Occurrence, Etiology and Molecular Characterization of Phytoplasma Diseases on *Solanum lycopersicum* Crop in Egypt

Eman A. Ahmed[1], Osama Y. Shalaby[2], Emad F. Dwidar[2], Samah A. Mokbel[1], Ahmed K. El-Attar[1]

[1]Virus and Phytoplasma Research Department, Plant Pathology Research Institute, Agricultural Research Center (ARC), PO Box 12619, Giza, Egypt.
[2]Plant Pathology Department, Faculty of Agriculture, Fayoum University, PO Box 63514, Fayoum, Egypt.

ABSTRACT

Surveys were carried over the course of the 2012 and 2013 in different areas of tomato-growing fields in Egypt (Giza, Ismailia, Bani-Sweif, Fayoum and Qena) in order to determine the occurrence and distribution of phytoplasma associated with diseased tomato plants (*Solanum lycopersycum* L.), and to identify and classify the phytoplasma involved. A detection of infected tomato plants, which showed symptoms of big bud, witches'-broom and phyllody, in all regions of the screened governorates, reacted positively when assayed by nested polymerase chain reactions (PCR) using universal phytoplasma-specific primer pairs P1/P7 and R16F2n/R16R2. Similar assays were used to detect phytoplasma interactions with experimentally host plant. Dienes' stain was also used for detection of natural infection of phytoplasma. The phloem of infected tissues showed scattered area stained bright blue. Different techniques for transmission of phytoplasma to healthy tomatoes and periwinkles in an insect-proof greenhouse were tested, including mechanical inoculation, wedge grafting, parasitic plant dodder (*Cuscuta campestris*), insects in the family *Cicadellidae* (leafhopper, *Empoasca decipiens*) and in germinated seeds within the fruit, suggesting mechanical inoculation and seed-transmissible were not feasible while the positive results were obtained by the other three techniques for host plants with numerous symptoms obtained later. Transmission electron microscopy (TEM) of experimentally inoculated samples, revealed phytoplasma in the phloem of most of tested samples. Phytoplasma were observed as rounded bodies, ranging in size from 200 to 600 nm. The molecular characterization was performed for three different samples representing the different symptoms of phytoplasma through cloning and direct sequencing. The DNA sequencing, phylogenetic analysis and the multiple alignments for the sequences of the Egyptian clones with each other and with the other sequences of phytoplasma strains on GenBank showed that we may have two different phytoplasma isolates infecting tomato plants in Egypt.

Key words: Phytoplasma, PCR, Sequencing, Dienes' stain, TEM, graft, dodder and insect transmission.

INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill., *Solanum lycopersicon* L.), belongs to *Solanaceae* family. Tomato is one of the most economically important crops in Egypt; grown in open fields and greenhouses. Egypt ranks fifth in the world for tomato production and has a total annual production 8,6 million tons (FAO, 2014). Tomato fruits are consumed fresh in salads and cooked in sauces or in processed forms such as juice, ketchup, and canned tomatoes.

Phytoplasmas, formerly called mycoplasma-like organisms (MLOs), are wall-less Gram-positive bacteria of the class Mollicutes that inhabit plant phloem and are known to cause disease in several plant species worldwide (Liefting *et al*.,

2004), non-cultivable outside their hosts in cell-free artificial culture media (Chang *et al*., 2004).

Tomato is susceptible to many pathogens, including phytoplasma, lead to considerable losses of production or diminished of fruit quality. Phytoplasma diseases of tomato (PDT) have been reported in several countries around the world and exhibited a range of symptoms such as big bud, proliferation and small leaves of lateral shoots, purplish top leaves, phyllody, enlarged pistil, hypertrophic calyxes, small and polygonal fruit, stolbur and witches'broom (Jones *et al*., 2005; Xu *et al*., 2013; Salehi *et al*., 2014), in Egypt, the first occurrence of tomato plants with symptoms of big bud was reported by El-Banna *et al*. (2007).

In previous decades, the inability to culture phytoplasmas has made it difficult to characterize these pathogens (Schneider *et al*., 1997) and phytoplasma detection was based traditionally on symptomatology through grafting of the infected plant to healthy plants or through the parasitic plant dodder (*Cuscuta sp*.) (Akhtar *et al*., 2009; Salehi *et al*., 2014) and also by insects in the families *Cicadellidae* (leafhoppers), *Fulgoridae* (plant hoppers) and *Psyllidae* (psyllids), which feed on the phloem tissues of infected plants (Shaw *et al*., 1993; Hanboonsong *et al*., 2002) until the establishment of electron microscopy (EM) which became an alternative approach to the traditional indexing procedure for phytoplasmas (Cousin *et al*., 1986). However, these methods are not suitable for revealing genetic relatedness among different phytoplasmas (Lee *et al*., 2000).

Lately, molecular DNA-based techniques such as, polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), analysis of PCR products, and sequence analysis used increasingly for differentiation and characterization of phytoplasma strains based on various conserved genes coding in the dispersed regions of the 16S rRNA which enabled the detection of numerous phytoplasma and classified into 28 groups (Lee, 1996 and Tran-Nguyen *et al*., 2000; Gundersen). Whilst, nested PCR most widely used to overcome the low concentration problem of phytoplasma in infected plants. The efficiency of nested PCR clearly show in amplifying of the direct PCR product where, the amplifications provide a simple, rapid approach to obtain rRNA that may either be sequenced directly or cloned and sorted through a recombinant genomic DNA library to find a specific gen (Giovannoni, 1991) however, this technique requires more than one PCR step which increasing the chances of contamination between samples (Olmos *et al*., 1999).

The objectives of this study to investigate the occurrence and etiology of tomato malformation recently observed in Egypt and illustrate the responsibility of associated phytoplasma. Also, the study was extended to characterize associated phytoplasma, based on molecular techniques.

MATERIAL AND METHODS

The investigations presented in the present study were carried at Plant Pathology Department, Faculty of Agriculture, Fayoum University during 2012-2013, whilst studies to characterize the phytoplasma using molecular techniques were carried out at Virus and Phytoplasma Research Department, Plant Pathology Research Institute, Agricultural Research Center, Giza.

Occurrence and distribution of phytoplasma:

A field survey was conducted to determine the disease incidence of five governorates in Egypt (Giza, Ismalia, Banisweef, Fayoum and Qena) based on visual symptoms related to phytoplasma during 2012 and 2013, and also to isolate and identify of the phytoplasma strain associated with the observed symptoms. In each governorate, a certain number of fields were selected and inspected from April to November where, incidence of symptom expression (% of plants with phytoplasma symptoms) was estimated for each field by visual inspection of 100 plants following a W pattern (crossing the rows), then were detected firstly using light microscope and verified by PCR assay.

Transmission studies and Etiology:

The responsibility of the associated phytoplasma was tested by different transmission methods. At the end of each test, developed test plants were examined for symptoms expression by visual inspection, electron microscope, and then verified by PCR assay.

Seed transmission:

One hundred seeds were collected from infected tomato fruits (positive PCR- test) and planted after drying in plastic pots (5 seeds/pot, with 1 cm deep) contain a 1:1 mixture of sand and peat moss. Pots were covered with transparent polyethylene bags and removed after germination occurs (1 week). Irrigation was followed using lightly tap water and keep consistently moist under greenhouse conditions till symptoms development in insect-proof cages.

Mechanical transmission:

Three gram of naturally infected tomato leaf tissues with associated phytoplasma was ground in (0.02M) phosphate buffer (pH 7.4, 1g/mL^{-1}) with a mortar and pestle. Fully expanded leaves of twenty healthy tomato seedlings (month-old) and periwinkle (*Catharanthus roseus* L.) plants were dusted with carborandum 500 mesh and then mechanically inoculated by homogenized inoculation buffer by using cotton pads. Plants were rinsed with a gentle stream of water after inoculation and kept in an insect-proof greenhouse till symptoms development. An equal number of healthy seedlings of the same species and age were left without inoculation as control treatment.

Dodder transmission:

Seeds of dodder (*Cuscuta campestris*) were surface sterilized with 1% sodium hypochlorite solution, and then were washed with distilled water and dried. Seeds were *in vitro* germinated on Petri-dishes with wetted filter paper. Germinated seeds were transferred to 20 infected tomato plants (5seeds/infected plant), sown in plastic pots containing a 1:1 mixture of sand and peatmoss. After three weeks, dodder strands were used to connect each infected plant to healthy one of both tomato and periwinkle plants. Connections were maintained for 4 weeks, irrigation was followed using tap water every two days, after which the test plants were freed of dodder strands and kept in an insect-free greenhouse till symptoms development. In check treatment, dodder stolons were allowed to parasitize on healthy source plants before parasitizing on their healthy ones as negative controls.

Grafting transmission:

Graft inoculation was used to transmit associated phytoplasma from naturally infected tomato plants (scions) to twenty healthy tomato and periwinkle plants (root stocks). Plants were paired

(one positive and one negative). Control plants (non-grafted) grown from phytoplasma positive and healthy plants were established as part of the trial. Following graft inoculations, inoculated plants were placed in an insect-free greenhouse till symptoms development.

Leafhopper transmission:

Approximately 450 of leafhopper were collected by sweep netting from tomato fields with associated phytoplasma, and about 15 leafhoppers were sent to Insect Classification and Surveying Department, Plant Protection Research Institute (PPRI), Dokki-Giza, for identification. After starving the remaining leafhoppers for 24 h, were allowed to feed on diseased plants for 7 days for disease acquisition. A batch of twenty insects per plant was released onto 20 caged of both healthy tomato seedlings (month-old) and periwinkle plants for an inoculation access period of 30 days. Insects were killed in a jar containing cotton pieces immersed in ethyl acetate, and then symptoms were monitored daily.

Light microscopy of Dienes' stained tissues:

Flower and petioles from infected plants and healthy dissected into approximately 1-2 mm sections, using scalpe containing phloem tissue. The prepared sections were transferred onto 70% ethanol, then stained using Dienes' stain as described by (Deeley *et al*., 1979), and examined by light microscope at x 330 times (Musetti, 2013).

Transmission Electron microscopy (TEM):

Tissues from inoculated tomato and periwinkle plants and healthy tomato plants (as a control) were fixed in 2% (vol/vol) glutaraldehide dissolved in 0.1 M sodium cacodylate buffer (pH 7.2), subjected to a vacuum for 1-4 minutes every 15 minutes for 2 hours at 4°C, and fixed again in 1% (vol/vol) osmium tetroxide for 1.5 hours at room temperature. The samples were washed with distilled water, treated with 5% uranyl acetate for 1.5 h, washed again with distilled water and then dehydrated with ascending concentrations of ethanol for 15 minutes each. After dehydration, ultra-thin sections (70 nm thickness) were cut using ultramicrotome Leica model EM-UC6, mounted on copper grids (400 mish), and doubled stained with 2% (vol/ vol) uranyl acetate for 10 min followed by 0.4% (vol/vol) lead citrate for 5 min, and then observed under T.E.M. (JEOL, JEM-1400) and images were captured by AMT's CCD camera.

Nested PCR for detection of phytoplasma:

The total DNA was extracted from infected tomato plant leaves using the standard assay developed by Dellaporta (Dellaporta *et.al.,* 1983). The phytoplasma DNA was detected in symptomatic tomatoes using nested PCR. The DNA extracted from healthy tomato plant was used as negative control for all reactions. Two pairs of universal primers, P1/P7 and R16F2n/R16R2 specific for spacer region of the phytoplasma genome, were used to amplify the 16SrRNA and 16S/23 in nested PCR (Bhat *et al*., 2006 and Mokbel *et al*., 2013). The primer pair P1/P7 was used - in the first step PCR- for the amplification of 1.8 kbp product of 16S rRNA gene while the primer pair R16F2n, R16R2 was used to amplify a 1.2 kbp fragment of 16S rRNA gene in the second step nested-PCR as described by Wang and Hiruki (2001). To run the first step PCR; 1 µl DNA extracted from tomato plants was used in 25µl total PCR mixture contained 25 pmol of each primer; 200 µM of each dNTP; 1x

polymerase reaction buffer; 2.5 mM MgCl2; 1.25 U of dream-Taq polymerase (Fermentas) and sterile water to a final volume of 25 µl. The DNA amplification was started with a denaturation step at 94°C for 2 min followed by 35 cycles consisting of denaturation at 94°C for 30 s, annealing at 55°C for 1 min, and primer extension at 72°C for 1.5 min. A final extension step was added for 10 min 72°C.

One µl of DNA amplified in the first step PCR was used at 1:10 dilution as template for second step, nested, PCR. The nested PCR was started with a denaturation step at 94°C for 2 min followed by 35 cycles consisting of denaturation at 94°C for 30 s, annealing for 2 min at 50°C, and primer extension at 72°C for 3 min. A final extension step was added for 10 min 72°C. All amplification reactions were carried out in 0.2 ml micro Amp PCR tubes using T-Gradient thermal cycler (Biometra, Germany). The PCR products were stained with gel star (Lonza, USA) and separated on 0.7 % agarose gel with 1x TBE buffer then analyzed using (Gel Doc 2000 Bio.RAD).

Molecular cloning and DNA sequencing:

Three different tomato samples infected with the phytoplasma those collected from different fields and representing the different symptoms of phytoplasma on infected tomato were used for the molecular cloning. The three PCR fragments those amplified in the nested-PCR were purified and cloned individually into pCR4-TOPO cloning vector (Invitrogen, Cat: K4530-20). The three recombinant plasmids were abbreviated as A1, B2 and C3 and transformed into DH5α E.coli cells using heat chock (Ausubel *et al*; 1999). The cloned plasmids were isolated from selected white colonies, and purified using the QIAprep Spin Miniprep Kit (Qiagen, Germany). The obtained recombinant plasmids were analyzed with restriction digestions using EcoRI enzyme and verified by automated DNA sequencing. The universal M13 (-20) forward and M13 reverse primers were used for DNA sequencing. The nucleotide sequences were assembled using DNAMAN Sequence Analysis Software (Lynnon BioSoft. Quebec, Canada) and submitted to the GenBank at National Center for Biotechnology Information (NCBI, Bethesda, USA). BLAST analyses were performed at NCBI to search for sequence similarity. To perform the phylogenetic tree; the nucleotide sequences of the three Egyptian clones (A1, B2 and C3) were aligned to each other's then aligned with selected, equivalent, partial sequences of the 16S ribosomal gene from representative strains of 'Candidatus Phytoplasma' available in GenBank.

RESULTS

Incidence and Symptoms of Phytoplasma:

A survey was conducted on tomato fields in different regions of five governorates in Egypt (Giza, Ismalia, Bani-sweef, Fayoum and Qena). Naturally infected plants with different symptoms of phytoplasma were observed in (29 and 42 field) of total tomato cultivating fields (120 and 145) that were surveyed in 2012 and 2013 respectively, starting from April to November, in all governorates which surveyed.

In most of the plants that were collected, the major disease symptoms were big or swollen flower buds with green petals (Fig.1.B), phyllody (Fig.1.C.D), stunting with heavy

proliferations and yellowing (Fig.1.E), witches'-broom (Fig.1.F), purplish of top leaf (Fig.1.G) and the plants formed a reduced number of hard green fruits that were deformed or polygonal with atrophy of seeds at the time of infection (Fig.1.H).

The percentage of incidence were varied greatly not only between regions but also between fields where, the disease frequency in most cases ranged between 1-7% in 2012 but, the percentage of incidence in the same locations increased in 2013 however, the highest disease incidence was recorded in Bani-sweef governorate and reached to 15 % as indicated in table 1.

The presence of phytoplasmas in these plants was firstly detected through combination of sectioned plant tissues with the application of Dienes' stain, observed as dark blue areas in the phloem region, using light microscope (Fig. 2), and then confirmed by PCR assay. Total DNA was extracted and used as a template for nested PCR. The results of the nested PCR using the universal phytoplasma-specific primers showed a clear band at the specific size 1200 bp in tomato samples that collected from Ismailia, Banisweef, Fayoum and Qena, while no results were obtained from samples that collected from Giza governorate (Fig. 3A&B).

Table 1. The incidence of phytoplasma disease of tomato plants in five governorates during 2012 and 2013.

Governorate	Locations	N.S./N.F[a]		Incidence %[b]		Symptomatic Fields[c]		Symptoms observed	PCR detection N./T.[d]
		2012	2013	2012	2013	2012	2013		
Giza	El-Iyaat	100/10	100/10	1	2	1/10	1/10	Y, SL	0/30
	El-Badrashin	100/10	75/15	1	3	2/10	3/15		
Ismailia	El-Kasasin	90/10	100/10	3	5	2/10	4/10	BB, Y, SL, S,WB	25/25
	El-Salhia	100/15	100/10	5	6	3/15	4/10	BB, Ph, WB	
Bani-Sweif	Sedmant	200/15	100/15	6	15	5/15	7/15	BB, Ph, PT, WB	20/60
	El-Fashn	90/10	120/15	3	5	2/10	1/15	Y, SL	
Fayoum	Senours	120/12	200/25	5	7	4/12	6/25	BB, Y, SL, S	25/50
	Ibshaway	100/10	70/10	3	4	1/10	2/10	Ph, Y, S, WB	
	Tamiya	90/8	100/10	7	`10	4/8	6/10	BB, WB	
Qena	Qena	100/10	120/15	4	6	3/10	5/15	BB, Ph, SL, WB	12/30
	Qeft	100/10	100/10	2	5	2/10	3/10	Y, S	

(a): Number of samples/number of fields. **(b):** Number of affected fields / total number of fields surveyed **(c):** Symptomatic plants/100 plants. **(d):** Number of symptomatic samples positive in PCR/total samples detected. **(Y)**= Yellowing **(SL)**=Small leaves **(BB)**=Big bud **(S)**=Stunting **(Ph)**=Phyllody **(PT)**= Purplish of top **(WB)**=Witches'-broom

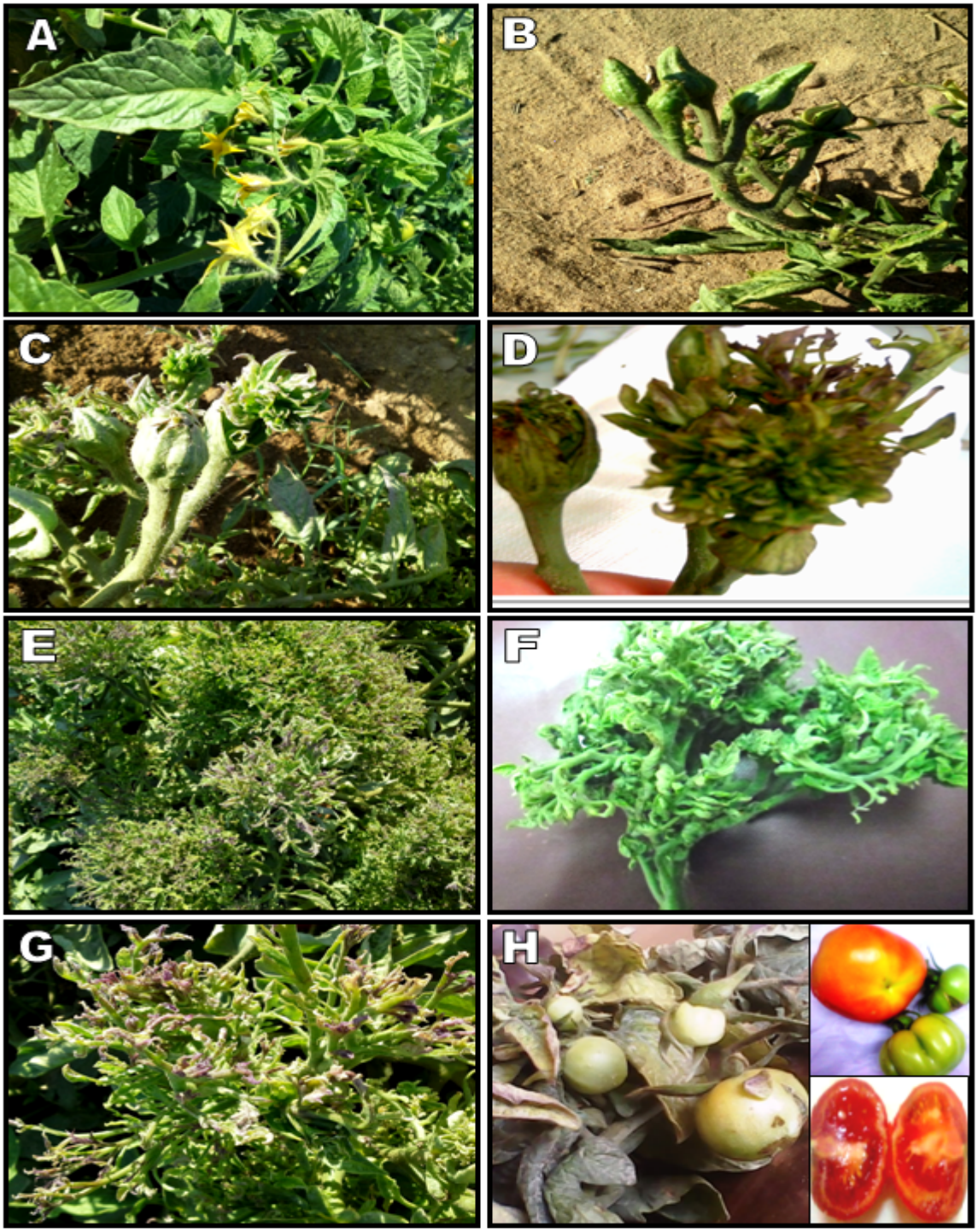


Figure 1. Healthy tomato plants **(A)** and symptoms of phytoplasma on naturally infected tomato plants, big or swollen flower buds with green petals **(B)** and phyllody **(C,D)**, Stunted of tomato plants with apical and branch proliferations **(E)**, witches'-broom **(F)**, purplish of top leaf **(G)** hard green and polygonal fruits with seeds atrophy **(H)**.

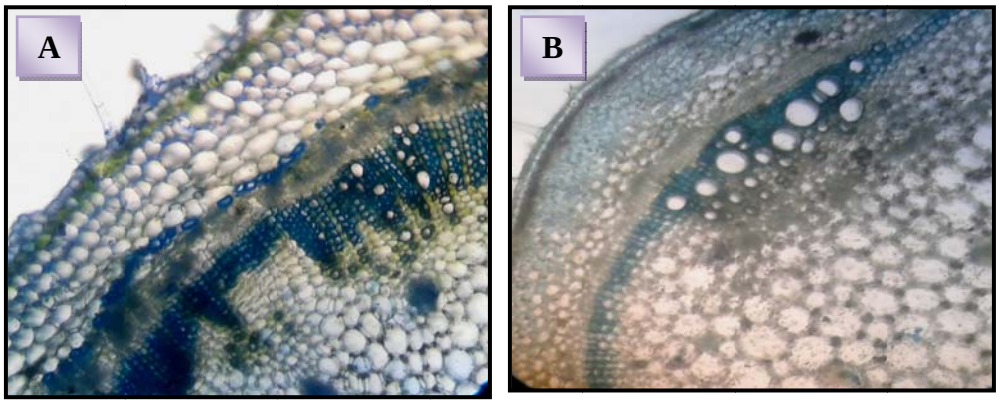
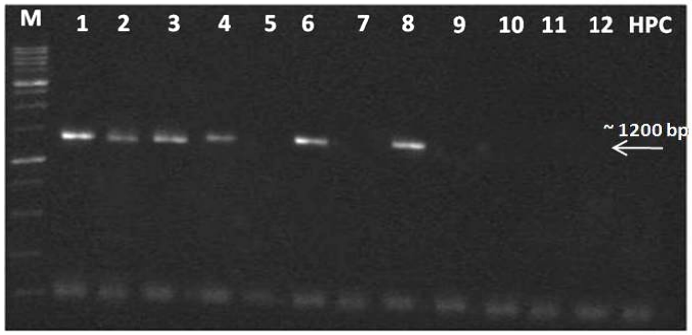
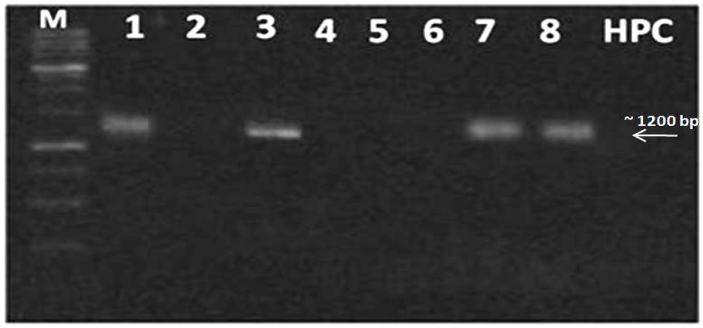


Figure 2. Micrograph of transverse section of infected tomato stained with Dienes' stain showing dark blue areas in phloem cells **(A)**, section of healthy plant with unstained phloem **(B)**.



(A)



(B)

Figure 3. Gel electrophoresis for the detection of the phytoplasma in infected tomato plants collected from Ismailia (Lanes 1-4), Banisweef (Lanes 5-8) and Giza (Lanes 9-12) **(A)**; Fayoum (Lanes 1-4) and Qena (Lanes 5-8) **(B)**. HPC: Healthy tomato plants. M: 1 Kb DNA Ladder**.**

Leafhopper identification:

In all surveys of tomato fields, one leafhopper specie was found on the underside of the leaves, insects were morphologically identified under the stereoscope, and the specimen was identified as *Empoasca decipiens* belonging to the family *Cicadellidae*, subfamily *Typhlocybinae*, which are about 3 mm in length and green in color.

Transmission studies and Etiology of tomato malformation:

Seed transmission and mechanical inoculation of phytoplasma affecting tomato plants could not be transmitted under greenhouse conditions, which indicates that phytoplasma affecting tomato is not mechanically or seed transmissible; however, the phytoplasma was successfully transmitted by grafting and dodder where, the rate of transmission reached to 60% and 40% respectively, in the case of inoculated healthy tomato plants while, reached to 40% and 30% respectively, in the case of transmission the agent associated with disease from infected tomato to healthy periwinkle. Identified leafhoppers (*Empoasca decipiens*) were collected in tomato fields and used as potential vectors for the phytoplasma transmission studies. The transmission trials with *Empoasca* spp. were successful where, the rate of transmission was 55% and 65% in the case of inoculated healthy tomato and periwinkle, respectively, as indicated in Table 2.

Transmission of phytoplasma under study by different involved methods from donor infected tomato to receptor healthy tomato plants (Fig.4.A) led to many kinds of leaf malformations and symptoms included big bud with malformed petals, phyllody or distortion and bunched of old leaves, similar to witches'-broom symptoms (Fig.4.B,C,D) while, to receptor healthy periwinkle (Fig.4.E) plants included mottling or yellowing with very short stalks and phyllody (Fig.4.F,G), and all the previous symptoms appeared during a period, ranged from 30 to 60 days from the beginning of the inoculation.

The presence of phytoplasma in the phloem tissue of inoculated tomato and periwinkle plants from different transmission methods including grafting, dodder, leafhopper, seed and mechanical were verified through examination of ultra-thin sections using transmission electron microscopy. Micrograph observations of the inoculated plants showed that numerous bodies of phytoplasma appear as separate or clustered units in the phloem sieve tubes and were mostly rounded or irregular in shape with a diameter of 200 to 600 nm or distorting the cell wall and became thicker (Fig.5) however, phytoplasmas were more abundant in the inoculated samples through transmission by leafhopper on the other hand, no bodies were observed in the phloem cells of seed and mechanical inoculated plants.

The tomato or periwinkle plants those inoculated with phytoplasma were tested using PCR to check the efficiency of the transmission mechanism. Samples collected from tomato plants those previously inoculated with phytoplasma using Wedge grafting, Dodder and Leafhopper showed positive PCR results at the expected band size of 1200 bp. Plants those inoculated mechanically showed negative PCR results. PCR results confirmed the unsuccessful seed transmission of the phytoplasma onto tomato seeds. Fig (6) showed the PCR results for the transmission studies.

Molecular characterization and phylogenetic analysis:

To molecularly characterize the phytoplasma strain, the PCR fragment of 16S rRNA gene that amplified from the three different samples representing the different symptoms of phytoplasma were cloned successfully into the TOPO cloning vector. The selected clones which showed positive results after EcoRI enzyme digestion (data not shown), were directly sequenced. The nucleotide sequences were submitted to the GenBank.

Multiple sequence alignment and phylogenetic analysis was performed to compare the three Egyptian clones with each other and to compare them with the corresponding sequences of the other phytoplasma strains on GenBank (Fig. 7). The isolates on GenBank those used for the alignments are, Alfalfa W.B (AB259169), Tomato big bud (EF193359), Eggplant phyllody (HQ423156), Eggplant big bud (JX441321), Tomato W.B (HM584815), Sesame phyllody (JX436204), Sweet potato little leaf phytoplasma (JQ067649), Tomato yellows phytoplasma (JX162606), Peanut W.B (JX871467) and Candidatus Phytoplasma- Australians strain (KM212951). As shown from Fig. 7A; the alignment of the three Egyptian clones showed that, the clone B2 and C3 are very close to each other with 99% identity, while both of them are different than A with a relatively low similarity (94%). Fig. 7B showed that; the alignment of the Egyptian clone A1 with the other sequences of phytoplasma strains on GenBank showed 94% identity, while B1 showed 95% identity when compared with the same strains and C3 showed 96% identity as well.

Table 2. Reaction of plant species to inoculation with phytoplasma.

Transmission mode	Host or feeding plant	Symptoms	Time taken For symptom Expression /days	PCR Positive samples	
				No.	%
Seed	Tomato	No symptoms	-	0	0
Mechanical inoculation	Tomato	No symptoms	-	0	0
	Periwinkle				
Wedge grafting	Tomato	Big bud	52	12	60
	Periwinkle	Mottling or yellowing leaves	40	8	40
Dodder	Tomato	Witches'-broom	49	8	40
	Periwinkle	Yellowing	30	6	30
Leafhopper *Empoasca decipiens*	Tomato	Phyllody	60	11	55
	Periwinkle	Phyllody	53	13	65

20 plant/ transmission test

Figure 4. Healthy tomato plant **(A)**. Development of big bud, witches'- broom and phyllody symptoms **(B, C and D)** in tomato plants with agent of tomato big bud phytoplasma through graft, dodder and insect transmission respectively. Healthy periwinkle plant **(E)**. Different degrees of mottling or yellowing symptoms through graft or dodder **(F)** and phyllody symptoms through insect transmission **(G)** with agent of tomato big bud phytoplasma in periwinkle plants.

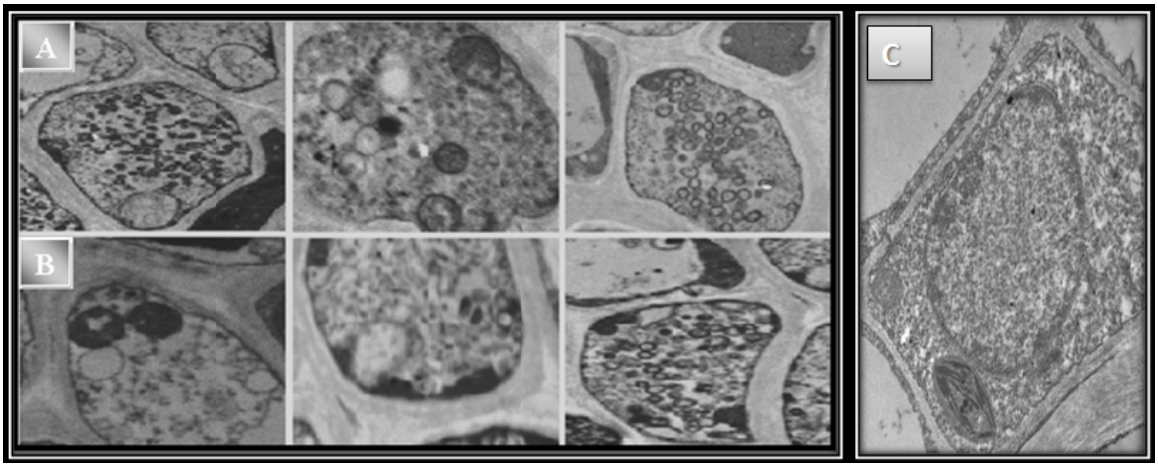


Figure 5. TEM micrograph of phytoplasmas transmitted by graft, dodder and leafhopper from left side respectively, to tomato **(A)** and periwinkle plants **(B)**, magnification (X 4.0000). Control healthy tomato plant **(c)**.

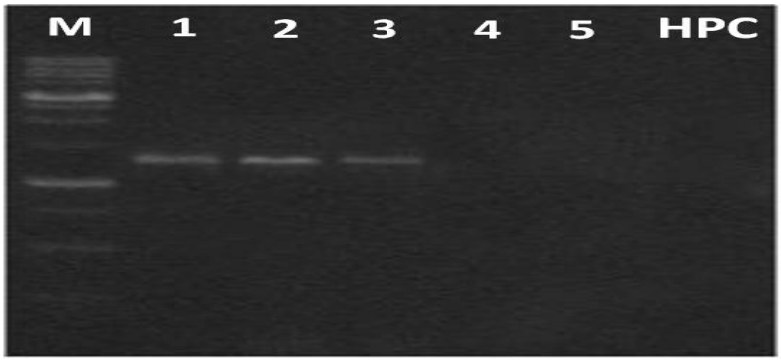


Figure 6. Gel electrophoresis for the detection of the phytoplasma in tomato or periwinkle plants to check the transmission efficiency. L1, L2 and L3: plants those previously inoculated with phytoplasma using Wedge grafting, Dodder and Leafhopper respectively. L4 and L5: Tomato plants those previously inoculated with phytoplasma using Seed transmission or Mechanical inoculation. HPC: Healthy tomato plants. M: 1 Kb DNA Ladder

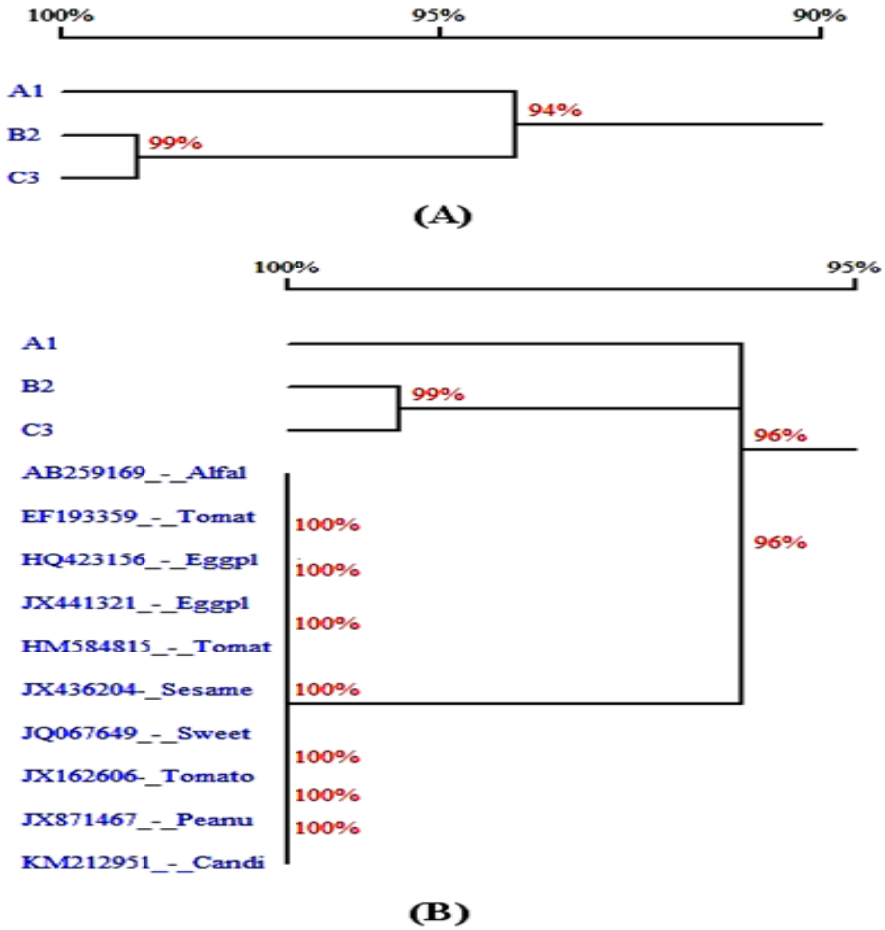


Figure 7. Phylogenetic analysis for the three Egyptian isolates aligned to each other **(A)** and to other different phytoplasma strains in the GenBank

DISCUSSION

In recent years, emerging phytoplasma diseases have increasingly become important in Egypt, due to their serious impact on economically important crops, therefore, precise diagnosis, including pathogen identification is very essential for disease control and prevents further infection spread.

In the current study, visual inspection of eleven regions of five different governorates (Giza, Ismalia, Bani-sweef, Fayoum and Qena) particularly dedicated to tomato production, showed several types of external symptoms related to phytoplasma diseases of tomato (PDT), the first visible symptoms are swollen flower buds with green petals or phyllody and hard green fruits, the other indications of infection are distortion or stunted growth, witches'-broom, yellowing and purple-colored leaves. The character of the observed symptoms in tomato plants, somewhat similar to those was described in Egypt (El-Banna *et al*., 2007) or in several countries including, USA (Shaw, *et al*., 1993), Brazil (Amaral *et al*., 2006), China (Xu *et al*., 2013) and Iran (Salehi *et al*., 2014). The results of the phytoplasma incidence in the surveyed field was somehow considerable (81.8%), considering that 9 regions out of 11 were PCR-positive with clear band at the specific size 1200 bp using the universal phytoplasma-specific primers which confirmed the natural infection of tomatoes with phytoplasma associated with big bud, witches'-broom and phyllody unlike, PCR amplified products from asymptomatic tomato plants as well as from samples that collected from Giza governorate however, more investigations and analysis need to be performed in the future to study the reason for the appearance of these symptoms in Giza governorate.

Investigations of phytoplasma in the present work were correlated with insect vector, and was identified as *Empoasca decipiens* that belong to the *Cicadellidae* family, and the only vector that found in all locations despite the differences in symptom expression where this small insect, play a role in spreading different types of phytoplasmas, Almond Witches'-Broom Phytoplasma (Dakhil *et al*., 2011), Stone Fruit Yellows phytoplasma (Pastore *et al*., 2004) or Chrysanthemum yellows phytoplasma (Galetto *et al*., 2011).

In Egypt, this insect has a very broad range of host plants, on cabbage and cauliflower, rice, marjoram, different bean species, cowpea, squash and other 57 hosts (Mahmoud *et al*., 2011) which makes them efficient vectors of pathogens residing in those crops where more than ten insects of *Empoasca* spp. cause severe bushy canopy and possibly witches'-broom symptoms (Licha, 1980) furthermore, tomato big bud phytoplasma, causes phyllody and an absence of normal flower production in *Catharanthus roseus* (periwinkle) (Padovan *et al*., 2000) due to disturbance in the normal balance of growth regulators with accumulation of sucrose in infected leaves (Bertaccini, 2007, Fletcher and Wayadande, 2002). Accordingly, the existence of these insects in tomato fields cannot exclude which mainly attracted to the sugar-rich phloem sieve tubes of infected plants then function as a vector therefore, be taken as an indication for the dispersion of phytoplasma infection in tomato fields which also corresponded with experimental transmission results as

evident from observed symptoms such as, phyllody.

Also, in order to confirm the responsibility of associated phytoplasma to reproduce these symptoms, has been achieved through other series of inoculations methods, were performed differently in the greenhouse including, parasitic plant dodder (*Cuscuta campestris*) or wedge grafting, on healthy tomato or host periwinkle plants which characterized by some degree of specificity (Marcone *et al*., 1997), while the mechanical inoculation and seed-transmissible were not feasible. These findings are further supported by reports of the possible transmission of phytoplasma through different methods (Shaw *et al*., 1993; Bertaccini 2007; Akhtar *et al*., 2009; Salehi *et al*., 2014), and confirm that grafting is the perfect method for re-infection of phytoplasma (Al-Zadjali *et al*., 2007) at the same time, the healthy dodder is one of the main ways by which phytoplasma infection is achieved under artificial conditions (Marcone *et al*., 1997), while, phytoplasma seed transmission is still a controversial issue due to the very poor connection of embryo with the mother plant however, the reduced seed production in infected plants is quite observed (Calari *et al*., 2011) although few studies confirmed the natural transmission of phytoplasmas through infected seed, that belonging to ribosomal groups 16SrI,16SrXII and 16SrII (Botti and Bertaccini, 2006).

In the present study, an association between observed phytoplasma in inoculated host plant and their detection were achieved using electron microscopy, phytoplasmas were demonstrated in high numbers in sieve elements of inoculated plants, especially through insect transmission that may be due to multiplication of phytoplasmas in the insect body (Boudon-Padieu *et al*., 1989; Liu *et al*., 1994), and were usually appear as rounded or irregular bodies, with a diameter of 200-600 nm, the reliability of phytoplasma diagnosis in phloem tissue by TEM technique, was reported and discussed by Fletcher and Wayadande (2002) and IRPCM (2004) in addition to, the morphology and size of observed structure of phytoplasma as well as their localization in sieve elements using TEM are broadly consistent with the phytoplasma structure (200-400 nm diameter) were observed in the phloem of lucerne plant fed on by *Austroagallia torrida* that was TBB phytoplasma positive (Pilkington *et al*., 2004) or correspond to other type of phyllody-phytoplasma diseases reported by Al-Saady *et al*. (2006) and Akhtar *et al*. (2009). Similarly, the internal damage induced by phytoplasma, as evident by distorting the cell wall and became thicker than in healthy ones similar to those observed by Akhtar *et al*. (2009) and Xu *et al*. (2013). Furthermore, phytoplasma was successfully detected using nested PCR instead of direct one, because they often occur at low levels in plants, making the detection of DNA by direct PCR is unreliable may be attributed to inhibitors present in host plant tissue (Cheung *et al*., 1993; Goodwin *et al*., 1994; Andersen *et al*., 1998) but actually, phytoplasma cannot be morphologically or ultra-structurally distinguished from one another using either electron or light microscopy (CABI, 2007) while, the molecular investigation carried out in this research on samples collected from natural infected tomato plants or different samples from experimental transmissions, showed the presence of different infections of phytoplasma, where the phytoplasma associated with tomato big

bud and/or witches'-broom were successfully identified and molecularly characterized that performed through the sequence analysis and phylogenetic tree to study the homology with the sequence of the 16S ribosomal RNA (rRNA) gene. The phylogenetic tree that constructed for the alignment of the three Egyptian clones clearly demonstrated that, two of them are very close to each other with 99% identity (B2 and C3) while the third (A1) is different isolate with only 94% similarity as well as the multiple alignments for the sequences of the Egyptian isolates with the other sequences of phytoplasma strains on GenBank showed 94% identity with the isolate A1, while B2 showed 95% identity when compared with the same strains and C3 showed 96% identity as well. Those results confirmed that we may have two different phytoplasma isolates infecting tomato plants in Egypt. Further researches and deeper sequence analysis are required in the future to focus on the full characterization and classification of the detected strains.

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