected different samples with phytoplasma-like symptoms at (1200 bp) and L6: Healthy plant negative control (HPC)

The results showed that the isolate was a member of the 16SrII group. Phylogenetic analysis revealed high similarity from 99.2% to 98.8% with Candidatus Phytoplasma 16Sr-II from Egypt and Croton bonplandianus phytoplasma, Candidatus Phytoplasma citri from India, respectively. Moreover, it showed 98.6%, 98.5%, 98.4% and 98.1% identities with Jute Phyllody phytoplasma (KM103728.1) from Turkey, Gerbera phyllody Phytoplasma (AB026155.1) from Australia and Australian tomato big bud and Lablab Bean Bud Proliferation Phytoplasmas 16Sr-IID (Y08173 and KP899065.1) from Australia and India respectively, which belong to the same subgroup (Fig 3 and 5 and table 2).

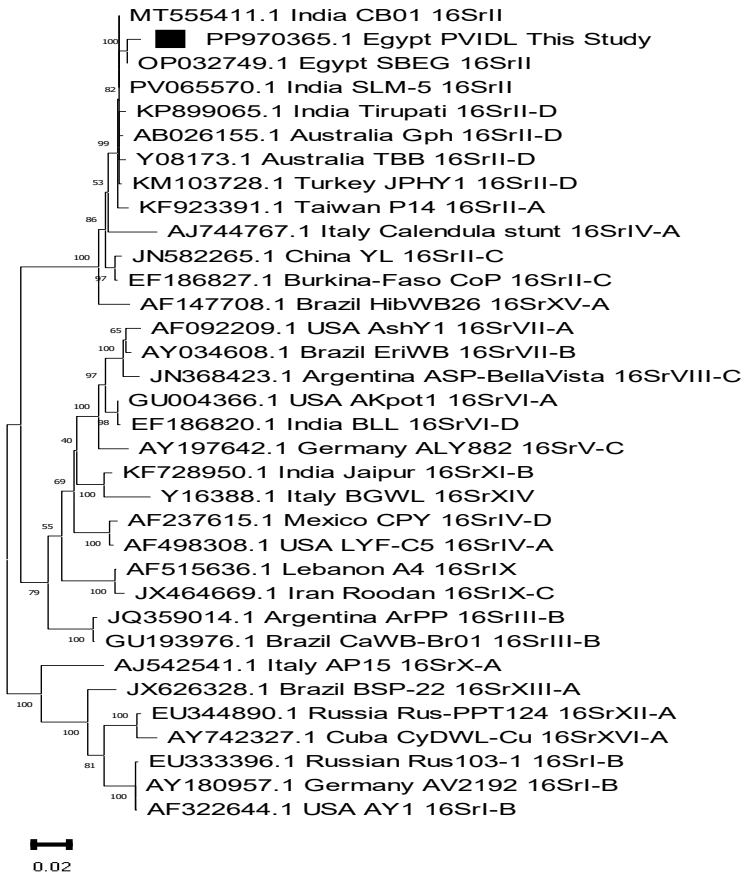

Fig. (4): The phylogenetic tree of Candidatus Phytoplasma 16S rDNA gene was constructed using MEGA11, with 33 previously identified sequences available in GenBank as reference strains showing the phylogenetic relationships for the partial 16S rDNA nucleotide sequences for the Egyptian PVIDL isolate (*Jasminum sambac* L.).

In addition, the comparison showed that the PVIDL isolate (PP970365.1) shared 98.1% sequence homology with stem curling phytoplasma from Taiwan. Identity with other subgroups ranged from 97.3% to 83.9% as shown in Table 2 and Fig.5. Sister taxa was It is represented in MT555411.1 india CB01 16rII and PP970365.1 Egypt PVIDL relationship isolate (Fig. 4). Polygenic relationship for each isolate within the same group (16r II). Information obtained using molecular methods such as sequencing of the 16S rRNA gene can be used to determine the appropriate management strategies for the phytoplasma disease. In this context, the current classification of phytoplasmas is primarily based on 16S rRNA gene sequences. Subgroups *Candidatus Phytoplasma* group II have a 98.8% similarity threshold, while subgroups IIA, IID, and IIC have a 97.1% similarity threshold. Further subgroups have lower thresholds depending on variation and characteristics; however, no species name exists, consistent with the findings of Bertaccini (2019).

Table (2). Details of the 33 phytoplasma strains reference and Egyptian *Jasminum sambac L.* isolate (PVIDL) (PP970365.1) phytoplasma isolates in the present study used in building the phylogenetic tree.

GenBank	Disease	16Sr	Host	Country	I.d %
PP970365.1	……	II	*Jasminum sambac*	Egypt	……
OP032749.1	*Candidatus Phytoplasma sp.*	II	*Sugar beet*	Egypt	99.2%
MT555411.1	*Croton bonplandianus phytoplasma*	II	*Croton bonplandianus*	India	98.8%
PV065570.1	*Candidatus Phytoplasma citri*	II	*Sesame*	India	98.8%
KM103728.1	*Jute Phyllody*	II-D	*Corchorus olitorius*	Turkey	98.6%
AB026155.1	*Gerbera phyllody*	II-D	*Gerbera jamesonii*	Australia	98.5%
Y08173	*Australian tomato big bud*	II-D	*Lycopersicon esculentum*	Australia	98.4%
KP899065.1	*Lablab Bean Bud Proliferation*	II-D	*Lablab purpureus*	India	98.4%
KF923391.1	*Stem Curling*	II-A	*Sesamum indicum*	Taiwan	98.1%
EF186827.1	*Cotton Phyllody*	II-C	*Gossypium hirsutum*	Burkina Faso	97.3%
JN582265.1	*Cactus WB*	II-C	*Opuntia spp.,*	China	97.1%
AF147708	*Hibiscus witches' broom*	XV-A	*Hibiscus*	Brazil	95.9%
AJ744767.1	*Calendula stunt*	IV-A	*Calendula arvensis*	Italy	95.4%
GU193976.1	*Cassava Witches' Broom*	III-B	*Manihot esculenta*	Brazil	89.1%
JQ359014.1	*Argentinean Peach Phytoplasma*	III-B	*Prunus persica L.*	Argentina	88.9%
AF498308.1	*Coconut lethal yellowing*	IV-A	*Cocos nucifera*	USA	88.0%
KF728950.1	*Jasminum leaf yellows and WB*	XI-B	*Jasminum sambac,*	India	88.0%
AF515636.1	*Almond witches' broom*	IX	*Prunus amygdalus,*	Lebanon	87.8%
GU004366.1	*Potato purple top-AK*	VI-A	*Solanum tuberosum*	USA	87.8%
AF237615.1	*leaf yellowing*	IV-D	*Carludovica palmata*	Mexico	87.7%
EF186820.1	*Brinjal little leaf*	VI-D	*Solanum melongena,*	India	87.6%
JX464669.1	*Eggplant Phyllody*	IX-C	*Solanum melongena,*	Iran	87.4%
AY197642.1	*Alder yellows phytoplasma*	V-C	*Alnus glutinosa*	Germany	87.1%
AY034608.1	*Erigeron witches'-broom*	VII-B	*Erigeron sp.*	Brazil	87.0%
AJ542541.1	*Apple proliferation*	X-A	*Malus domestica,*	Italy	86.9%
JN368423.1	*Argentinean Strawberry Phyllody*	VIII-C	*Fragaria x ananassa*	Argentina	86.5%
AF092209.1	*Ash Yellows*	VII-A	*Fraxinus americana,*	USA	86.4%
JX626328.1	*Broccoli Stunt*	XIII-A	*Brassica oleracea*	Brazil	86.2%
Y16388	*White leaf*	XIV	*Cynodon dactylon,*	Italy	85.9%
AY180957.1	*Aster yellows*	I-B	*Callistephus chinensis*	Germany	85.2%
AF322644.1	*Aster yellows*	I-B	*Catharanthus roseus*	USA	85.1%
EU333396.1	*Potato Purple Top*	I-B	*Solanum tuberosum*	Russia	85.0%
EU344890.1	*Potato Purple Top-RU*	XII-A	*Solanum tuberosum*	Russia	84.8%
AY742327	*Cynodon white leaf*	XVI-A	*Cynodon dactylon*	Cuba	83.9%

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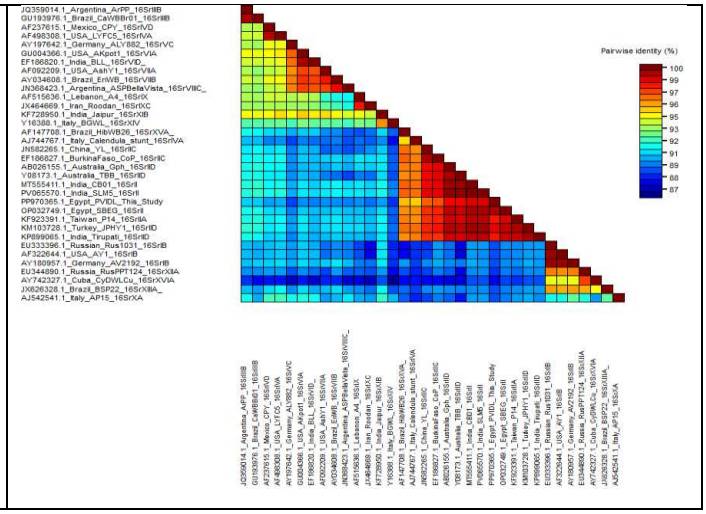

Fig. (5). The percentage of nucleotide identities of the Candidatus Phytoplasma 16S rDNA gene (Egyptian PVIDL isolate) used in the present study (PP970365.1) was calculated using the Sequence Demarcation tool (SDT v1.3).

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Electron Micrscope

Phytoplasma particles

In Figures (6a-c), electron microscopy observations revealed that phytoplasma bodies are characterized by a very high degree of polymorphism. They were observed as high-density concentrations, appearing as dense spherical aggregates with budding forms undergoing binary fission or fragmentation. These are common phenomena and appear either as bead-like structures (6a) or as chain-like formations of phytoplasma particles resembling octopus-like chains (6c) (Lee *et al.,* 2000; Christensen *et al.,* 2004; Bertaccini *et al.,* 2014). In some cases, heteromorphic structures were observed that are capable of passing through narrow pores in the sieve plates. As shown in Figure (6b), this represents the process of phytoplasma penetration of the cell wall and entry into the plant cell was evident. In addition, their systemic movement within the host may be irregular, depending on the pattern of colonization within the tissue and/or the type of plant host, which is consistent with the findings reported by Christensen *et al.,* (2004) and McCoy et al., (1989). This contrasts with observations by Berges *et al.,* (2000) who noted that phytoplasma concentration is sometimes low or very low, particularly in woody hosts. In general, phytoplasmas display marked morphological variability, and these observable morphological differences likely represent different developmental stages of phytoplasma, facilitating systemic movement and adaptation to host conditions. These variations depend on multiple factors, including nutritional status, particle abundance, and age, in agreement with Marcone *et al.,* (1996). Similarly, Musetti and Pagliari (2019) suggested that this polymorphism represents an adaptation to environmental conditions, resulting from the absence
of a rigid cell wall.

Microscopic anatomical changes

Regarding the histopathological changes observed in Egyptian *Jasminum sambac* L. isolate deposited in the GenBank database under accession number PP970365.1, the observed changes are illustrated. Typical phytoplasma were detected in the phloem cells in semi and ultrathin sections of petiole leaf examined by electron microscopy. Phytoplasma infection leads to significant ultrastructural changes in plant tissues, including deterioration of cells components.

Histopathological affects

In figure (7a–c), phytoplasmas are localized within the sieve tubes, where they actively multiply and move systemically within the host plant, thereby interfering with the transport of water and nutrients throughout the plant. In addition, the accumulation of phytoplasma components may lead to an

increase in the thickness of phloem cell walls (Christensen et al., 2004). Their distribution within the sieve tubes may be irregular, and their concentration is often low, particularly in woody hosts (Berges et al., 2000).

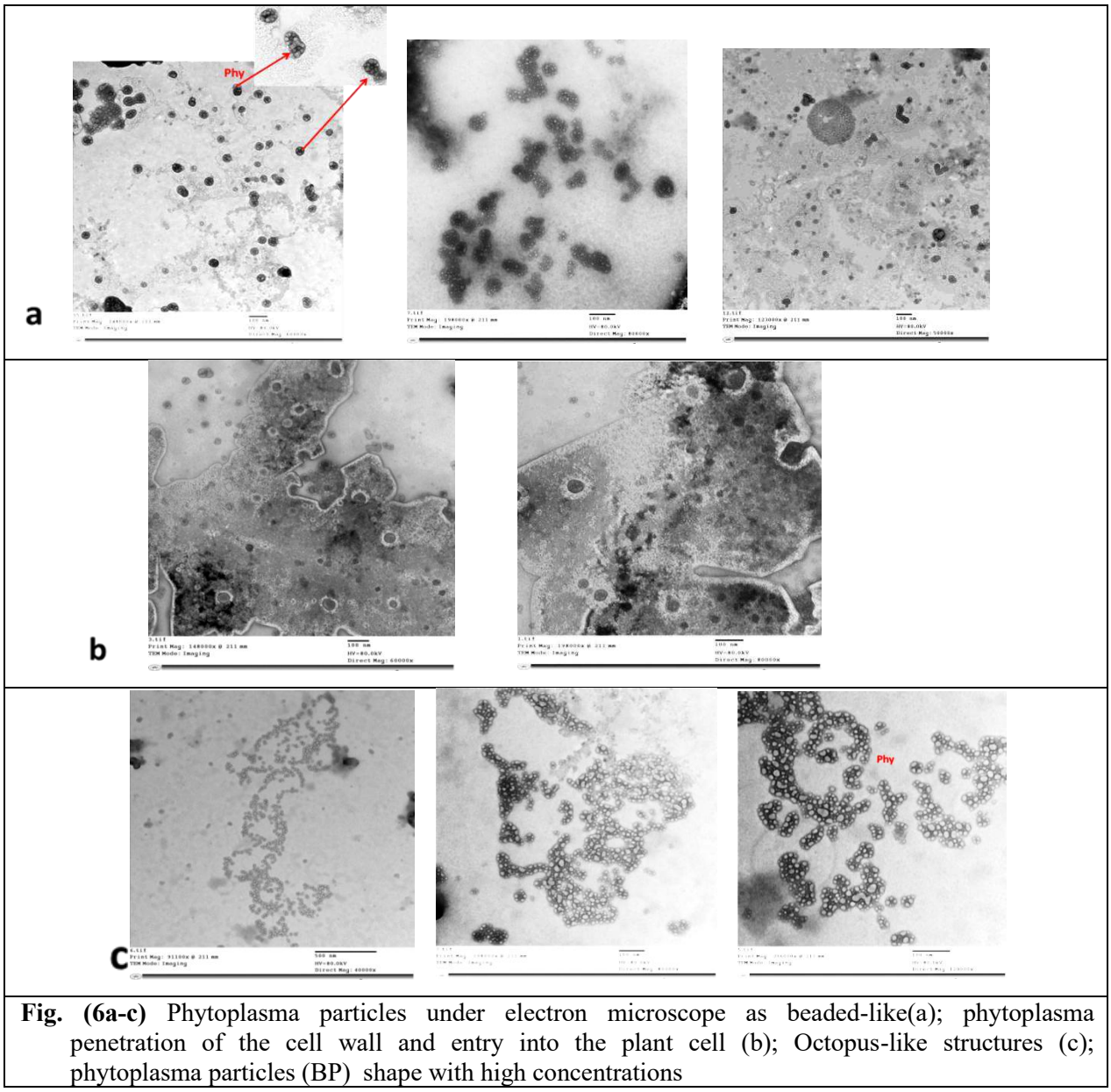

Fig. (6a-c) Phytoplasma particles under electron microscope as beaded-like(a); phytoplasma penetration of the cell wall and entry into the plant cell (b); Octopus-like structures (c); phytoplasma particles (BP) shape with high concentrations

On the other hand, alterations in the arrangement of vascular bundles were observed, and in some cases abnormal vascular tissues were formed (Figure 7c). This phenomenon was first demonstrated by Doi *et al.,* (1967). Since then, numerous studies have used electron microscopy to detect phytoplasmas in phloem tissues and to investigate the associated tissue necrosis (Favali *et al.,* 1990; Musetti *et al.,* 1992; Musetti and Favali, 1999; Musetti *et al.,* 2000; Musetti *et al.,* 2002). Parenchyma cells exhibited clear disturbances, as xylem parenchyma cells differed from one another

in shape and structure, while the phloem elements appeared irregular. In addition, sieve tubes varied in both shape and number.

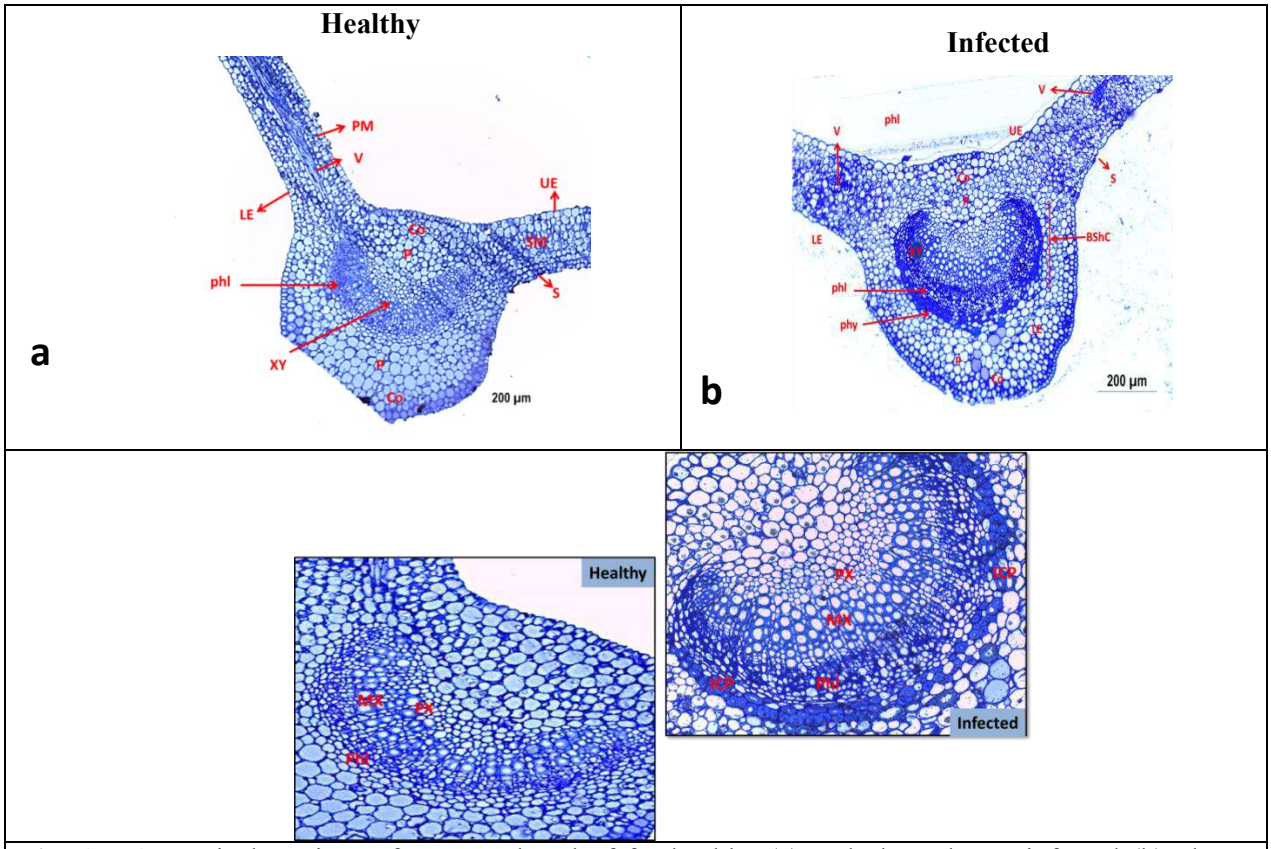

Fig. (7a-c). vertical sections of pepper plant leaf for healthy (a) and phytoplasma-infected (b) plants. ICP= infected cells with phytoplasma' PM = palisade mesophyll, SM = spongy misophil , Co= Collenshyma, P= Paranshyma, Xy= Xylem, PX= protoxylem, MX= metaxylem, phl= phleom, S= Stoma, LE= lower epiderm, UE= uper epiderm, BShC= Bundell sheath cell and V = Vein.

Anatomical measurements of phytoplasma-infected plants of *Jasminum sambac* L. also revealed an increase in all studied parameters compared with healthy plant leaves as presented in Table (3), Percentage increase in the width and length of the midrib reached 29.3% and 30.2%, respectively. The vascular bundle showed an increase of 51.22% in width and 48.32% in length, while xylem length increased by 43.36% and phloem length by 52.13%. An increase in leaf blade thickness was also observed, reaching 34.39%.

These results are consistent with the findings reported by Nassar *et al.,* (2017), Islam and Khatoon (2020), El-Sgai *et al.,* (2020), and Ahmed *et al.,* (2022), who indicated that phytoplasma infection affects leaf thickness and causes changes in the anatomical proportions of vascular tissues in the midrib. The data also revealed an increase in mesophyll tissues, including palisade parenchyma and spongy parenchyma, in response to phytoplasma infection. These findings agree with those reported by Boghdady *et al.,* (2012), Mikhail *et al.,* (2012), and Atala and Moya-Urrutia (2014).

Table (3). Mean of Anatomical differences betwen healthy and phytoplasma infected Jasminum sambac L stems.

Parameters (μm)	Infected	Healthy
Midrib width	723.86	511.75
Midrib length	723.16	504.95
Vascular bundle width	478.54	219.78
Vascular bundle length	227.6	117.63
Xylem length	114.12	64.64
Phleom length	66.04	31.61
Plate thikness	203.46	133.49

Cytotopathological affect:

The effect of phytoplasma on jasmine (*Jasminum sambac*) leaf cells was studied using an electron microscope. The main observations included the distribution and arrangement of phytoplasma particles, cytoplasmic deformations, and ultrastructural changes in organelles. The cytopathological effects (Fig. 8a–d) can be summarized as follows: since phytoplasma inhabits the phloem, its presence may hinder the transport of sugars and starch, leading to blockage and accumulation in the chloroplasts as shown in picture 8a, which may interfere with the photosynthetic process, according to wei *et al.,* (2022). Accumulation plastoglobuli appeared in picture (8a) as dense spherical bodies attached to the thylakoid membrane within the chloroplasts. These structures typically arise in response to stress, regulate membrane components, participate in lipid storage and recycling, and contribute to the biosynthesis of vitamin E (Bréhélin and Kessler, 2008; Lundquist *et al.,* 2012; Rottet *et al.,* 2015). Also, the appearance of central vacuoles (lysosomes) in the 8c segment, which engulf foreign bodies through autophagy, leads to the cleaning, maintenance, breakdown, and recycling of cellular materials within the cell, thus balancing energy needs during periods of stress. This is achieved through phagocytosis. In 1963, Christian René de Duve introduced the term autophagy and provided the first evidence of the role of lysosomes in the autophagy process.

Although the nucleus is not the primary site of phytoplasma infection, it is nevertheless noticeably affected in several ways. These include the degradation of the nuclear envelope and the release of its contents into the cytoplasm. The nucleus may also change shape from spherical to oval, becoming irregular and flattened, and phytoplasma units inside vesicles extended from the cell membrane and penetrating it (Fig. 8d) as a result of osmotic pressure and cytoplasmic shrinkage. This agrees with

observations reported by Marcone *et al.,* (1997), Musetti *et al.,* (2002), and Bertaccini and Duduk (2014). In other regions of the cells, the nucleus may become smaller and shrink due to the deterioration of cellular functions, loss of water, and cytoplasmic contraction and cell wall thickening is evident. This is consistent with the findings of Lee *et al.,* (2000) in their study of phytoplasma infection in apple tissues. Reactive Oxygen Species (ROS) are highly reactive molecules produced inside the cell as a result of normal metabolic processes such as respiration, as well as pathological infection or environmental stresses (heat, salinity, and drought). Living cells produce large quantities of reactive oxygen species (ROS) as a natural byproduct of cellular metabolism. Under conditions of extreme stress, organisms eventually evolve a series of response mechanisms to adapt to exposure to these species, as well as to utilize them as signaling molecules. ROS molecules catalyze oxidation processes (He *et al.,* 2017).

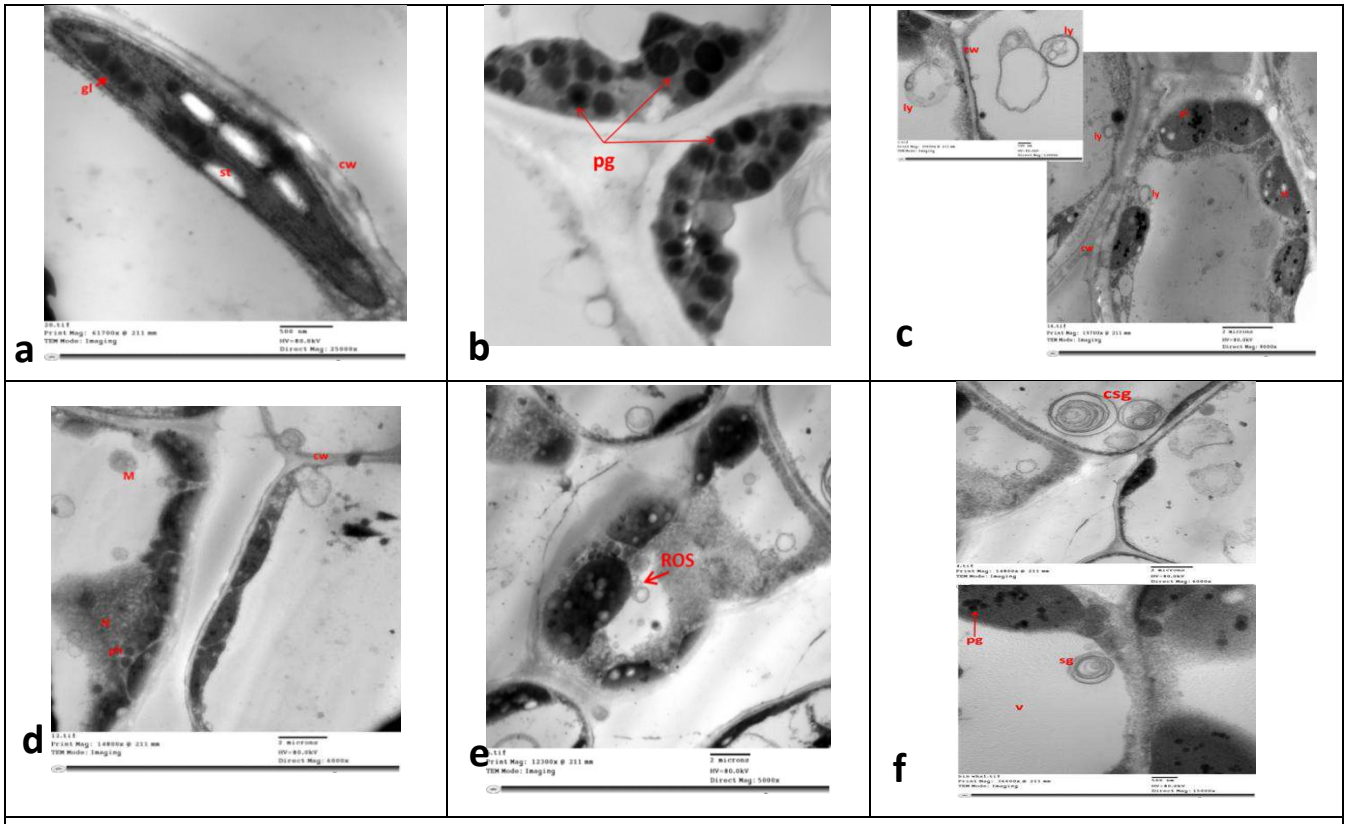

Fig. (8a-f). Electron micrograph of a ultra thin section through pepper infected phytoplasma views as Plastoglobuli (pg), Starch (st; csg and ssg), Chloroplas (Chl), Lysosome (Ly), Mitocondria (M), Nnucleus (N) and (ROS)

These molecules play a dual role; they act as a defensive weapon and as signaling molecules that activate defense-related genes, which is reflected in the increased activity of antioxidant enzymes such as superoxide dismutase (SOD) and peroxidase (POD), as shown in Table (6). However, their excessive accumulation can cause oxidation and damage to proteins, lipids, and DNA, leading to tissue destruction, membrane disintegration, plastid deformation, and protein degradation.

This is clearly observed under the electron microscope, as shown in Fig. e8, which illustrates their substantial accumulation in phytoplasma-infected chloroplasts, along with mitochondria and peroxisomes, which are major sources of reactive oxygen species (ROS) production within the plant cell due to their high oxidative metabolic activity (Apel & Hirt, 2004; Foyer & Noctor, 2005). This accumulation is also reflected in the symptoms observed in phytoplasma-infected plants, as shown in Fig. (1), such as leaf chlorosis, stunted growth, reduced leaf size and deformation, and deterioration of phloem transport. Therefore, reactive oxygen species (ROS) are considered a key factor in explaining the cellular damage caused by the infection. This is consistent with the findings of previous studies (Ehya *et al.,* 2013; Rasool *et al.,* 2020; Wang *et al.,* 2024), which demonstrated that ROS have a dual role in plants: at low concentrations, they act as signaling molecules that regulate growth and activate defense pathways, whereas high concentrations lead to oxidative stress and cellular damage, prompting the cell to rely on antioxidant systems to maintain its balance and protect its vital components. Starch is formed in the cytoplasm, including compound starch granules and simple starch granules as illustrated in Fig (8f). (Horner *et al.,* 2007; Weise *et al.,* 2011). Chloroplasts, along with mitochondria and microorganisms, are major sources of reactive oxygen species (ROS) production within the plant cell due to their high oxidative metabolic activity

Biochemical analysis:

The biochemicals within plants are an important aspect of understanding plant responses to diseases and environmental threats. Changes in the levels of certain plant hormones during infection help plants adapt to and combat infection. Biochemical test aimed to study the biochemical effect of phytoplasma and the plant defense mechanism via Phytohormons, enzyme activities and secondary metabolite production.

Phytohormons analysis

Plant biochemicals are essential for understanding plant responses to various diseases and environmental stresses. Changes in plant hormone levels during infection contribute to plant adaptation and enhance its resistance to infection.

The biochemical tests in this study aimed to evaluate the effect of phytoplasma on plant hormones and defense mechanisms, using HPLC to determine auxin, jasmonic acid, salicylic acid, and abscisic acid. The analysis of phytoplasma-infected *Jasminum sambac* L. samples showed that auxin, jasmonic acid, saliclic acid, and absesic acid represented in table (4) were significantly increased, with higher values of auxin (9.14 µg/gm), salicylic (19.03 µg/gm), jasmonic (5.69 µg/gm) and absisic (17.23 µg/gm) in infected samples combared of healthy samples (7.05, 7.65, 4.32 and 5.18 µg/gm, respectively), indicating the extent of plant resistance

to infection. Salicylic and jasmonic acid are hormones that regulate the plant's immune system against biotic and abiotic stresses through morphological, physiological, and biochemical mechanisms **(**War *et al.*, 2011) Including antioxidant enzyme activities (Yap *et al.,* 2021), it is an important component of various signaling pathways involved in plant defense against pathogens, insect pests, and abiotic stresses, participating in the activation of acquired systemic resistance (SAR) and the regulation of defense-related gene expression, thus enhancing plant resistance to pathogens and pests. (Chen *et al.,* 2009; War *et al.,* 2011; Vicent and Plasencia 2011; Gao *et al.,* 2015). They also affects nutrient uptake, water relations within the plant, and the stomatal regulation, improving photosynthetic efficiency. These effects were reflected in the present study through a marked increase in the activity of the POD and PPO enzymes, along with a high accumulation of phenolic compounds, especially flavonoids, which are an important part of the plant defense system, acting as antioxidants and inhibitors of pathogen growth. As for abscisic acid (ABA), its increased levels have been shown to play an adaptive role in the plant's stress response, contributing to the regulation of water balance and stomatal closure, and potentially interfering with the signaling pathways of both SA and JA to enhance the overall defensive response. In general, the complex changes in plant hormone balance resulting from phytoplasma infection contribute to a complex regulatory network of defensive signals that work together to enhance the plant's natural immunity and mitigate the effects of infection. These changes may also affect overall plant growth and development.

Table (4). Mean of analysis evaluation growth regulators (**µg/gm**) of *Jasminum sambac* L. healthy and phytoplasma infected samples

Sample	Absesic	Salicylic	Jasmonic	Auxin
Healthy	**5.18**	**7.65**	**4.32**	**7.05**
Infected	**17.23**	**19.03**	**5.69**	**9.14**

Enzyme activities

With respect to enzymatic activity, the results (Table 5) revealed a significant increase in the activities of superoxide dismutase (SOD; 374.09), peroxidase (POD; 43.25), and polyphenol oxidase (PPO; 47.81) in infected plants compared with healthy controls (187.28, 29.66, and 21.71, respectively). This elevation is consistent with the findings of Sahar *et al.,* (2018) and reflects a defensive response of the plant against infection. Phytoplasma infection induces substantial biochemical alterations in plants by affecting various metabolic pathways. In response to infection-induced stress, plants may experience oxidative stress due to the excessive accumulation of reactive oxygen species (ROS), such as superoxide radicals, hydrogen peroxide (H_2O_2), and hydroxyl

radicals. This is often associated with phloem dysfunction and vascular blockage (Bertaccini *et al.,* 2014; Hogenhout *et al.,* 2008). These ROS are highly harmful as they can cause severe cellular damage, including membrane disruption, lipid peroxidation, degradation of proteins and DNA, and impairment of chloroplast structure and function, as confirmed by microscopic observations. In this context, SOD plays a crucial protective role by catalyzing the conversion of superoxide radicals into less harmful molecules, such as molecular oxygen (O_2) and hydrogen peroxide (H_2O_2). Subsequently, other antioxidant enzymes, including POD and PPO, contribute to further detoxification processes, particularly through the breakdown of H_2O_2 (Gill and Tuteja, 2010). PPO is important in plant defense, as it oxidizes phenolic compounds into reactive quinones that possess antimicrobial properties and reduce the digestibility of plant tissues by insects. High activity of this enzyme is often associated with increased plant resistance to infection (Mayer, 2006). .Peroxidase, in particular, is a key enzyme involved in plant growth and development, as it participates in cell wall formation and lignification, as is evidenced in the cytological examination, thereby enhancing tissue resistance against pathogen invasion (Passardi *et al.,* 2005). Therefore, the increased activity of SOD in infected plants can be considered a strong indicator of the plant's attempt to counteract oxidative stress and defend itself. However, under severe infection conditions, antioxidant production may not be sufficient to mitigate damage due to the exhaustion of defense mechanisms, mitochondrial dysfunction, and reduced enzymatic efficiency. Similar findings have been reported in studies on jasmine, citrus, and grapevine (Musetti, 2010; El-Beltagi and Mohamed, 2013; El-Beltagi *et al.,* 2017; Kuar *et al.,* 2022).

Table (5). Spectroscopic Evaluation antioxidant enzymes (U/gm) of *Jasminum sambac* L. healthy and phytoplasma infected samples

Sample	Polyphenol oxidase (PPO)	Peroxidase (POD)	superoxide dismutase (SOD)
Healthy	**21.71**	**29.66**	**187.28**
Infected	**47.81**	**43.25**	**374.09**

Secondary metabolite

Phytoplasma infection in *Jasminum sambac* L. caused significant biochemical changes affecting both secondary metabolites and photosynthetic pigments. Essential oil content showed a remarkable increase from 0.0658% in healthy plants to 0.1674% in infected plants, representing an increase of approximately 154%. This elevation may be attributed to the activation of secondary metabolic pathways induced by phytoplasma stress due to metabolic disturbance and the redirection of energy toward defense rather than normal physiological processes (Tan *et al.,* 2020). Flavonoid compounds

play an important role in plant defense mechanisms and may accumulate in response to infection. In the present study, flavonoid content increased significantly from 16.03 to 29.06 mg/g (about 81%), which is consistent with the activation of the phenylpropanoid pathway as a defensive response against pathogen invasion. Flavonoids also function as antioxidants that help alleviate infection-induced stress. Similarly, anthocyanin content exhibited a pronounced increase from 5.01 to 11.69 mg/g (about 133%), reflecting enhanced defense responses against oxidative stress and cellular damage. This increase may also contribute to the abnormal purple or reddish coloration observed in infected flowers, as shown in Figure (1). In general, flavonoids are secondary phenolic compounds characterized by their polycyclic structure, including anthocyanins and flavonols, and they play a central role in enhancing plant resistance to pests and diseases. They are an essential part of the plant's chemical and physical defense system, providing antioxidant protection as well as structural support through lignin formation (Dias *et al.,* 2021; Ramaroson *et al.,* 2022). Regarding photosynthetic pigments, both lutein and β-carotene showed moderate increases (approximately 13% and 17%, respectively), suggesting an adaptive protective response to maintain photoprotection and membrane stability under stress conditions. However, total carotenoids decreased slightly by 1.6%, indicating partial disruption of carotenoid metabolism. In contrast, total chlorophyll content declined from 0.801 to 0.694 mg/g (about 13%). This reduction may be attributed to chloroplast degradation and inhibition of chlorophyll biosynthesis. Such a decrease is a common symptom of phytoplasma infection and is often associated with leaf yellowing (Bertaccini & Duduk, 2010; Negro *et al.,* 2020).

Table (6). Secondary metabolite (mg/g) and Oil% of *Jasminum sambac* L. healthy and phytoplasma infected samples

	Total chl.	**Anthothyanin**	**Carotineoide**	**β-car**	**Lutine**	**Flavonoides**	**Oil%**
Healthy	**0.801**	**5.01**	**12.583**	**0.397**	**0.659**	**16.03**	0.0658
Infected	**0.694**	**11.69**	**12.380**	**0.463**	**0.745**	**29.06**	0.1674

Conclusion

Phytoplasmas represent a serious and often underestimated threat to ornamental plants worldwide. Therefore, accurate diagnosis, a better understanding of phytoplasma biodiversity, and the application of integrated management strategies are essential factors to limit disease spread. The present study provides a comprehensive investigation and represents the first detailed characterization of phytoplasma infection affecting *Jasminum sambac* L. in Egypt. The obtained sequence was deposited in GenBank as a reference record for Egypt. This study establishes an important scientific foundation for accurate diagnosis, phytoplasma classification, and the

development of future disease management strategies to protect jasmine production. Moreover, under current climate change conditions, the increasing abundance of insect vectors may enhance the spread of phytoplasma-associated diseases, making the development of sustainable control measures even more important in the future. Since phytoplasmas are phloem-limited pathogens, the results provide clear evidence that phytoplasma infection in *Jasminum sambac* L. leads to hormonal imbalance, degradation of plant pigments, oxidative stress, and strong activation of biochemical defense responses. These biochemical and physiological markers may serve as reliable tools for early detection of infection and for improving our understanding of plant–phytoplasma interactions.

Recommendations

- The present study indicated that phytoplasma infection of *Jasminum sambac* L. induced oxidative stress as a result of increased production of reactive oxygen species (ROS), as observed in cytological examination. Therefore, further studies are recommended to identify and characterize the specific ROS involved, such as superoxide radicals, hydrogen peroxide, and hydroxyl radicals.
- Conduct epidemiological surveys to determine the occurrence and distribution of the disease, as well as to identify the insect vectors and the associated phytoplasma strains
- Continued scientific research and the implementation of strict phytosanitary measures are essential for protecting economically important ornamental crops.
- This study primarily classified phytoplasmas on jasmine plants based on the 16S rRNA gene sequence. However, group and subgroup classifications are not fully standardized, and further research is needed to refine classification, as some closely related strains are difficult to distinguish using 16S rRNA alone.
- Use additional genes alongside 16S rRNA to improve the accuracy of phytoplasma classification. The classification system should also be regularly updated using modern genomic data to better distinguish closely related strains.

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