Ultrastructural changes in tomato plant induced by phytoplasma infection and attempts for its elimination using tissue culture techniques

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

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

ABSTRACT:

Light microscopy was used to compare the anatomical characteristics of stem, flower petiole and leaves of infected tomato with phytoplasma with healthy ones in order to reveal anatomical modifications caused by the infection process. The results obtained showed that infection of tomato plants with phytoplasma led to an increase in stem diameter by 10.23% as well as greatly increase in measurements of the other stem components while the diameter of pith was decreased by 38.46%. This infection was led to an increase in the diameter of petiole by 109.2% and also the other components of flower petiole. At the same time, tomato leaves were greatly affected as a result of the infection with phytoplasma. The thickness of leaf blade was remarkably increased by 200% as well as thickness of either palisade or spongy tissues by 212.5% or 275%, and significant malformation in leaflet midvein was observed which consequently led to increase in both length and width of midvein by 15.19% and 5%. Electron microscopy was used to recognize the internal changes in cell organelles due to phytoplasma infection. The results obtained showed that, general disorganization of phloem tissue and thickness of cell wall resulted from high concentration of phytoplasma units; necrosis starts in companion cells; severe damage in chloroplasts with abnormal membrane and thylakoid system was absent. The xylem vesicles were characterized by deforming the secondary walls. Three methods were done towards the production of phytoplasma-free tomato plantlets through tissue culture using antibiotic compound (tetracycline hydrochloride in three concentrations 25mgL-1, 50mgL-1 and 75mgL-1), irradiation by three doses (3, 5 and 10 Gy) of gamma ray and natural compound (1ml of garlic juice). All treatments proven to be a very useful effect against phytoplasma, except concentration 25 mgL-1 of tetracycline hydrochloride. The findings of this study have proven treatment at lower dose of gamma rays (3 Gy) of efficient methods on growth promotion.

Key words: Phytoplasma; Light microscopy; Electron microscopy; Tissue culture; Anti-phytoplasmal activity.

INTRODUCTION:

Tomato is one of the most important vegetable crops in Egypt and considered as number one in terms of total production and cash value. Tomatoes are grown in three seasons' winter, summer and autumn on about 3 percent of Egypt's total planted area (Baka, 2014).

Phytoplasmas (class *Mollicutes*, genus Candidatus Phytoplasma) are among the smallest bacterial plant pathogens that caused severe malformation on infected plants, their flowers turn into leafy shoots or their petals into green, dwarfism (general stunting), phyllody, virescence, rapid senescence and flower sterility that transform plants into zombies or unable to produce offspring, this transformation attracts the sap-sucking insects that carry the pathogen to new hosts (Hogenhout *et al*., 2008). Such symptoms were observed in tomato plants during phytopathological surveys in the main agricultural areas during 2012-2013 (Ahmed *et al*., 2014). Set of diagnostic procedures and transmission electron microscopy (TEM) technique were performed to analyze the pathogens in collected samples prior the molecular techniques to confirm the results, and also to

Correspondence: dr.samahmokbel@gmail.com

formulate early disease management strategies.

Plant tissue culture is a major tool of biotechnology and of particular importance with vegetatively propagated crops in which infected planting materials transmit the pathogen to the new crop or has a great role in improving productivity of crops through rapid availability of healthy plants to avoid the great yield losses caused by phytoplasmas. Micropropagation of phytoplasma-free plants was successfully introduced using treatment of plant tissues with antibiotics like tetracycline hydrocloride (Wongkaew and Fletcher, 2004; Singh *et al*.,2006; El-Banna *et al*., 2007; Gribaudo *et al*., 2007; Mahrous, 2012) or with antimicrobial substance like garlic (Durairaj *et al*. 2009 and Mahrous, 2012). Recently, radiotherapy has appeared as a new approach for producing phytoplasma-free plants (Mokbel and El-Attar, 2014).

The objectives of this study are: (i): To investigate and recognize the internal and abnormalities impacts induced by phytoplasma infection in the tomato host according to recent studies have shown that the association between plants and phytoplasmas can result in anatomical alteration in phloem tissues of infected plants, and great differences between healthy and diseased samples using microscopic examination of longitudinal, cross or ultra-thin sections of leaf blade, leaf petiole and stem (El-Banna *et al*., 2007). (ii): To determine the efficiency of different techniques toward production of phytoplasma-free tomato plantlets and mitigation of phytoplasma disease.

MATERIALS AND METHODS

Plant materials:

The plant materials selected for present study were examined using nested PCR and have been widely investigated in previous study (Ahmed *et al*., 2014). The molecular investigation results of previous studies on samples collected from natural infected tomato plants, suggest that the presence of three infections of phytoplasma in different areas of tomato-growing fields in Egypt where, witches' broom, phyllody and big bud phytoplasma were successfully identified, molecularly characterized and accessioned by GenBank under accession numbers KT225548, KT230865 and KT225545 respectively. In the present study we focused on the following points:

Histopathological studies:

Ligh microscopy:

Plant materials of infected tomato (stem, flower petiole and leaflets) were fixed and preserved in F.A.A., dehydrated, embedded in paraffin wax, then serially sectioned at 20-µ-thick and finally, stained according to the conventional method (Sass, 1958) with the crystal violet–erythrosin combination, cleared in carbol xylene and mounted in Canada balsam. Control pieces from healthy plant were also prepared for comparison. The investigations were carried at Plant Pathology Department, Faculty of Agriculture, Fayoum University.

Electron microscopy (ultrathin-sections):

Ultra-histopathological changes due to phytoplasma infection on the cell components of infected tomato were studied using electron microscopy (EM) according to Hanschke and Schauer (1996) and carried out in Electron Microscopy Lab, Faculty of Agriculture, Cairo University.

Phytoplasmas elimination:

Antimicrobial agents:

One tablet (250 mg) of tetracycline hydrochloride ($C_{22}H_{24}N_2O_8$.HCl), produced by SEDICO Pharmaceutical Company (Egypt) was used within the recommended period. Stock solutions were prepared by dissolving tablet using 250 ml of autoclaved

liquid MS medium to produce 1mg/ml solution.

Garlic extract, prepared as a juice from crushed garlic cloves, and then centrifuged at 5,000 rpm for 5 min. The pure liquid juice was carefully removed from the top of the liquid by a syringe.

***In vitro* treatment processes:**

The phytoplasma-infected plants (scions) were introduced to the greenhouse, and graft inoculated (Fig.1) to twenty healthy tomato plants (root stocks). Stem segments (2-3 cm long) were taken from the plants grown under greenhouse conditions and then surface-sterilized as described by (Mokbel and El-Attar, 2014).

Three single node cuttings of 1.0 cm were cultivated in culture jar containing 25 ml Murashige and Skoog (1962) medium (full strength MS) with $30gL^{-1}$ sucrose and $8gL^{-1}$ agar. Shoots were micro-propagated by repeatedly sub-culturing in a second medium with the same basal composition but without agar using filter paper bridge, thus giving rise to stocks of plant for different treatments. In a first treatment, explants were cultivated on medium with three concentration of tetracycline hydrochloride (25, 50 and 75 mgL^{-1}) and were applied separately. In a second treatment, tomato explants were transferred to fresh medium with liquid garlic juice (1 ml/25ml MS). Antimicrobial agents were applied through sterilized filter 0.22 µm Millipore onto culture medium after autoclaving. Twenty infected explants with phytoplasma were used per each treatment. Control experiments free of antimicrobial agents were also set up. In third treatment, five jars with three explants were irradiated with each dose 3, 5 or 10 Gy of gamma irradiation for 30 min. five non-irradiated culture jars were served as control treatment. The source of gamma irradiation was ^{60}Co gamma cell 3500, from the Middle Eastern Regional Radioisotope Center for the Arab countries, Giza, Egypt.

The cultures at all growth stages were incubated under artificial conditions 25±1ºC, 16h photoperiod, 3000 Lux for three weeks, and then were daily observed and survival percentages were investigated. The survived explants of each treatment were sub-cultured for 3 weeks on fresh MS medium, and then transferred onto rooting liquid medium contain naphthalene acetic acid ($0.4\ mgL^{-1}$) for 3 weeks.

The presence of phytoplasma was assayed by nested PCR as following: For each condition, DNA was extracted from treated fresh shoots as well as diseased ones (control treatments) using the standard assay developed by Dellaporta *et al*. (1983). Two pairs of universal primers, P1/P7 and R16F2n/R16R2 were used for the first and second PCR, amplifying fragments of 1.8 Kb and 1.2 bp, respectively, was performed according to the protocol described by Wang and Hiruki (2001).

Treatment Efficiency (TE):

At the end of each *in vitro* treatment, treatment efficiency (%TE) was determined according to the rate of success in eliminating phytoplasma and percentage of surviving plantlets as follows:

$$TE = \frac{\text{Number of survival phytoplasma-free plants}}{\text{Total number of plants in each treatment}} \times 100$$

Fig.1. Transmission of phytoplasma from donor infected tomato to receptor healthy tomato through wedge grafting. Healthy tomato plants as root stock and the infected tomato plants as scions (A). Symptoms of big bud and witches' broom were developed on graft inoculated tomato plants after 52 and 45 days from grafting, respectively (B and C). Healthy tomato plants as control (D).

RESULTS

Histopathological studies:

Histopathological studies using light and electron microscopy revealed that dramatic changes occurred in anatomical structure of tomato plants stem, flower petiole and leaflet.

Data presented in Table (1) show that the infection of tomato plants with phytoplasma led to an increase of the diameter of the stem, thickness of the cortex, thickness of the vascular tissues and thickness of the inner phloem tissues by 10.23%, 32.35%, 37.5% and 525%, respectively while, the diameter of the pith decreased by 38.46% as compared to those in healthy plants (Fig.2[1]).

Data presented in Table (2) as well as the cross section of the infected tomato flower petiole (Fig.2[2]) reveal that the infection with phytoplasma led to an increase of the components of flower petiole. The diameter of the petiole was markedly increased by 109.2 %, the cortex thickness by 120%, the thickness of the vascular tissues by 51.89% and greatly increases in the thickness of the inner phloem by 333.33%.

The changes on the anatomical structure of the infected tomato leaflet with phytoplasma were clearly observed in Figure (2[3]) and Table (3). The infection of tomato plants with phytoplasma led to increase the thickness of the leaf blade by 200% and thickness of both palisade and spongy tissues by 212.5% and 275%, respectively. In addition, greatly malformation in leaflet midvein was observed which consequently led to increase both length and width of midvein by 15.19% and 5% respectively. Also, the number of the xylem vessels was remarkably increased by 126% and both length and width of the vascular bundle by 25% and 5.7%, respectively.

On the other side, the investigation of ultra-thin sections by transmission electron microscopy were carried out to recognize the ultrastructure of both healthy and infected tomato plants, and resulted in highly cytopathological changes of the infected plants as compared with that in the healthy cells including, general

disorganization of phloem tissue and cellular abnormalities of the infected tomato plants (Fig.3), thickness of cell wall and irregular in shape, high concentration of phytoplasma units in the sieve element of phloem cell (Fig.4), in addition, gradual degradation and dissocition of the adjacent cell wall followed by complete lysis (Fig.5), necrosis starts in companion cells (Fig.6) and changes on the plasma membrane of phloem cells was clearly observed as well as shattering of its structure (Fig.7). It was also observed that the chloroplasts of the infected cells became malformed and large in size if compared with healthy ones, with abnormal membrane and thylakoid system was absent (Fig.8) similarly, the xylem vesicles were characterized by deforming of secondary cell walls (Fig.9).

Table 1: Effect of phytoplasma infection on the anatomical structure of tomato stem.

Plant paramiters	Healthy tomato	Infected tomato	% Change
Stem diameter (µm)	5080	5600	+ 10.23
Cortex thickness (µm)	340	450	+ 32.35
Number of cortex layers	11	9	- 18.18
Vascular cylinder thickness (µm)	3900	4250	+ 8.974
Vascular tissues thickness (µm)	800	1100	+ 37.50
Xylem zone thickness (µm)	725	525	- 27.58
Number of xylem vessels	220	200	+ 9.090
Pith diameter (µm)	3250	2000	- 38.46
Inner phloem zone thickness(µm)	80	500	+ 525.0

Table 2: Effect of phytoplasma infection on the anatomical structure of tomato flower petiole.

Plant paramiters	Healthy tomato	Infected tomato	% Change
Flower petiole diameter (µm)	1525	3190	+ 109.2
Cortex thickness (µm)	125	275	+ 120.0
Number of cortex layers	8	8	0
Vascular cylinder thickness (µm)	1175	2590	+ 120.4
Vascular tissues thickness (µm)	370	562	+ 51.89
Xylem zone thickness (µm)	220	160	- 27.27
Number of xylem vessels	100	308	- 208.0
Pith diameter (µm)	795	1500	+ 88.67
Inner phloem zone thickness (µm)	90	390	+ 333.3

Table 3: Effect of phytoplasma infection on the anatomical structure of tomato leaflet.

Plant paramiters	Healthy tomato	Infected tomato	% Change
Midvein length (µm)	1020	1175	+ 15.19
Midvein width (µm)	1000	1050	+ 5.000
Blade thickness (µm)	100	300	+ 200.0
Palisade layer thickness (µm)	40	125	+ 212.5
Spongy layer thickness (µm)	40	150	+ 275.0
Number of xylem vessels	23	52	+ 126.0
Vascular bundle length (µm)	240	300	+ 25.00
Vascular bundle width (µm)	520	550	+ 5.769

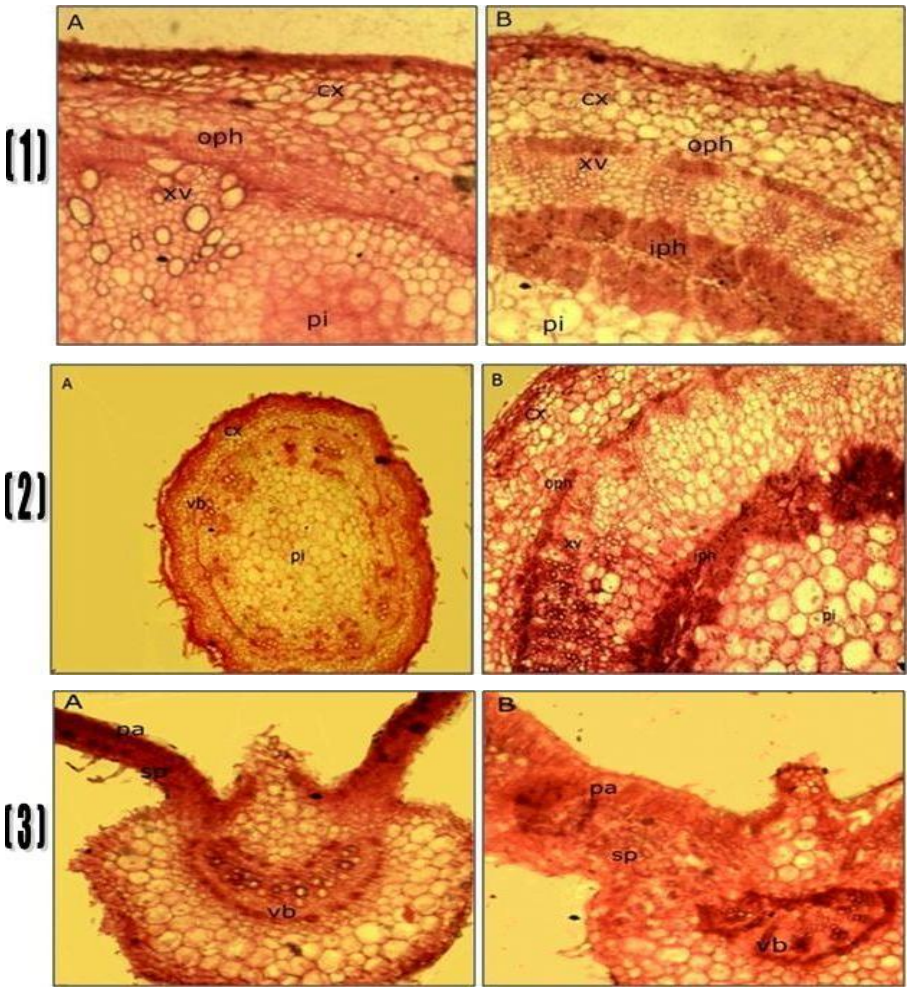

Fig.2. Transections of tomato stem (1) flower petiole (2) and leaflet (3) as affected by phytoplasma infection:

(A) Healthy plant. (B) Infected plant.

vb = vascular bundle. **xv** = xylem vessels. **cx** = cortex.
oph = outer phloem. **iph** = inner phloem. **pi** = pith.
pa = palisade tissue. **sp** = spongy tissue. X =70

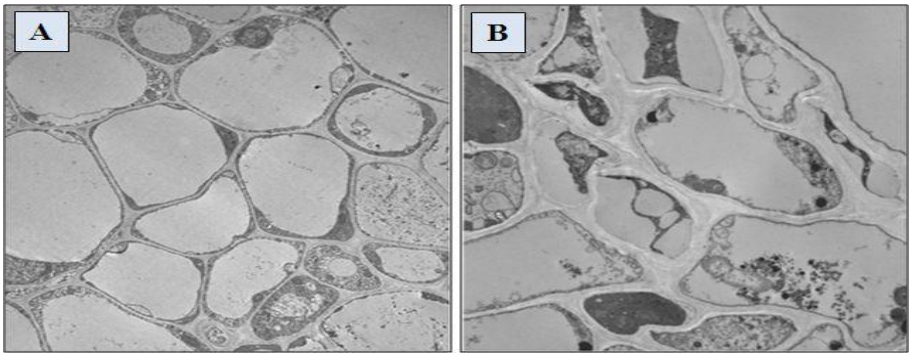

Fig.3. Transmission electron micrographs of the phloem tissues from the healthy and infected tomato plants. Phloem tissues of the healthy tomato plant (A). Disorganization of the phloem tissue and cell abnormalities of symptomatic tomato plants (B). X=4000.

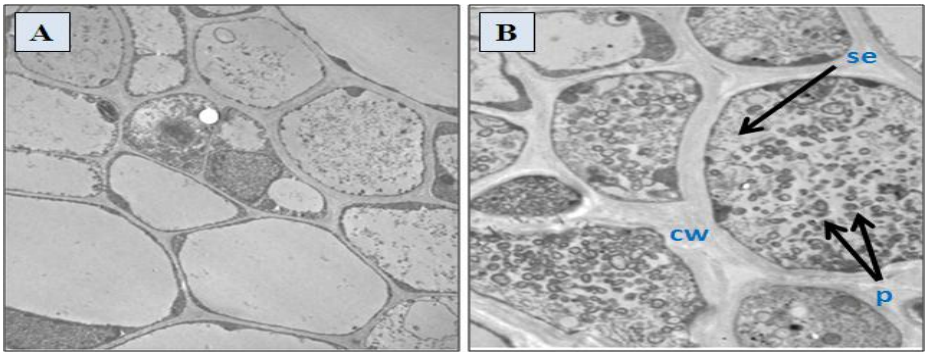

Fig.4. Transmission electron micrographs of the phloem tissues from the healthy and infected tomato plants. Phloem tissues of the healthy tomato plant (A). Sieve element of the phloem filled with high concentration of phytoplasma and uneven thinking of cell wall of symptomatic tomato plants (B). X=8000. **cw** = Cell wall, **se** = Sieve element, **p** = Phytoplasma units.

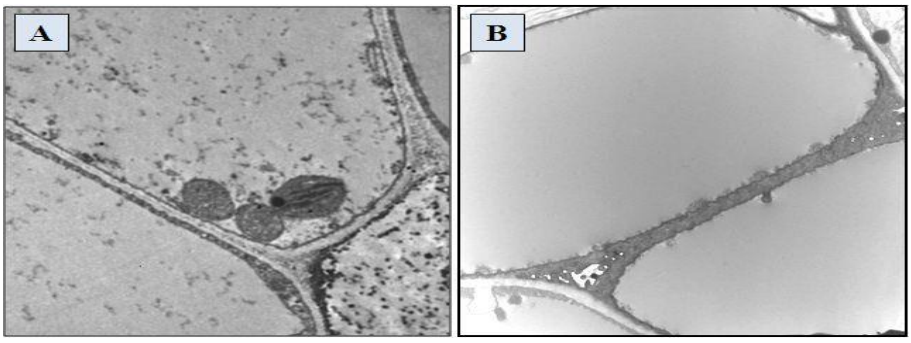

Fig.5. Transmission electron micrographs of the phloem tissues from the healthy and infected tomato plants. Cell wall of the phloem cell of the healthy tomato plant (A). Gradual degradation and dissociation of the cell wall of the infected cell (B). X=15000.

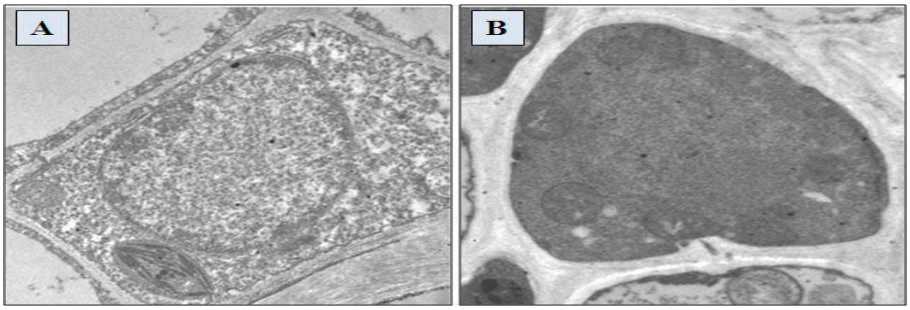

Fig.6. Transmission electron micrograph of the phloem tissues from the healthy and infected tomato plants. Phloem parenchyma cell of the healthy tomato plant (A). Phloem cell of symptomatic tomato plant became necrotic (B). X=15000.

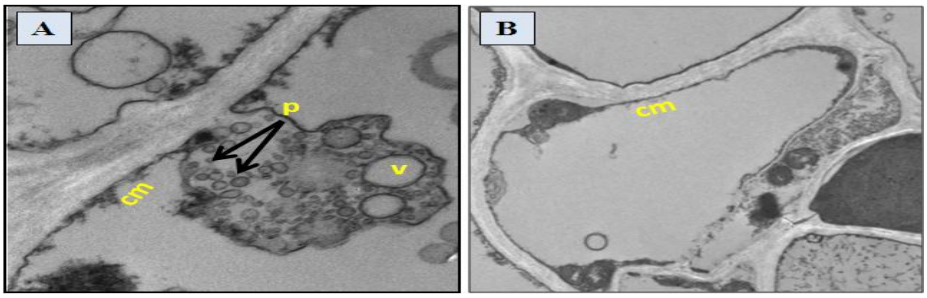

Fig.7. Transmission electron micrographs of the phloem tissues from the infected tomato plants with phytoplasma. Plasma membrane of the phloem cell affected as its structure was distributed (A and B). X=20000. **cm** = Cell membrane, **v** = Vacuole, **p** = Phytoplasma units.

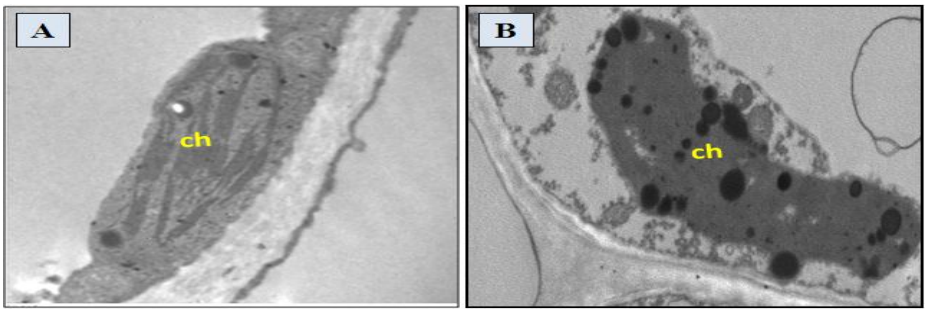

Fig.8. Transmission electron micrograph of the phloem tissues from the healthy and infected tomato plants. Chloroplast (ch) of the phloem parenchyma cell of healthy tomato plant (A). Phloem parenchyma cells of symptomatic tomato plant showing malformed chloroplast (B). X=30000.

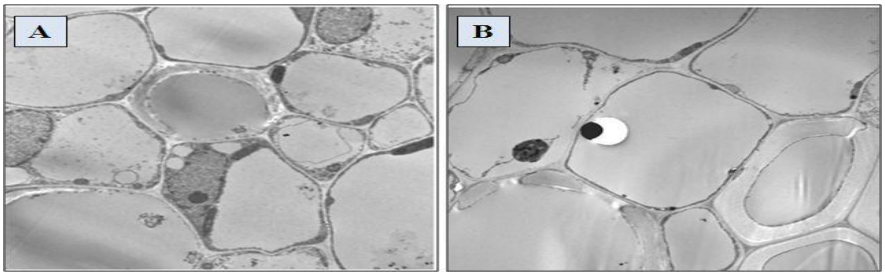

Fig.9. Transmission electron micrograph of the xylem vesicles from the healthy and infected tomato plants. The xylem vesicles of the healthy tomato plant (A). Deformation of secondary wall development on xylem vesicles of symptomatic tomato plant (B). X=4000

Controlling of phytoplasma disease:

Data presented in Table (4) indicated that the percentage of phytoplasma-free tomato plantlets was achieved by increasing the concentration of tetracycline hydrochloride in culture medium. These percentages were 22.2% and 70.5% upon using 50 and 75 mgL^{-1} tetracycline hydrochloride with the highest percentages obtained 90% and 85% of survival respectively, as compared with control 0%.

While, the addition of the tetracycline hydrochloride at a concentration of 25 mgL^{-1}, had no apparent effect on eliminating of phytoplasma. A concentration of 75 mgL^{-1}, was found optimal in this study (Fig.10), based on the detection for the presence or absence of phytoplasma by PCR (Fig.11).

On the other side, three different doses of gamma radiation 3, 5 and 10 Gy (for 30 min in each) were investigated on controlling phytoplasma, survival rate and development of tomato shoots. Data presented in Table (5) demonstrated that the percentage of phytoplasma-free shoots as determined by nested PCR and the number of survival shoots after been submitted to radiation treatment varied with the dose of treatment applied where, the best culture evolution (Fig.10) and survival rate (66.6%) obtained with 3 Gy dose, but their treatment with 5 Gy dose reduced the survival rate to 53.5% with growth retardation. Reduction was particularly evident in stem cuttings that were subjected to 10 Gy which led to the loss of a large number of explants being 73.4% and only four plantlets still survived.

Regarding the various treatments, the nested PCR using the universal phytoplasma-specific primers conducted after re-cultured of the treated explants on fresh liquid medium and at the end of incubation period. The results confirmed the absence of phytoplasmas in all tested shoots regenerated from stem cuttings while showed a clear band at the specific size 1200 bp only when the template DNA was extracted from the non-irradiated tomato samples (Fig.12).

Finally, data in Table (6) demonstrated that the treatment with concentrated garlic juice (1ml/jar) was more distinguished and optimal for eliminating phytoplasma in the present study where increased the percentage of phytoplasma-free plantlets to 87.5% where a total number of 16 tomato plantlets were tested for the presence or absence of phytoplasma by PCR and 14 tomato plantlets did not yield any fragment specific for phytoplasma infection (Fig.11). In addition, the fourteen tomato plantlets propagated well in subsequent sub-cultures (devoid of garlic) without any phytoplasma symptomatic (Fig.10).

Treatment Efficiency (%TE):

According to the treatments and data demonstrated in tables (4,5,6), the *in vitro* treatment with natural product proved to be more effective (70%) in our study in terms of controlling phytoplasma and the high percentage of survival rate followed by irradiation with dose of 3 Gy of gamma ray (66.6%) and finally a concentration 75 mgL^{-1} of tetracycline hydrocloride (60%). However, our results showed that the treatment with dose of 3 Gy of gamma irradiation had a positive effect on viability and growth ability of tomato plantlets (Fig.10).

Rooting stage:

All phytoplasma-free plantlets from those efficient treatments were sub-cultured on liquid rooting medium contain 0.4 mgL^{-1} of NAA. The results found that roots initiated within few days. With time, well developed and long intensive roots were also formed at the end of incubation period (Fig.13).

Table 4: Influence of different concentrations of tetracycline hydrochloride on the percentage of survival rate and phytoplasma-free tomato plantlets.

Tetracycline hydrochloride concentration	S	%S	PCR detection		Phytoplasma free plantlets %	%TE
			In	H		
25 mgL^{-1}	16	80	16	0	0	0
50 mgL^{-1}	18	90	14	4	22.2	20
75 mgL^{-1}	17	85	5	12	70.5	60
0 (Control)	15	75	15	0	0	-

Twenty Plantlets per each treatment, In= Infected plantlets, H=Healthy plantlets, S=Survival explants. TE= Treatment Efficiency.

Table 5: Effect of different doses of gamma radiation on the percentage of survival rate and phytoplasma-free tomato plantlets.

Radiation dose (Gy)	S	%S	Phytoplasma free plantlets %	%TE
3	10	66.6	100	66.6
5	8	53.5	100	53.5
10	4	26.6	100	26.6
0 (Control)	12	80	0	-

Data are based on PCR assay, 15 Plantlets per each treatment, S=Survival explants. TE= Treatment Efficiency.

Table 6: Influence of liquid garlic juice on the percentage of survival rate and phytoplasma-free tomato plantlets.

Garlic treatment	S	%S	PCR detection		Phytoplasma free plantlets %	%TE
			In	H		
1ml/ 25ml MS	16	80	2	14	87.5	70
0 (Control)	17	85	17	0	0	-

Twenty Plantlets per each treatment, In= Infected plantlets, H=Healthy plantlets, S=Survival explants. TE= Treatment Efficiency.

Fig.10: *In vitro* treatment. (A): Treatment of the infected shoot tips of tomato plants with antibiotics compound (tetracycline hydrochloride), radiotherapy (gamma ray) and antimicrobial substance (garlic juice) from left side, respectively. (B): Healthy *in vitro* plantlets developed after treating with 75 mgL^{-1} of tetracycline hydrochloride, dose of 3 Gy of gamma irradiation and 1ml of garlic juice from left side, respectively.

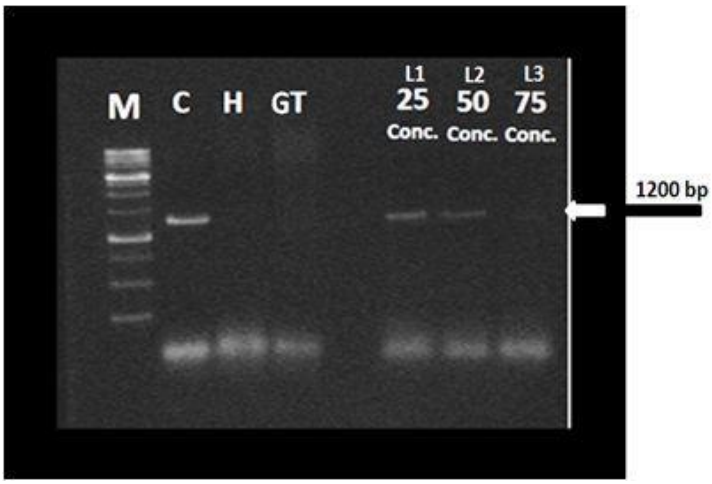

Fig.11: Electrophoresis analysis for the PCR products amplified from treated *in vitro* tomato plants with tetracycline hydrochloride and garlic juice. C: None treated infected tomato plant. H: Healthy tomato plant. GT: infected tomato plants treated with 1ml of garlic juice. L1, L2 and L3: infected tomato plants treated with 25, 50 and 75 mgL^{-1} of tetracycline hydrochloride, respectively. M: 1 Kb DNA Ladder.

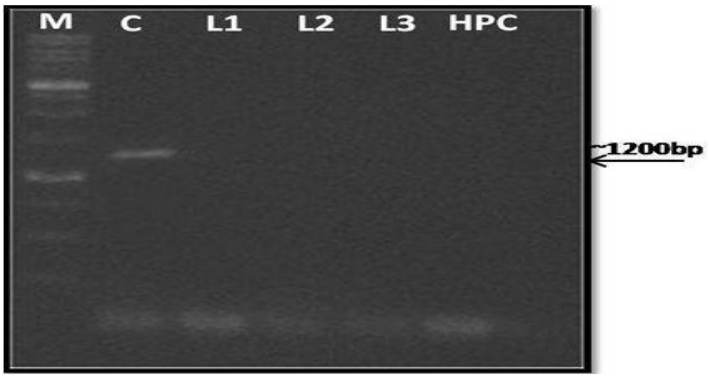

Fig.12: Electrophoresis analysis for the PCR products amplified from irradiated and none irradiated tomato plants. C: None irradiated infected tomato plant. L1, L2 and L3: irradiation treatments of *in vitro* explants with 3, 5 and 10 Gy doses of gamma ray. HPC: Healthy plant as control. M: 1 Kb DNA Ladder.

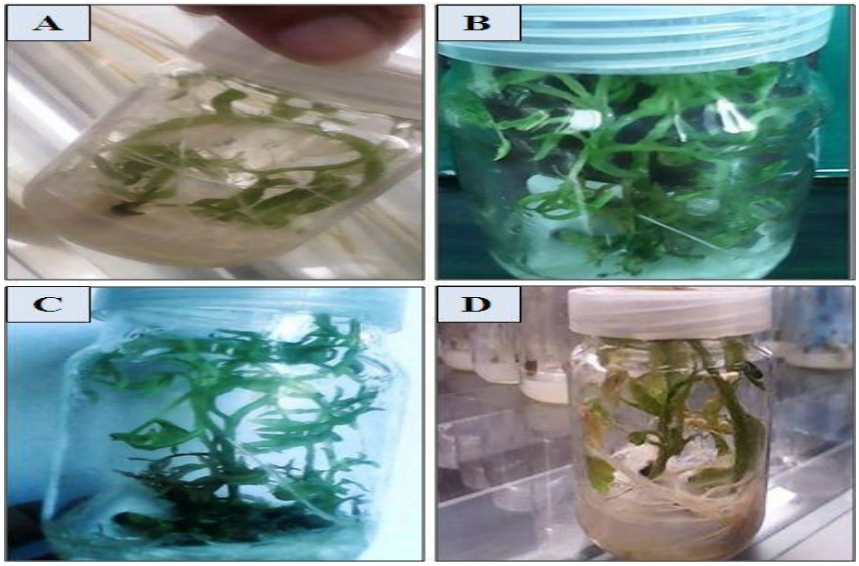

Fig.13: Roots development from healthy tomato plantlets at the end of incubation period (21 days) from efficient treatments with 75 mgL^{-1} of tetracycline hydrochloride (A), dose of 3 Gy of gamma ray (B) and 1ml of garlic juice (C). Roots development from infected tomato explants, as control (D).

DISCUSSION

In this research work, the aim was to investigate and recognize the potential damage induced by phytoplasma infection by studying histopathological and cytological changes in tomato host, and also to elucidate the possible mechanism of phytoplasma elimination through tissue culture techniques.

Light and electron microscopic examination on the effect of phytoplasma infection on anatomical and ultrastructural changes in tomato plants revealed deterioration effects on stem, flower petiole or leaflets tissues, and important histological variations included general disorganization and deformation of phloem tissue, that mainly due to the adhesion of phytoplasma units with the inner surface of the cell plasma

membrane that represent a functional aspect of the phytoplasma which uses the sterols of the cell membrane in order to satisfy their energy needs for growth and division (Christensen *et al*., 2005 and Mou *et al*., 2013).

The obtained results through the investigations into the fine structure of sieve tube contents in the present study agree with that reported by El-Banna and El-Deeb (2007) and Randall *et al*. (2011) who found that considerable differences between healthy and malformed samples (leaf blade, leaf petiole and stem) and the most important changes are disorganization of phloem cells that accompanied with an increase in cell wall thickness, middle lamella and size of spaces between cells, that may be due to the accumulation of sugars and starch concentration as a result of phloem malformation which, remarkably affected in translocation of carbohydrates and other photosynthesis molecules.

Similarly, the histopathological and cytological changes of infected tomato plants with phytoplasma like thickening of cell wall or irregular shape, malformation of chloroplasts, deformation of the xylem vesicles and the changes on the plasma membrane of phloem cells as well as shattering of its structure, have been reported by other authors (Carpita and Gibeaut 1993, Siddique *et al*. 1998, El-Banna *et al*. 2007 and MacLean *et al*. 2011).

Further, Esau (1977) reported that infection with phytoplasma led to anatomical alteration in spinach phloem tissues such as phloem degeneration and necrosis of sieve tubes as well as abnormal cell proliferation.

Three methods were successfully done towards the production of phytoplasma-free tomato plantlets through tissue culture using antibiotic compound (tetracycline hydrochloride), gamma ray and natural product (garlic juice), the results indicated that *in vitro* treatment with tetracycline that routinely used for controlling of different strains of phytoplasma led to a strong recovery of infected tomato explants especially with concentration 75 mgL^{-1} used in the present study as evidenced by the high percentage of elimination (70.5%) and the survival percentage of explants (85%), which may be due to inhibits protein synthesis in microorganisms (El-Banna *et al*., 2007) and thus inhibit the growth or replication of phytopalsma. Also, Mahrous (2012) has shown that the tetracycline proved to be effective in eliminating phytoplasma from 70% infected tomato plantlets using $75mgL^{-1}$, and also failed to eliminate phytoplasma using $25mgL^{-1}$. Similarly, Singh *et al*. (2006) was reported that 50% of infected plants cultivated on MS media and subjected to concentration of 75 mgL^{-1} of an oxytetracycline for two weeks were phytoplasma free, and they remained healthy for more than 3 years. In general, several investigators reported that adding of tetracycline to the culture media allowed permanent phytoplasma elimination as well as other microorganism, highly sensitized to tetracycline antibiotics, like Spiroplasma (Saglio *et al*., 1973 and Davies and Clark 1994).

Obtained results through tissue culture technique coupled with radiotherapy proved that gamma ray had a positive effect on phytoplasma elimination. *In vitro* treatment with different doses of gamma irradiation (3, 5 and 10 Gy) have been successfully controlled phytoplasma especially, dose 3 Gy as evidenced by the high percentage of elimination (100%) and the survival percentage of explants (85%).

The successful controlling of phytoplasma using gamma ray are supported also by Mokbel and El-Attar (2014) who completely succeeded in eliminating witches' broom phytoplasma with percentage 100% using various doses (5, 10, 15, 20 and 25 Gy)

of gamma ray with survival percentages (96, 73.3, 73.3 20 and 13.3%, respectively) from infected hibiscus plantlets through tissue culture technique. Moreover, several investigators reported that gamma irradiation led to successful inactivation of some plant viruses like citrus tristeza virus (*CTV*), necrotic ring spot virus (*NRSV*) and prune dwarf virus (*PDV*) (Megahed and Moore, 1969 and Ieki and Yamaguchi, 1984). Also, consistent with many studies in previous years, have shown that irradiation as effective means of controlling human pathogens such as *Escherichia coli* (Thayer and Boyd, 1993) as well as food-borne pathogens (Beuchat, 1996 and Sumner and Peters, 1997) and the fungal pathogens (*Rhizoctonia solani* Kuhn and *Sclerotium rolfsii* Sacc) that attacked sugar beet plant (Moussa and Rizk 2003). Moreover, gamma irradiation has been also used to sterilize agricultural products in order to increase their conservation time or to reduce pathogen when being traded from a country to another (Melki and Salami, 2008).

The successful treatment with gamma ray in the current study may be a quite similar to mechanism of gamma ray during sterilization processes for the control of food contaminating microorganisms like bacteria, however it would not be a sterilizing step as it is for bacteria that has a cell wall and are ten times larger than phytoplasma, and the presence of cell wall is a step for irradiation resistance, taking in account the phytoplasmas are very small prokaryotes which are related to bacteria, but in contrast to bacteria, they do not have a cell wall and thus collapsed with the effect of high-energy gamma photons (1.33 MeV) given off by Cobalt-60. This energy can penetrate plant cell and hit and break down the double helix of the pathogen-DNA or split the water molecules and generate free hydrogen (H^+), hydroxyl (OH^-) and oxygen (O^{-2}) radicals that capable of killing the pathogen by deactivating and damage pathogen-DNA, which in turn causing defects in the genetic instructions and disrupting its function and is therefore inhibiting pathogen reproduction through a safe and nontoxic treatment, without any radioactivity even if the high doses are used up to 10 kGy (Who, 1981 and Mokbel and El-Attar, 2014).

On the other side, the influences of irradiation on plant growth and development depend mainly on doses of gamma irradiation used in the present study, and completely consistent with many studies carried out on the stimulation and inhibition of plant growth by applying gamma irradiation where, increasing the doses of gamma irradiation lead to severe effects on the plant development and the surviving plants percentage, which decreased linearly in our study with increasing doses of gamma irradiation that may be attributed to the inhibition of DNA synthesis, destruction of the membrane system of mitochondria and chloroplasts, or other physiological damages and complications after irradiation treatments, like disruption of protein synthesis, hormone balance, enzyme activity (Ladanova, 1993; Kovacs and Keresztes 2002; Wi *et al*., 2006; El Sherif *et al*., 2011; Shekari, *et al*., 2011; Hasbullah *et al*., 2012 and Minisi *et al*., 2013).

Concerning garlic juice effect on the percentage of phytoplasma-free plants; the concentration 1 mg/l increased the percentage to 87.5%, as compared with the control. Similarly, Mahrous (2012) mentioned that using grinded garlic bulbs in distilled water by a rate of (1:2 w/v) inhibited witches' broom phytoplasma from 83.3% infected tomato plants. Furthermore, Durairaj *et al*. (2009) and Olaiya *et al*. (2011) reported that the raw juice of garlic was effective against many strains of pathogenic bacteria that have become resistant to antibiotic, and attributed the antibacterial activity of garlic bulbs to *allicin* where interferes with RNA production

and lipid synthesis and cause damage of cell membranes.

In conclusion, the same procedures of nested polymerase chain reaction (Nested-PCR), are successfully carried out for the detection of phytoplasma in the current study as our previous study (Ahmed *et al*. 2014), but the main difference between these two studies: the samples used in current research came from tissue cultured plants, while the samples used in previously came from natural infection and experimentally inoculated samples. Also, our results demonstrated that the EM technique is a highly valuable in detection of phloem inhabiting pathogens, and is a very important tool in correct diagnosing of phytoplasma diseases prior the molecular techniques to prove the results in addition to the anatomical features represent a reliable source of information about the effect of phytoplasma on the nature of different organs of tomato plant (stem, flower petiole and leaflet). The use of new tools based on tissue culture technique and gamma irradiation for controlling of phytoplasma diseases may help to propose effectively strategies for obtaining healthy planting material and prevent further their prevalence.

Received: Oct. 2015; Accepted: Dec. 2015; Published: Jan. 2016

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