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Identification and Molecular Characterization of Phytoplasma Associated with Carrot Plant (Daucus carota L.) in Qalyubia Governorate, Egypt

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Abstract

In recent years, phytoplasma diseases have increased and become serious in Egypt. **S**amples of carrot plants exhibiting resemble symptoms of phytoplasma diseases were collected from seven different cultivated areas in El Qalyubia governorate during the growing seasons 2021 and 2022; to detect phytoplasma infection used Light microscopy, Dienes' stain, transmission electron microscopy (TEM), and Nested-PCR. Dodder (*Cuscuta campestris*), leafhopper (*Hebata decipiens*), and seed transmission were used to study the transability of phytoplasma. The obtained results showed pleomorphic bodies in infected phloem tissues treated with Dienes' stain as irregular patches of intensely dark blue stained cells. However, a light microscope observed significant abnormal changes in infected tissues compared with healthy ones. Transmission electron microscopy (TEM) that observed phytoplasma units in the phloem tissue and deterioration in the ultrastructure of infected plants including malformed cells, thickness, and abnormal shape of the cell wall. But, due to inconsistent distribution within the plant, low titer, and fluctuation often hamper accurate phytoplasma detection. Therefore, a PCR was used to detect phytoplasma infection using the universal primer pairs P1/P7 and R16F2n/R16R2 that produce fragments at 1200 bp. For transmission of phytoplasma in carrot, dodder, and leafhoppers gave positive results and mechanical transmission gave negative results. Our results will provide a better understanding of the effects of phytoplasma infection in carrots, helping us to develop effective strategies for overcoming such diseases.

Keywords: Phytoplasma, carrot, Dienes' stain, light microscopy, TEM, dodder, leafhopper, sequencing, PCR.

Introduction

Plants are continuously exposed to certain biotic and abiotic stress, causing serious crop losses every year **(Ul Haq and Ijaz 2020).** Recently, the number of diseases caused by fungal, bacterial, nematode, and viral has increased **(Nazarov *et al*., 2020).** Carrots (*Dacus carota*) are the most important dietary source of vitamin A and are considered a safe way to prevent heart disease and eliminate the risk of cancer diseases. Also, helps in strengthening the immune system and protecting bones from osteoporosis. Additionally, carrots are rich in vitamins that support the body with useful essential nutrients. It is recommended to consume carrots daily. The world carrot cultivated area was 1,147,155 ha and production was estimated at 42,831,958 tons, achieving an average yield per square meter of 8.89 kg. The cultivated area in Egypt in 2022 year (36.831 thousand hectares) and the production is 294.191 thousand tons, according to **FAOSTAT, 2022.** Commercial production of carrot plants is impeded by a wide range of diseases (e.g., fungal, oomycete, bacterial, viral, nematodes, two parasitic plants, insect pests, mite infestations, and phytoplasma) causing severe yield-reduction worldwide **(Selvakumar and Kalia 2022).** Phytoplasmas are among these diseases that attack several crops causing huge losses worldwide **(Ahmed *et al*., 2022)** are arguably one of the most impactful diseases affecting carrots. Symptoms associated with phytoplasma including aster yellow, reddling, bronze foliage, purple foliage, witches'- broom, phyllody, little leaf, and hairy root were observed. Phytoplasma refers to cell wall-less prokaryotes, microscopic, and phytopathogenic of 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 **(Park *et al*., 2021 and Vishnyakov, 2022)** that were previously classified as mycoplasma-like organisms (MLOs) which discovered by **Doi *et al*., 1967.** It is considered an obligate parasite that colonizes the phloem tissue of the host plant as well as the gut, hemolymph, and

salivary glands of the insect host **(Wei and Zhao 2022 &Prabha *et al*., 2023)** and is transmitted among plants through grafting, dodder, and phloem-sucking insects, such as leafhoppers, planthoppers, and psyllids **(Huang *et al*., 2023, Boopathi *et al*., 2023 and Yang *et al*., 2023).** Phytoplasma cannot be cultured in cell-free media **(Wei and Zhao 2022).** Therefore, the methods of its detection and diagnosis are restricted. For these reasons, several techniques including Dienes' stain and light microscopy are used as a primary detection of phytoplasma. Furthermore, Transmission electron microscopy (TEM) was used to investigate the presence of phytoplasma units in infected phloem tissues and the accumulation of phytoplasma-like bodies in sieve elements **(De Oliveira *et al*., 2020 and Chen *et al*., 2021).** Recently, due to the low concentration problem of phytoplasma in infected plants, PCR and nested PCR approaches have been used to overcome and identify groups and sub-groups of phytoplasma **(Nair and Manimekalai, 2021; Kazeem *et al*., 2021 and Ahmed *et al*., 2022).** Many researchers have reported that phytoplasma is associated with carrot in many countries **(Mitrovic *et al*., 2021, Clements *et al*., 2021, Wulandari *et al*., 2021, Zelyüt *et al*., 2022 &Randa-Zelyüt *et al*., 2023).** Limited and scarce information is available regarding phytoplasma of carrot in Egypt; therefore, our study was conducted.

Materials and Methods

2.1. Field survey and samples collection

Carrot plants showing resemble symptoms suspected to be naturally infected with phytoplasma were collected from open fields in 2021 and 2022 from (Moshtohor-1, Moshtohor-2, Qaha, El Hesa, Senhera, Namol, and Sandanhor) within El Qalyubia governorate.

2.2. Incidence of the Phytoplasma Disease

The percentage of phytoplasma disease incidence in fields was determined based on the visual symptoms developed on diseased plants using the formula by (**Chaudhary *et al*., 2003)**

$$\text{Percentage of disease incidence (D. I. \%)} = \frac{\text{No. of infected plants (n)}}{\text{Total no. investigated plants (N)}} \times 100$$

2.3. Pathogenicity test and Transmission studies.

The pathogenicity of the suspected infection of phytoplasma was verified using dodder, leafhopper, and seed.

2.3.1. Dodder transmission

In vitro, seeds of dodder (*Cuscuta campestris*) were surface sterilized in 5% (w/v) sodium hypochlorite solution (NaOCl) for 3 min and then rinsed three times with sterile distilled water and dried on Petri dishes (12 cm in diameter) bottomed with wetted filter paper No. 2. After approximately 7 days seeds were germinated and formed haustoria and germinated seeds were transferred to infected carrot plants (10 seeds/ infected plant), sown in plastic pots containing a 1:1 mixture of sand and peat moss which used as bridge to transmit carrot associated phytoplasma. The dodder bridge was left to parasite for 30 days on infected carrot plants to acquire the pathogen. Subsequently, the stolons were allowed to parasitize on healthy carrot and periwinkle red flowers (*Catharanthus roseus*) plants sown in plastic pots containing a 1:1 mixture of sand and peat moss as (1 plant/ pots) and were kept in an insect-free cage under greenhouse conditions (22-25 °C) till the appeal of typical symptoms to check the transmission of the phytoplasma according to **Mikhail, *et al*. 2012**.

2.3.2 Insect transmission

Leafhopper insects *Empoasca decipiens* (*Hebata decipiens*) were collected from carrot fields in Moshthohor and about 15 leafhoppers were carefully stored in plastic vials at 4 °C in 70% ethanol for further identification. The identification of leafhoppers was carried out in the Department of Piercing-Sucking Insects, Plant Protection Research Institute (PPRI), Agriculture Research Center (ARC), according to their morphological characteristics using available description and identification keys to start a stock colony for the present work. Adult leafhopper individuals were reared on carrot plants in insect-proof cages and allowed to oviposit. The hatching nymphs were transferred to healthy carrot plants in cylindrical cages to maintain a leafhopper colony free from viruses or phytoplasma. Nested PCR confirmed the results. After starving for 24 hours, phytoplasma-free insects were allowed to feed on infected carrot plants for one week **(Ahmed *et al*., 2014)** as the acquisition period. About 20 insects/plants were placed on healthy carrot plants for an inoculation access period of 30 days. The plants were then sprayed with pesticides Sivanto® prime 200SL (flupyradifurone 20%) to kill adult insects. Finally, the plants were monitored until symptoms appeared.

2.4. Seed transmission

One hundred seeds were collected from infected carrot plants (positive PCR- test). Then, they were dried and planted in plastic pots (2 seeds / pot with 1 cm deep) containing a mixture of 1:1 sand and peat moss. Pots were covered with separate plastic cages which were removed after 7 days of germination. Furthermore, the pots were kept in insect-proof cages under greenhouse conditions and observed till symptoms developed. Samples of leaves were repeatedly taken for molecular testing.

2.5. Preparation of freehand section for Diene's stain

Free hand-cut cross sections of approximately 1-2 mm were prepared from both infected and healthy carrot leaves. Sections were transferred to distilled water using a razor blade edge. The sections were treated with Dienes' stain for 5 min as

described by **Deeley *et al.*, 1979.** After staining, sections were washed with distilled water and the slices were examined immediately by a light microscope and photographed using a digital camera (LEICA ICC50 HD).

2.6. Histopathological Changes

Light and electron microscopy were used to study and explain the anatomical and ultrastructural changes in tissues and cell components of carrot plants as a result of the infection of phytoplasma. This work was carried out in Fac. Agric. Res. Park (FARP), Transmission Electric Microscope (TEM) Lab. Faculty of Agriculture Cairo University.

2.6.1 Light microscope

The leaf midribs of healthy and infected carrot plants (6 weeks- old) which were collected from the third leaf at the beginning of symptoms appearing were sectioned with ultra-microtome (Leica model EM-UC6) roughly (500- 900 nm thick), killed, and fixed for at least 48 h in formalin glacial acetic acid (F.A.A), washed in 50% ethyl alcohol. Before being dehydrated in normal butyl alcohol series and embedded in paraffin wax, they were serially sectioned by a rotary microtome at 20 µ thickness. Finally, sections were double stained with a crystal violet erythrosine combination, cleared in carbol xylene, and mounted in Canada balsam **(Sass, 1958, Nassar and El-Sahhar, 1998).**

2.6.2 Transmission electron microscopy (TEM)

Healthy and infected carrot leaf midrib was examined using transmission electron microscopy. In brief, tissues were cut, prepared, and fixed in 2% (v/v) glutaraldehyde dissolved in 0.1M sodium cacodylate buffer (pH7.2) for 2 h at 4 ˚C then, fixed again for 1.5 h in 1% (v/v) osmium tetroxide (OsO_4). After removing the fixative solution samples were dehydrated with ascending concentrations of ethanol, 15 min for every concentration except the concentration of 100% which was repeated twice. The samples were sectioned at roughly a thickness of 90 nm using an ultra-microtome Leica model EM-UC6 (LEICA EM-UC6, Leica Microsystems, Wetzlar, Germany) and mounted on thin-bar 400 mesh copper grids. Finally, the sections were double stained with Uranyl acetate 2% for 10 min followed by 0.4% lead citrate for 5 min and examined using JEOL JEM-1400 transmission electron microscope JEOL (JEM-1400 TEM, Tokyo, Japan) at the specified magnification as described by **El-Banna *et al.*, 2007.**

2.7. Molecular biology studies

2.7.1. Nucleic acid extraction

The total DNA was extracted from about 1g of fresh leave samples of healthy and infected carrot, periwinkle, and leafhopper. As well as, from mechanically carrot inoculated by dodder, leafhopper, and seed using the modified was extracted as **Dellaporta *et al.*, (1983)** extraction method**.** Nucleic acid was precipitated by adding 2.5 volumes of absolute ethanol and was collected by centrifugation, washed with 70% ethanol, dried, and re-suspended in 50 µL TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). DNA was used immediately and/or stored at -20°C before use in nested PCR.

2.7.2 PCR primers and amplification

Phytoplasma DNA was detected in the infected carrot samples, mechanically infected plants, and leafhoppers using specific amplification of the 16S-23S rRNA gene by PCR. Using the universal phytoplasma primer pair P1/P7 to amplify an 1800 bp in direct PCR followed by primer pair R16F2n, R16R2 to amplify a 1200 bp (**Table1)** in nested PCR (second PCR) **(Deng and Hiruki, 1991, Schneider *et al.*, 1995).**

Table 1. Primer sequences and the size of the amplified PCR product.

Primer	Sequence	PCR Product (bp)	Reference
P1 (forward)	5'AAGAGTTTGATCCTGGCTCAGGATT-3'	1800	**Deng and Hiruki, (1991) Schneider *et al.*, (1995)**
P7 (reverse)	5'- CGTCCTTCATCGGCTCTT -3'		
R16F2n (forward)	5'- GAAACGACTGCTAAGACTGG -3'	1200	
R16R2 (reverse)	5'- TGACGGGCGGTGTGACAAACCCCG-3'		

2.8. Nested PCR for Phytoplasma Detection:

First PCR was carried out using the primer pair P1/P7 to amplify about 1800 bp product. However, in the second PCR R16F2n/R16R2 was conducted to amplify 1200 bp.

One microliter of extracted DNA was used in 25 µL of PCR mixture in 0.2 mL Eppendorf tube including the following reaction mixture: 12.5 µL ama*R* OnePCR™ mix (GeneDireX, Inc.), 10 nm of each primer R16F2n, R16R2 also, sterile deionized water up to 25µL total volume. The product of the first PCR after diluted 1:10 was used as a template for the second PCR round, the amplification-optimized PCR assay for the first and second PCR, started with a denaturation step for 3 min at 94 ºC followed by 35 cycles involving denaturation for 30 s at 94 ºC, annealing for 60 s at 53 ºC, and extension for 1 min at 72 ºC, followed by extension step was added at 72 ºC for 10 min. The PCR products were stained with gel star (Lonza, USA) that was analyzed

by electrophoresis in 1% agarose gel with 1x TAE buffer and visualized by UV illumination using Gel Documentation System (Gel Doc2000 Bio. Rad, USA) **(El-Banna *et al.*, 2013 and Hamed *et al.*, 2014).**

2.9. PCR sequencing and analysis

PCR product of the positive samples was clarified with a Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech Ltd., Taipei, Taiwan). The DNA fragments were sent to Macrogen Inc. - South Korea for sequencing. The results of the obtained sequences were analyzed and compared with other phytoplasma sequence isolates available on GenBank by performing a BLAST comparison using Phylogenetic and molecular evolutionary analyses conducted using MEGA version 11 **(Tamura *et al.*, 2021**).

Results

3.1 Incidence and symptoms of phytoplasma:

This study was carried out in carrot fields in seven localities of El Qalyubia governorate, Egypt (Moshtohor-1, Moshtohor-2, Qaha, El Hesa, Senhera, Namol, and Sandanhor). Naturally symptomatic plants were observed in all carrot cultivated areas which were surveyed during 2021 and 2022 respectively and collected as (30 plants/ field). The major symptoms of phytoplasma were characterized by leaf reddening, Aster yellow, bronze leaf, reduction in size in taproot, stunting and little leaf **(Fig. 1)**. The percentage of disease incidence varied significantly between investigated fields. In 2021 the disease frequency ranged between 16.7 – 43.3%. However, the percentage increased in the same fields in 2022 and the highest incidence was recorded in Moshtohor-1 reaching 83.3% based on collection of symptomatic plants as shown in **Table2**.

Table 2. The percentage of disease incidence of phytoplasma infection in carrot plants in seven locations in El Qalyubia governorate from 2021 to 2022seasons.

Location	Incidence%				Main symptoms	PCR detection N./T.
	2021	n	2022	n		
Moshtohor-1	43.3	13	83.3	25	R, AY, RZ, BL, S, LF	38/60
Moshtohor-2 (2)	20	6	30	9	R, AY, BL	15/60
Qaha	30	9	40	12	R, AY, RZ	21/60
El Hesa	16.7	5	23.3	7	R, AY	12/60
Senhera	20	6	30	9	AY	15/60
Namol	20	6	26.7	8	R, AY	14/60
Sandanhor	23.3	7	30	9	R, AY, RZ, BL, S	16/60

(%Incidence): Number of infected samples / total number of inspected plants. **(PCR detection N. /T.):** Number of symptomatic samples positive in PCR/total number of detected samples. **(n):** Number of Infected plants. **(R):** Reddening, (**AY):** Aster Yellow, **(RZ):** reduction size in taproot, **(BL):** Bronze Leaf, **(S):** Stunting, **(LF):** Little Leaf.

Fig. 1: Host plants showing symptoms of infection with phytoplasma. (A and D) Yellow and bronze foliage discoloration. (B) Formation of chlorotic adventitious shoots. (C and H) Reduction in taproot size and quality. (E) Purple foliage discoloration of infected plants. (F) Reddening leaves. (G) Little Leaves, (I) compared with healthy leaves.

3.2 Histopathological studies

Histopathological studies using light and electron microscopy techniques showed dramatic changes occurred in the anatomical structure of carrot plant leaves. Infected tissues were stained with Dienes' stain which was observed as dark blue areas in the phloem of infected tissues using a light microscope (LM) **(Fig. 2)**. These results were also confirmed through PCR assay as a clear band at the specific size 1200 bp was produced **(Fig. 3)**.

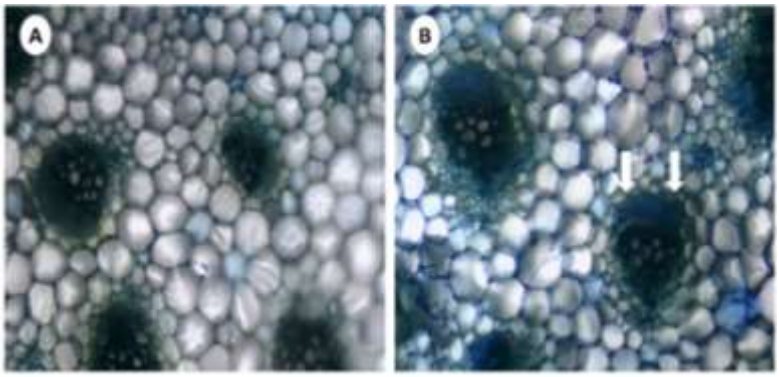

Fig. 2: Micrograph free hand sections of petioles carrot hand cut stained with Diene's stain. (A) Section of healthy plant with unstained phloem, (B) Phloem cells are stained in section obtained from an infected plant. White arrows show the dark blue area indicating phytoplasma presence.

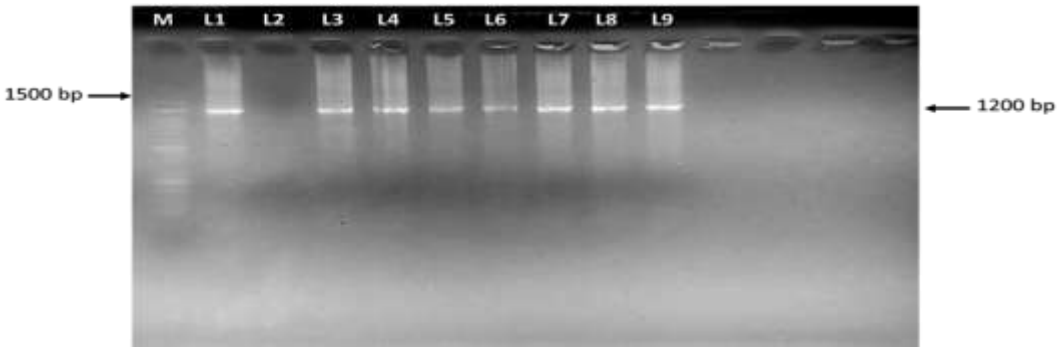

Fig. 3: Agarose gel electrophoresis of polymerase chain reaction (PCR) products from the 16S rDNA gene used for the detection of phytoplasma in carrot. L1: Positive control sample. L2: Negative control sample. L3: Moshtohor-1. L4: Moshtohor-2. L5: Qaha. L6: El Hesa. L7: Senhera. L8: Namol. L9: Sandanhor. M: 100bp DNA Ladder

In response to phytoplasma infection, the anatomical structure of carrot leaf was markedly affected as presented in **Table 3 and Fig. 4.** As the thickness of leaf blade was reduced by 21.2% compared with healthy ones. In the mesophyll layer, the spongy cells and palisade cells were affected in shape and size in response to the infection. Also, in addition to the spongy tissue being malformed, the palisade tissue was irregular in shape, with a wide intercellular space, as well as lost its normal shape due to the deformation of the cell wall. Moreover, the leaf midvein was negatively affected and showed a high degree of malformation that showed up normal shape and malformation and enlargement of the vein as clear in comparison with a normal leaf due to the increase of its width and length. Most notably, the main anatomical changes were observed in the bundle tissue vascular and phloem tissue.

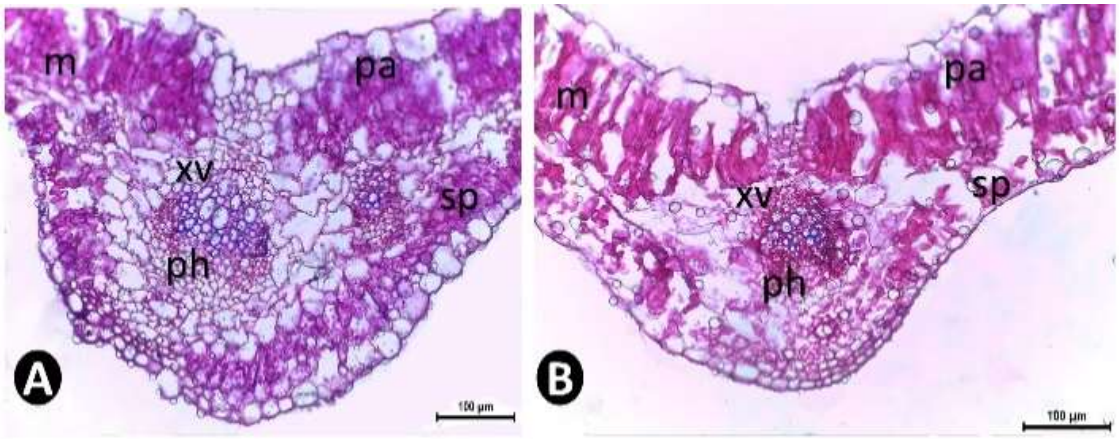

Fig. 4: Transactions of carrot plant leaf for healthy (A) and phytoplasma-infected (B) plants. **m** = mesophyll, **mv** = midvein, **pa** = palisade tissue, **sp** = spongy tissue, **xv** = xylem vessels and **ph** = phloem. Scale bar for **(A, B) = 100 µ.**

Table 3. Leaf anatomical structure of healthy and infected carrot plants.

Parameter (µm)	Healthy	Infected	Change (%)
Thickness of upper epidermis cuticle layer	13.50	9.00	− 33.3
Thickness of Lower epidermis cuticle layer	7.20	5.40	− 25
Thickness of the upper epidermis layer	45.00	42.30	− 10.4
Thickness of the Lower epidermis layer	30.60	15.30	− 50
Palisade tissue thickness	131.40	112.50	− 14.3
Spongy tissue thickness	90.00	65.70	− 27
Thickness of blade	317.70	250.20	− 21.2
Thickness of phloem tissue in the vascular bundle in	59.40	41.40	− 30.3
Length of midrib vascular bundle	187.20	140.40	− 25
Thickness of leaf midrib	468.00	360.00	− 23
Vascular bundle width	135.00	175.50	+ 30
Midvein width	450.00	630.00	− 20

$^{\Delta}$ Percentage change compared with those of healthy plants.

The examined of ultra-thin sections using transmission electron microscopy (TEM) were used to explain the ultrastructure changes in infected and healthy carrot plants and to verify the presence of phytoplasma units in the infected phloem tissues. Highly cytopathological changes were observed in infected plants compared to healthy ones involving the presence of phytoplasma units with different shapes that completely fill the infected tissue **(Fig. 5 A and B).** The secondary cell walls of xylem vesicles were malformed **(Fig. 5 C)** compared to healthy **(Fig. 5 D).** Thickness of cell wall, abnormal in shape, and the sieve element of phloem tissue containing units of phytoplasma **(Fig.5 E)** compared with healthy **(Fig. 5 F).**

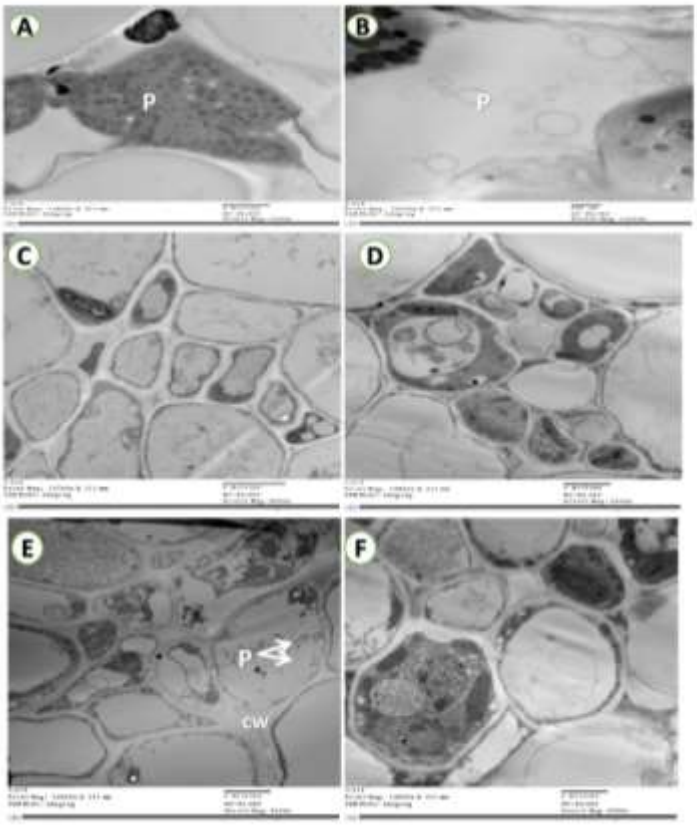

Fig. 5: Transmission electron micrograph of the phloem cells from the healthy and infected carrot leaves, with Phytoplasma. (A) Infected tissue fills up phytoplasma (P) units. (B) Phytoplasma (P) units with different shapes. **P** = Phytoplasma units. (C) Malformation of cell walls of xylem. (D) Xylem vesicles of the healthy tissue of carrot plants. (E) The sieve element of the phloem tissue containing phytoplasma units and the cell wall became thick and abnormal in shape. (F) Phloem tissues of the healthy carrot plant. P = Phytoplasma units, cw = Cell wall.

Moreover, piecemeal degeneration and detachment of the neighboring cell wall followed by complete lysis of the cell **(Fig. 6 G)** compared to healthy **(Fig. 6 H)**, the companion cells start to become necrotic **(Fig. 6 I)** compared to healthy **(Fig. 6 J)**. As well as it was also noted that the chloroplasts became deformed, larger in size, and had abnormal membranes in infected cells **(Fig. 6 K)** compared to healthy cells **(Fig. 6 L).**

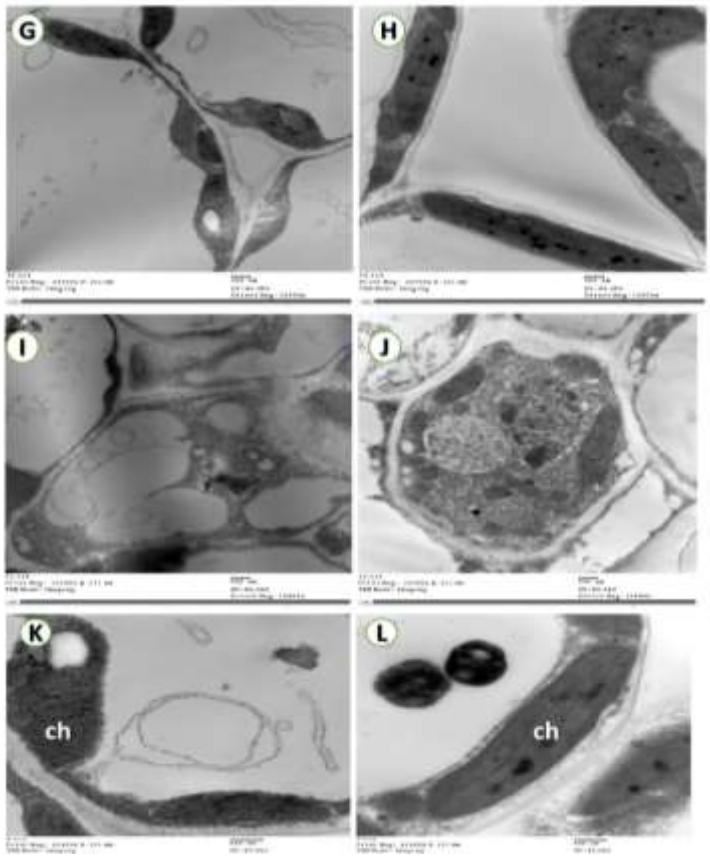

Fig. 6: Transmission electron micrograph of the phloem cells from the healthy and infected carrot leaves. (G) Infected cells observed piecemeal degeneration and detachment of the cell wall. (H) The cell wall of healthy carrot plants. (I) The phloem cells of the infected carrot plant became necrotic. (J) Phloem parenchyma cell of the healthy carrot plant. (K) Phloem parenchyma cells of infected carrot plants observed deformed chloroplast. (L) Chloroplast (Ch) of phloem parenchyma cells of healthy carrot plants.

3.3 Leafhopper identification

Several specimen leafhoppers were collected to determine whether the insects can play a role in phytoplasma transmission or not. These leafhoppers were tested as a vector of phytoplasma associated with carrot during the survey of carrot fields. All collected leafhopper species couldn't transmit the infection except one leafhopper species that was identified as *Empoasca decipiens* (*Hebata decipiens*) which belonged to the family Cicadellidae, subfamily Typhlocybinae, and located in order Hemiptera. It was 3 mm in length and green in color.

3.4 Transmission studies of phytoplasma

Dodder, Leafhopper insects, and seed transmission were used to test the transmission of the phytoplasma associated with carrot plants**.** The infection of phytoplasma was successfully transmitted by dodder and leafhopper insects where the rate of transmission reached 20% and 90% respectively in the case of inoculated healthy carrot plants. However, the percentage reached 40% and 90% respectively in the case of transmission of the pathogen associated with disease from infected carrot plants to healthy periwinkle plants. On the other hand, seed transmission of phytoplasma affecting carrot plants cannot be transmitted under greenhouse conditions. The obtained results were confirmed with nested PCR as shown in **Table 4**.

In the present study, transmission of phytoplasma associated with carrots led to symptoms appearing including reddening leaf and aster yellow (**Fig. 7 A and B**). Meanwhile, symptoms of phyllody and yellowing were observed on inoculated periwinkle **(Fig 7. C and D).** These symptoms appeared after inoculation during the period from 30 – 60 days.

Table 4. The percentage of transmission of phytoplasma by dodder, leafhopper, and seed to healthy carrot and periwinkle plants detected by PCR.

Method of Transmission	Plant	Total no. of plants		No. of infected/total plants	Days taken for the appearance	%infected Plants	Nested PCR test
		Inoculated	Infected				
Dodder	Carrot	10	2	2/10	60 – 65	20%	+
Leafhopper		10	9	9/10	30- 60	90%	+
Seed		100	0	0	-	0%	-
Dodder	Periwinkle	10	4	4/10	60 – 65	40%	+
Leafhopper		10	9	9/10	30- 60	90%	+
seed		100	0	0	-	0%	-

Fig. 7: (A and B) Symptoms of phytoplasma infection on carrot. (C and D). Symptoms of phytoplasma infection on periwinkle.

3.5 Molecular characterization of phytoplasma

Total nucleic acid was extracted from infected and healthy carrot, periwinkle, leafhopper, and mechanically transmission plants and used as a template for direct PCR was using the universal primers pair P1/P7 and nested PCR amplification using R16F2n/R16R2. The infected samples that were used as a template because of the nested PCR amplification produced amplicons of about 1200bp. On the other hand, no visual bands were observed in the healthy samples **(Fig 8).**

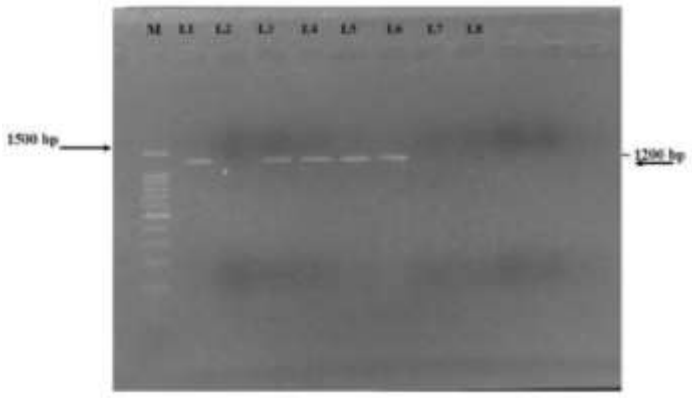

Fig. 8: Agarose gel electrophoresis of polymerase chain reaction (PCR) products from the 16S rDNA gene used for the detection of phytoplasma in carrot, periwinkle, and leafhopper. L1: Positive control sample. L2: Negative control sample. L3: Infected periwinkle. L4: Leafhopper. L5 and L6: Carrot plants previously inoculated with phytoplasma using dodder, and leafhoppers. L7: seed transmission. L8: Healthy periwinkle. M: 100bp DNA Ladder.

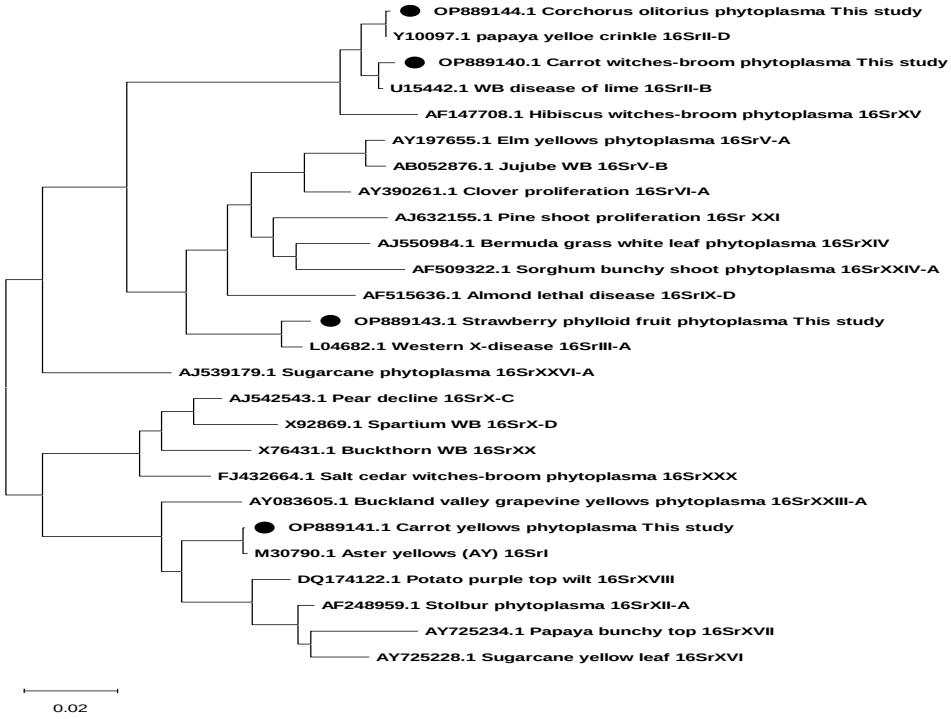

Fig. 9: Phylogenetic tree constructed from partial 16S rDNA sequences for Egyptian isolate OP889140 and OP889141 compared with other isolates available in the GenBank database.

3.6 Sequence Analysis

Phylogenetic analysis **(Fig. 9)** was performed to compare our Egyptian isolates which were deposited in the National Center for Biotechnology Information (NCBI) GenBank under accession numbers OP889140 and OP889141 with the sequences of other phytoplasma strains available in the GenBank database. The presence of phytoplasma in different carrot samples was detected and confirmed by the results, showing 99.6% identity with an isolate of U15442, 98.8% identity with an isolate of Y10097 which belongs to the same subgroup, 97.5% identity with an isolate of AF147708 from France, Australia, and USA respectively. Additionally, 92.3% identity with isolate L04682, 91.7% identity with isolate AF515636, 91.4% identity with the isolates AJ550984 and AJ632155, 91.3% identity with isolate AF509322, 91.1% identity with isolate X76431, while the homology with other subgroups ranged between 88.6% and 90.6%. The results indicate that our isolate (OP889140) was a member of the group 16SrII.

On the other hand, the isolate submitted with accession number (OP889141) had a 99.7% identity with isolate M30790, 96.9% identity with isolate AY083605, 96.4% identity with isolate AF248959, and 95.4% identity with isolate AY725228 and DQ174122 from USA, Australia, USA, USA, and Cuba respectively. Whereas 94.4% identity with isolate AY725234, and 93.9% identity with isolate FJ432664, AJ539180, and AJ539179 respectively. 93.6% identity with isolate AJ542543, 93.5% identity with isolate X92869, 93.0% identity with isolate X76431, and the homology with other subgroups ranged between 89.8% and 91.5%. This indicates that our isolate (OP889141) was a member of the group 16SrI. These results were confirmed as shown in **Fig 10**.

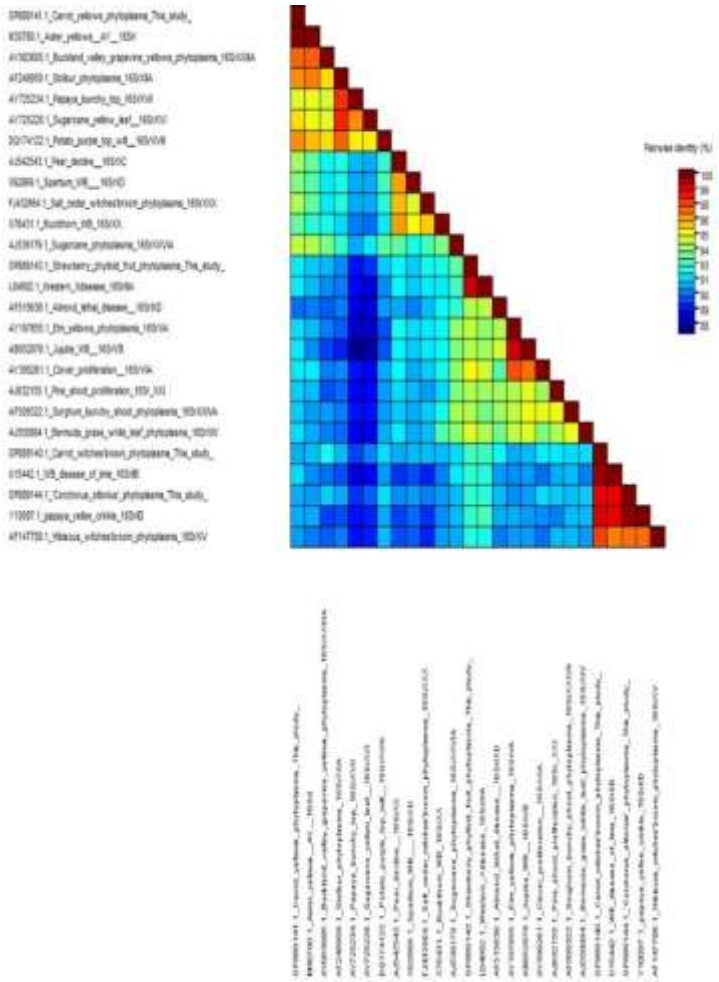

Fig 10. Accession number and similarity matrix for isolates of phytoplasma at the gene bank and the carrot Egyptian isolates based on nucleotide sequences.

Discussion

In recent years, phytoplasma diseases have increased and become serious in Egypt, due to their major effect on economic crops and its host range has become wide, so accurate diagnosis and surveys of the pathogen and identification are very important to find the appropriate methods for preventing pathogen spread and control. In the present study, a phytoplasma survey was conducted in seven carrot-cultivated areas in El Qalyubia governorate. All these fields observed different symptoms of phytoplasma associated with carrot diseases. Aster yellow, redding, bronze foliage, purple foliage, Witches'-broom, phyllody, little leaf, and hairy root symptoms are similar to those described by **Zelyüt *et al.*, 2022** in Turkey and **Everaert *et al.*, 2023** in Belgium. Collected samples were examined using a Nested-PCR assay; positive samples showed a clear band at the specific size 1200bp using the universal phytoplasma-specific primers R16F2n/R16R2 which proved naturally infected of phytoplasma in carrot associated with previous symptoms. In contrast, the negative samples did not observe any bands in the PCR test.

Different saps-sucking leafhopper insects were collected to determine whether the insects can play a role in phytoplasma trans-ability, only one species proved its ability to transmit phytoplasma and was detected as a vector of the pathogen despite the differences in symptom expression and was identified as *Empoasca decipiens* (*Hebata decipiens)* that belong to family Cicadellidae. Results showed that it plays an effective role in the spreading and transmission of phytoplasma among the different crops in different countries i.e., *Ca*. *Phytoplasma trifolii* associated with faba bean phyllody in Jordan **(Salem *et al.*, 2023)**, Aster yellows phytoplasma associated with carrot (16Sr-I, AYp) in Turkey **(Zelyüt *et al.*, 2022)**.

Several attempts of inoculation were performed to verify the responsibility of associated phytoplasma to reproduce these symptoms. These methods were confirmed under greenhouse conditions including dodder (*Cuscuta campestris*) as a parasitic plant,

leafhopper insect, and seed. The trans-ability of phytoplasma was confirmed through dodder and leafhopper from infected carrot plants to healthy and periwinkle plants **(Wulandari *et al.*, 2021 and Clements *et al.*, 2021)** while seed transmissible and mechanical inoculation were not suitable methods for transmission. These findings are further supported by reports of the possible transmission of phytoplasma through different methods **(Salehi *et al.*, 2022, Huang *et al.*, 2023, Boopathi *et al.*, 2023)**. Most notably, the transmission of phytoplasma through seeds is still a controversial issue because of the very poor connection of embryo with the mother plant, although little research proved the transmission of phytoplasma via naturally infected seeds **(Bunwaree *et al.*, 2023 and Mateeti *et al.*, 2023).**

Dienes' stain, light, and electron microscope were used to study cytological and histopathological changes in carrot plants. Visualized phytoplasma units using a light microscope of Dienes' staining section showed Pleomorphic bodies in phloem tissues as irregular patches of intensely dark blue stained cells. In contrast, no intense color was observed in similar stained sections of healthy tissues. Our results correlate with those of **Gupta *et al.*, 2022, and Boopathi *et al.*, 2023.**

Examination of infected tissues using light and microscopic electrons revealed noticeable changes in ultrastructural and anatomical in carrot plants revealed deterioration effects on leaf compared with healthy ones. These histological variations changes involve distortion and disorganization of phloem tissues, which mainly occurred due to the linking of phytoplasma units with the inner surface of the cell plasma membrane that consumes the cell membrane's sterols to meet their energy needs for growth and division **(Christensen *et al.*, 2005 and Mokbel & El-Attar 2016).**

Moreover, these changes included irregular palisade and spongy tissues, disorganization, and malformation of vascular bundle tissues. The obtained results agree with those previously reported by **El-Banna and El-Deeb 2007 and Randall *et al.*, 2011** who found a huge difference between healthy and infected tissues of the leaf.

The reduction of the leaf blade's thickness was a result of a reduction in palisade and spongy tissues by 14.3% and 27%, respectively. Also, the change of the mesophyll layer was due to the decrease, malformation, and irregular shape of sponge and palisade cells. As well as phytoplasma infection also leads to the disorganization of cells. The obtained results are like those reported before in tomatoes **(Ahmed *et al.*, 2016)**, apple trees **(Gallinger *et al.*, 2020),** sesame **(Ahmed *et al.*, 2022 and El-Aziz *et al.*,2023)** who observed that phytoplasma infection leads to marked changes between healthy and infected tissues including leaf, leaf blade, and the proportions of midvein's vascular tissues. Most notably, the main changes and differences are disorganization of phloem tissue that is attached with an increasing in size of spaces between cells, middle lamella, and cell wall thickness may occur due to accumulation of starch and sugars because of phloem tissue malformation that affected in the transmission of photosynthesis molecules, carbohydrates, and disturbs the function of sieve tube **(Maust *et al.*, 2003, El-Banna *et al.*, 2015).** Furthermore, the infection of phytoplasma affects stroma lamellae and grana destroying its structure, because of the downregulation of genes involved in photosynthesis **(Xue *et al.*, 2018).**

Conclusions

Phytoplasma infection associated with carrot was detected and isolated from naturally symptomatic fields in the El Qalyubia governorate and then transferred to healthy carrot and periwinkle plants by *Hebata decipiens* and dodder. Microscopic and molecular techniques confirmed the infection of phytoplasma in carrot plants. Phylogenetic analysis showed that our isolates (OP889140 and OP889141) were members of the 16SrII group 16SrI respectively. A phytoplasma infection led to several anatomical changes in the infected carrot's tissues including thickness, abnormal shape of the cell wall, irregular palisade, spongy tissues, disorganization, malformation of vascular bundle tissues, and abnormal shape of a carrot. We recommend further research to manage the disease. This can be achieved through understanding their adaptive mechanisms and response to phytoplasma infection.

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التعرف والتوصيف الجزيئي للفيتوبلازما المرتبطة بنبات الجزر (*Daucus carota* L.) في محافظة القليوبية، مصر

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في السنوات الأخيرة، زادت الاصابة بأمراض الفيتوبلازما وأصبحت خطيرة في مصر. تم جمع عينات من نباتات الجزر التي تظهر عليها أعراض مشابهة لأمراض الفيتوبلازما من سبع مناطق مختلفة في محافظة القليوبية خلال موسمي النمو 2021 و 2022 مزروعة بالجزر للكشف عن الاصابة بالفيتوبلازما. لذلك تم استخدام الفحص المجهري الضوئي، صبغة دينيس، المجهر الإلكتروني النافذ (TEM) وPCR–Nested. كما تم استخدام الحامول (*Cuscuta campestris*) ، نطاط الأوراق (*Hebata decipiens*) ، البذور لدراسة قابلية نقل الفيتوبلازما. أظهرت النتائج التي تم الحصول عليها أجساما متعددة الأشكال في أنسجة اللحاء المعالجة مع صبغة دينيس كبقع غير منتظمة من الخلايا المصبوغة باللون الأزرق الداكن بشكل مكثف. ومع ذلك، لوحظ باستخدام المجهر الضوئي تغيرات غيرطبيعية كبيرة في الأنسجة المصابة مقارنة بالسليمة. تم تأكيد هذه النتائج باستخدام المجهر الإلكتروني النافذ (TEM) ولوحظ وجود وحدات الفيتوبلازما في أنسجة اللحاء وتدهور في بنية النباتات المصابة وكذلك الخلايا المشوهة والسمك والشكل غير الطبيعي لجدار الخلية. ولكن بسبب التوزيع غير المتسق داخل النبات، فإن المعيارة المنخفضة والتذبذب غالبا ما يعيقان الكشف الدقيق عن الفيتوبلازما. لذلك، تم استخدام اختبار تفاعل البوليميراز المتسلسل للكشف عن عدوى الفيتوبلازما باستخدام أزواج البادئات العالمية universal primer pairs P1/P7 and R16F2n/R16R2 التي تنتج شظايا واضحة عند 1200 نقطة أساس. لانتقال الفيتوبلازما في الجزر، حيث أعطى الحامول، ونطاطات الأوراق نتائج إيجابية وأعطى النقل الميكانيكي نتائج سلبية. هذه النتائج ستوفر فهما أفضل للعدوى بالفيتوبلازما في الجزر، مما يساعدنا على تطوير استراتيجيات فعالة للتغلب على مثل هذه الأمراض.

الكلمات المفتاحية: الفيتوبلازما، الجزر، صبغة دينيس ، الفحص المجهري الضوئي ، TEM ، الحامول ، نطاط الأوراق ، التسلسل ، تفاعل البوليميراز المتسلسل.