Anatomical and Ultrastructural Changes in Tomato and Grapevine Leaf Tissues Infected with *Tomato ringspot virus*

Om-Hashem M. El-Banna[1], Maisa A.E. Awad[2], M.S. Abbas[3], Hoda M. A. Waziri[2] and Huda S. A. Darwish[2]

[1]Department of Plant Pathology, Faculty of Agriculture, Cairo University.
[2]Virus and Phytoplasma Research Department, Plant Pathology Researches Institute, Agricultural Researches Center.
[3] Natural Resources Department, Institute of African Research and Studies, Cairo University.

ABSTRACT

Tomato ringspot virus (ToRSV) is one of the most serious viruses affecting grapevine, tomato and other hosts. The virus was isolated from infected grapevine which was characterized by ring spots, mottling and shortening of internodes. And from tomato exhibiting necrotic rings and sinuous lines symptoms. The virus was checked in the suspected samples by ELISA using the specific antiserum. Anatomical and ultrastructure changes in leaf tissues of both Tomato and Grapevine artificially infected with *Tomato ringspot virus* were studied by light and electron microscopy. By light microscopy viral infection resulted in the presence of amorphous inclusion bodies in the cytoplasm of infected leaves.Phloem tissues were also affected by infection. Investigations of leaf tissues of both tomato and grapevine by Transmission Electron Microscope (TEM) revealed several ultrastructure changes in each of chloroplast, mitochondria, and the nucleus. The ultrastructure changes expanded also to the cell wall, cell membrane and the endoplasmic reticulum.

The chloroplast exhibited different degrees of malformation and lysis. The mitochondria were highly vacuolated and the nucleus was misshapen. On the other hand the cytoplasm contained large crystalline inclusion bodies. These anatomical and ultrastructure changes were not observed in the tissues of healthy plants, indicating the effect of the virus of these tissues.

Key words; *Tomato ringspot virus*, Electron microscopy, Ultrastructure, Inclusion bodies.

INTRODUCTION

Tomato ring spot virus (ToRSV), a member of the *Nepovirus* group which includes about 46 viruses, is widespread in the temperate regions of North and South America, Europe, and Asia (Anonymous, 2005). ToRSV is considered one of the most devastating members of the genus *Nepovirus* (Stace –Smith 1996 and Card *et al.,* 2007). The virus is distributed in most parts of the world and found in herbaceous ornamental and woody species including grape, apple, peach, cherry, apricot and raspberry. It also infects many herbaceous plants as tomato and tobacco (Moini. 2010). In Egypt it was isolated from vine yards grown in Giza , Menoufia , and Qualubia Governorates by Darwish (2005).

Symptoms on grapes are distinguished by many winter-killed buds and weak, stunted shoot growth. By about 9 weeks after the start of vine growth, shoot and foliage symptoms are conspicuous on one or more shoots. Leaves which develop ring spots and mottling, are reduced in size, and rosetted due to the shortening of internodes (Yang *et al.*, 1986, Darwish 2005 and Jovel *et al.,*2011). Fruit

clusters are reduced in size with many berries aborting. Removal of bark from trunks and stems of diseased vines may reveal thickened, spongy phloem tissue with numerous necrotic pits **(**Pourrahim *et al.*,2004 and Moini. 2010).

On field-grown tomatoes there is a conspicuous curling and necrosis of the terminals of one or more actively growing shoots. The basal portions of younger leaves develop brown, clearly defined, necrotic rings and sinuous lines. The petioles of the necrotic leaves and adjacent stem tissue are often marked with necrotic streaks and rings. The fruits develop faint to conspicuous, grey to brown, corky, superficial and frequently concentric rings or portions of rings (Brunt *et.al.,*1996 and Ben Moussa *et al.,* 2000).

Several methods are available for the identification of ToRSV. ELISA, including DAS-ELISA and TAS / indirect ELISA, are widely employed for the detection and identification of grapevine viruses (Boscia *et al.,* 1995, El-Banna, 1998 and Moini, 2010).

ToRSV is transmitted mechanically to herbaceous plants and by either grafting and/ or by nematode to grapevine and woody plants (Pourrahim *et al.,* 2004).

Light microscopy is still used to investigate and detect characteristic inclusion bodies and cell deformities (Overman *et al.,* 1992). As the nepoviruses are existed deeply in phloem, transmission electron microscopy of ultrathin sections is very valuable for detection the virus and/or inclusion bodies at very large scope, and investigate the ultrastructural changes in virus infected cells (Zhaosen *et al.,* 2009 and El-Banna *et al.,* 2013).

The aim of the present work was to confirm the identity of ToRSV to throw some light on the anatomical and ultra-structural changes in both tomato and grapevine plants infected with ToRSV by both light and electron microscopy.

MATERIALS AND METHODS

1-Virus transmission:

Tomato ringspot virus (ToRSV) was transmitted from infected tomato plants to healthy ones by mechanical transmission into tomato transplants cv. Castle Rock. The mechanical inoculation was applied on 30 days old tomato plants. The newly formed leaves representing symptoms were taken 18 days after inoculation and prepared for microscopy. Side to side grafting was carried out between diseased and healthy grapevine shoots (v. Early Superior seedlings one year old). Four weeks later, graft union success the symptomatic grapevine leaves were also collected. The symptomatic leaves of both tomato and grapevine were checked for ToRSV with specific antiserum by ELISA as described by Boscia *et al.,* (1995), El-Banna, (1998) and Darwish (2005).

2- Inclusion bodies examination:

A- Light microscopic examination:

Light microscopy was applied to detect the inclusion bodies formed by ToRSV in infected plants. Epidermal strips of *Necotiana tabacum* L. artificially inoculated with ToRSV were removed with forceps from the underside of midribs and petioles of young leaves exhibiting severe symptoms. The strips were stained after treatment with 5% triton x-100 solution and then the strips were stained by Mercuric bromophenol blue stain for 15 min. according to Ahmed (1994) the treated strips were put in 0.5% acetic acid for 15 minutes, washed in tap water

for 15 minutes and then mounted in water on glass slides examined by OPTIKA B-350 light microscope and photographs were captured using AIPTEK-HD-DV 1080P. About 20 strips were examined to be sure from the results.

B-Electron microscopic examination:

Small portions of grapevine and tomato leaves venules 1x6-7mm from healthy and virus infected plants including the midrib and small part of leaf tissue from the two sides were cut with a razor blade, then the samples were fixed overnight in cold 2.5% glutaraldehyde prepared in 0.1M potassium phosphate buffer (PH 7.4) and post fixed in 1% osmium tetroxide (OSO4) in the same buffer for 3 hr. After staining over night in 1% uranyl acetate, the specimens were dehydrated in the ehtanol-acetone series and imbedded in spurr's medium according to the method described by (Rocchetta *et al.,* 2007) and adopted by Hamed (2011) and El-Banna *et al.* (2013). Ultrathin sections were cut with diamond knife ultramicrotome (Leica model EM-UC6), mounted on copper grids (400 mish) and stained for 10min. with a mixture of an equal volume of saturated uranyle acetate and acetone followed by lead citrate. The stained sections were examined by transmission electron microscope (TEM Joel- 1400) at the candidate magnification. At least 10 sections of each of the prepared samples were examined. This work was done in The Electron Microscope unit, Faculty of Agriculture, Research Park, Cairo University, (FARP). Electron micrographs were captured using CCD camera model AMT, Optronics camera with 1632x1632 pixel format as side mount configuration.

RESULTS

1-Virus transmission:

Tomato ringspot virus was transmitted into tomato plants mechanically inoculated with the virus preparation. The inoculated tomato plants exhibited downword rolling and chlorotic spots on the newly formed leaves 18-21 days after mechanical inoculation(Fig.1). The chlorotic spots turned to brown with advance of the disease. On the other hand it was transmitted into one year old grapevine seedlings by side to side grafting. The newly formed grapevine leaves were reduced in size, with yellow mottling, chlorosis and cup shape (Fig.2). Samples of symptomatic leaves of both tomato and grapevine gave positive reaction by ELISA using the specific ELISA kit against ToRSV.

Fig.1

Fig.1: Tomato leaves artificially inoculated with ToRSV showing symptoms of chlorotic spots (ChS) and beginning of necrotic lesions and down word rolling of leaves.

2- Inclusion bodies examination:

A- Light microscopic examination:

Epidermal strips from tobacco (*Necotiana tabacum* L.) leaves representing symptoms of ToRSV were used to visualize the inclusion bodies.

Light microscopy of the prepared strips revealed the presence of cytoplasmic amorphous inclusion bodies (Fig.3 a). No similar inclusion bodies were observed in epidermal strips prepared from healthy leaves (Fig.3b).

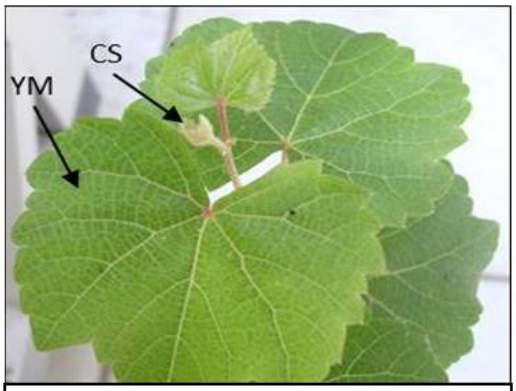

Fig.2

Fig.2: Grapevine leaves exhibiting yellow mosaic (YM), chlorosis and cup shape (CS) on newly formed leaves after graft transmission.

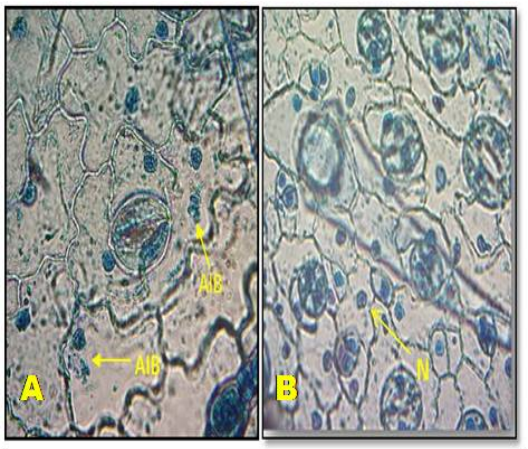

Fig.3 A: Epidermal cells of tobacco leaves showing amorphous inclusion bodies (AIB) formed by ToRSV, B: Epidermal cells of healthy tobacco leaves. (N) = Nucleus

B-Transmission electron microscopy:

Both healthy and *Tomato ringspot* infected tomato plant and grapevine tissues were inspected by transmission electron microscope. The inspected leaf tissues of infected tomato revealed ultra structural changes as a result of virus infection. The changes were pronounced in chloroplast, nucleus, mitochondria and cell wall. The ultrastructural changes were inspected in the phloem area. An over-all view of phloem tissue in both healthy and infected tomato plants are illustrated in (Figs.4and5). The cylopathic effects were phloem limited. The sieve elements of healthy tissues were filled with proteins which appeared in the form of short fibers (Fig.6), while it seemed necrotized in virus infected tissues (Fig.7). The chloroplast of ToRSV-infected plants were drastically affected. The outer membrane of the chloroplast showed degradation (Fig.8). The chloroplast was generally disorganized.

Virus like particles were observed in one polar of the chloroplast which contained one starch granule if compared with these healthy ones which were filled with several starch granules (Fig.9).The ultrastructural changes expanded to the mitochondria (Fig.10), big vacuole was observed in the center of the mitochondria and several more or less small others were also observed. The nucleus was misshapen and enlarged (Fig.11), the chromatin was arranged peripherally (Fig.12).

The cytoplasm contained several abnormal vacuoles if compared with the nucleus of healthy cell (Fig.13).

The phloem parenchyma cells of ToRSV infected tissues (Fig.14) showed large number of mitochondria, enlarged but lysed nucleus, large vacuole and deposits

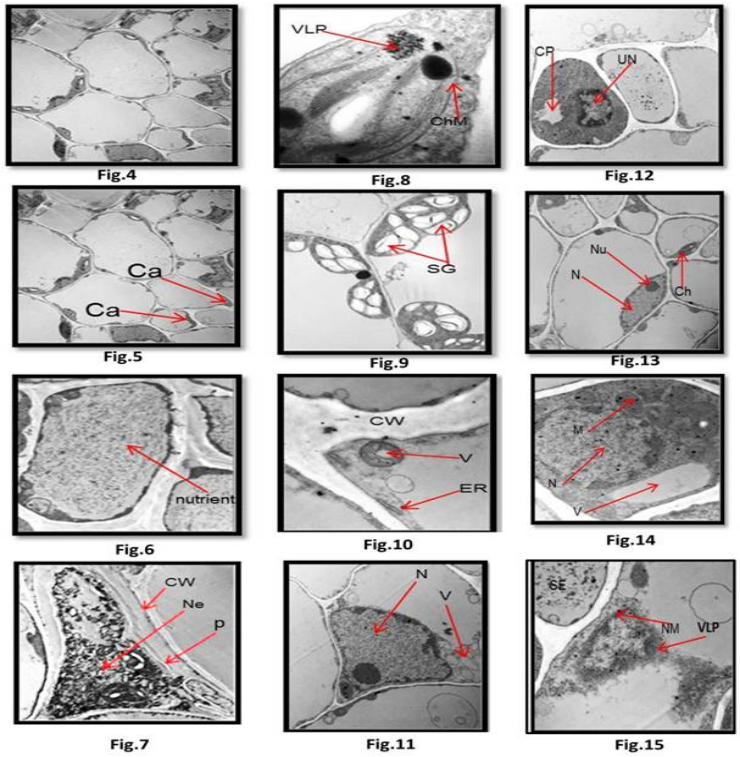

Fig.(4) An electron micrograph showing over-all view of phloem tissue of healthy tomato plant. The sieve elements (SE) are filled with nutrition materials. Fig. (5)An electron micrograph showing over-all view of phloem tissue of infected tomato plant. The sieve elements are characterized by callus (ca) formation in form of collar. Fig.(6)Normal phloem parenchyma cell in healthy tomato leaf filled with proteins and nutrients arranged in a form of filament. Fig.(7)An electron micrograph showing one sieve element SE in which plasmodesmata (P) was detached from the cell wall(CW) and the cytoplasm in necrotized (Ne) in virus infected tissues. Fig.(8)Transmission electron micrograph showing chloroplast of infected tomato tissues virus like particles(VLP) are observed and degradation of the chloroplast membrane(ChM) is remarkable. Fig.(9)Multiple starch granules (SG) in chloroplast of healthy tomato cell. Fig.(10)Transmission electron micrographs showing big vacuole(V) in mitochondria and enlarged endoplasmic reticulum(ER) thickening of the cell wall(CW). Fig.(11) Enlarged nucleus(N) and many small vacuoles(V) in the cytoplasm of phloem parenchyma cells of tomato leaves infected tissues. Fig.(12)Uneven nucleus shape(UN) and partial plasmolysis of the cytoplasm(CP). Fig. (13) An electron micrograph showing healthy phloem parenchyma cell with normal nucleus (N) nucleolus (NU). Fig.(14)Phloem parenchyma cell of infected tomato tissues containing large number of mitochondria(M) enlarged but lysed nucleus(N) and large vacuoles(V). Fig.(15)High degree of nucleus lysis and disappearance of nuclear membrane(NM). Virus like aggregates (VL) are also observed in sieve element (SE).

of some electron dense granules**.** In some phloem parenchyma cells the whole nucleus was degenerated and aggregates of virus particles were observed (Fig.15).The tonoplust in phloem parenchyma cells was almost disappeared and replaced by many unusual overlapped circular structures (Fig.16). The ultrastructural abnormalities extended into the cell wall as in many cells it lost its integrity (Fig.17), the plasma membrane also disappeared and virus like particles are observed adjacent to the laysid cell wall.

The formation of aphaki (abnormal circular electron dense structure) in sieve elements was observed (Fig.18), plasmolysis of the companion cell, sieve elements invaginations were also pronounced. The mitochondria was degenerated and the chloroplast as well. Moreover ToRSV infection resulted in the formation of crystalline inclusion bodies in the cytoplasm adjacent to and inside the chloroplast which seemed abnormal in structure (Fig.19).

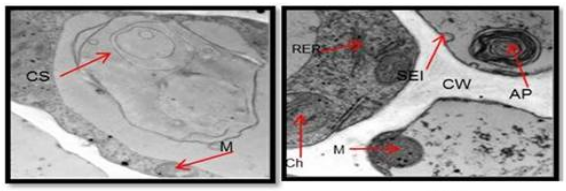

Fig. 16

Fig. 18

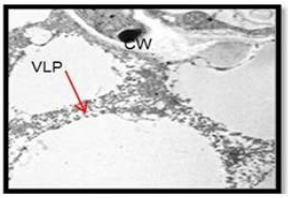

Fig. 17

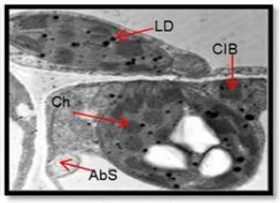

Fig. 19

Fig.(16)Transmission electron micrographs showing phloem parenchyma cell as the chloroplast(Ch) almost disappeared and replaced with large overlapped circular structures(CS), (M)=mitochondria.. Fig.(17)Cell wall (CW) lost its integrity and almost disappeared allowing virus like particles(VLP) to be free. Fig.(18) Phloem unit exhibiting numerous ultrastructure changes Aphaki (AP) and invaginations in the sieve elements (SEI), cell wall (CW) enlargement, plasmolysis of the companion cell (CC) and degeneration of the mitochondria (M) and chloroplast (Ch) of the phloem parenchyma cell with multiple rough endoplasmic reticulum (RER) unit. Fig.(19)Crystalline inclusion bodies in the cytoplasm and inside the chloroplast of phloem parenchyma cell of infected tomato cell. Lipid droplets (LD) are observed in the chloroplast and abnormal structure (AbS) is attached to the cell membrane.

Examination of ultra-thin sections prepared in grapevine infected tissues revealed numerous ultrastructural changes. Generally, the phloem area showed disorganization (Fig.20). As expected and according to the external symptoms observed on infected grapevine, the chloroplast showed different degrees of degradation especially those cells of the palisade layer (Figs 21a,b and c). at the same time the cell wall of the palisade cells was dissociated indicating the beginning of cell lysis (Fig.21b). It was also observed that electron dense materials were arranged along the cell membrane of the infected cell (Fig.21 c). The ultra-structure changes in the chloroplast expanded to those in the phloem parenchyma cells (Fig.22 a,b), as the chloroplast was shown to be enlarged, swollen, containing uneven vacuoles and different sized micro bodies were found adjacent to it (Fig.22b). The ultra-structure abnormalities expanded also to the nucleus of the mesophyll and phloem parenchyma cells (Fig.23).The nucleus was deformed, lysed, the nuclear membrane disappeared in more than one site and the nucleolus as well. On the other hand, the most pronounced feature was the formation of crystalline inclusion bodies which were observed scattering in the cytoplasm of phloem

parenchyma cells and in the cytoplasm of companion cells (Figs. 24,25and 26). The crystalline inclusion bodies were observed in different sizes but its structure was the same (Figs.24and 25). At high magnification the structure of the crystalline inclusion body was very clear as laminated lines parallel to each other as aggregates of virus like particles (Fig.26). Added to these, small spherical virus particles were observed in sites next to the large inclusion body (Fig.27). The cytopathic affects expanded to the xylem vessels (Fig.28) as their cell walls were completely degenerated and lysed.

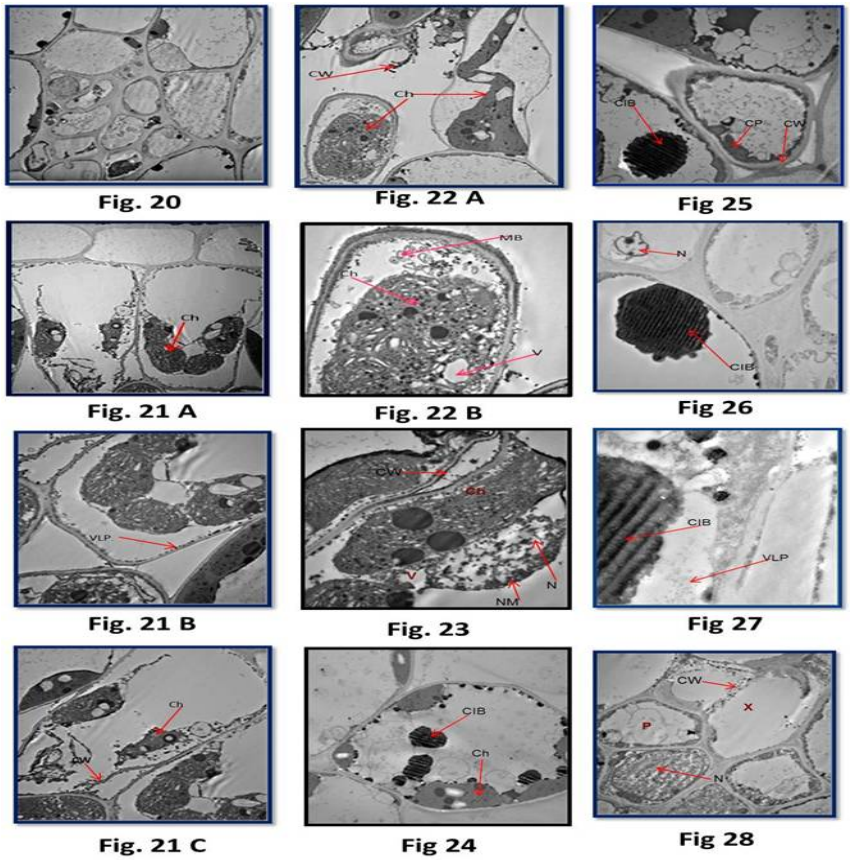

Fig.(20)An electron micrograph representing the disorganized phloem area. Fig.(21a) An electron micrograph showing palisade layer and the degradation of the chloroplast is obvious, different degrees of chloroplast degradation and cell wall (cw) dissociation (Fig.21 b), degenerated chloroplast (Ch) and electron dense materials(Fig.21c). Fig.(22 a) An electron micrograph showing misshapen and entarged chloroplast (Ch), plasmolysis of the cell wall (Cw) and cytoplasm , magnified part of (Fig.22 a) as the chloroplast (Ch) seems enlarged swollen, vacuolated (V) with micro bodies(MB) (Fig.22 b). Fig. (23)Transmission electron micrographs showing lysis of the nucleus (N) and its membrane (NM) and disappearance of the nucleolus, cell wall (Cw) dissociation was also observed. Fig. (24)An electron micrograph showing phloem parenchyma cell of ToRSV infected grapevine different size crystalline inclusion bodies (CI) are observed in the cytoplasm. The infected cell also contained numerous vessels (V). Fig.(25)An electron micrograph showing large crystalline inclusion body (CI) in the cytoplasm of companion cell of grapevine phloem tissue. Callus precipitations (CP) on the inner side of the cell wall of sieve element was also observed. Fig.(26)The fine structure of the crystalline inclusion body (CI) in the phloem tissue. Degenerated nucleus (N) is also observed. Sieve element cell wall appositions (CWaP) are very clear. Fig. (27)Spherical virus like particles (VLP) in the cytoplasm next to the large crystalline inclusion body(CI). Fig.(28)An electron micrograph in which the cell wall(Cw) of xylem (X) vessels is fully degenerated, and the nucleus of one phloem parenchyma cells enlarged filling the whole cell cavity.

DISCUSSION

Tomato ringspot virus (ToRSV) is one of the most important members of the nepovirus group. It is distributed worldwide and infects a wide range of economically important plants (Moini. 2010). The pathogenicity of ToRSV was verified by the success of mechanical inoculation into tomato plants and by graft transmission into grapevine.

As typical symptoms of the virus on both of tomato and grapevine were observed after 18-21 days and 4 weeks after inoculation respectively.

The virus was detected in the symptomatic plants by ELISA, using the polyclonal antibodies against ToRSV as described by Boscia *et al.* (1995). Means as host rang, symptomatology, serology and cytopathology act as diagnostic tools for diagnosis of grapevine viruses. (Pourrahim *et al.*2004 and Jovel *et al.* 2011).

Investigation of the epidermal strips of tomato and grapevine leaves was not easy , so strips of tobacco (*Necotiana tabacum* L.) plants mechanically inoculated with the virus under study were inspected. Light microscopy of the epidermal strips revealed the presence of amorphous inclusion bodies typical to those formed by nepoviruses **(**Overman *et al.,*1992, Ahmed, 1994, and Brunt *et.al.,*1996). These inclusion bodies were not observed in cells in healthy tobacco leaves. Light microscopy of semi thin-sections 700- 900 µm revealed obvious lysis and necrosis of the phloem area (data not shown). It is well known that the phloem is the transmission route for photo assimilates in plants, but it is also preferred destination for plant pathogen (including viruses) because it is a pathway for their movement and spread inside the host. In advanced stages of infection, phloem tissues are affected showing necrosis and disturbance (Simonetta *et al.,* 2013). In the present work, the anatomical and ultra structure changes in leaves of both of tomato and grapevine artificially infected with ToRSV were investigated and compared with tissues of healthy plants.

Electron microscopy of ToRSV-infected tomato and grapevine leaf tissues showed numerous cytopathic effects observed in chloroplast, nucleus, cell wall, cell membrane and cytoplasm of phloem infected cells. Chloroplast showed malformation, accumulation of starch granules, degradation and abnormal vacuolation. It was not strange observation as the external symptoms observed on the leaves in which specimens were taken were characterized with chlorosis and yellowing. Moreover the chloroplast is one of sites of virus replication (Matthews, 1993). The most pronounced was the disorganization and degeneration of chloroplasts in the palisade cells, it seems as a reflection of the yellowing symptoms of newly formed ToRSV-infected grapevine leaves.

The nucleus in cells of ToRSV infected plants was in some cases enlarged and swollen and in others degenerated and lysed allowing virus RNA to release into the cytoplasm after disappearance of the nucleolus and the nuclear membrane Regarding necrotized phloem units were observed and plasmalema detachment was obvious. The cell walls of the sieve elements were clearly thick. Accumulation of callose of the sieve plates led to partial occulation of them, this lead to impairment of their function. This resulted in accumulation of starch granules in chloroplasts of companion cells. Cell wall degradation, cell wall appositions were also cytopathic effects

of ToRSV infection of both grapevine and tomato. It is well known that cell wall appositions are reaction of the host cells to infection by several plant pathogens including viruses (Zhaosen *et al.,* 2009 and El-Banna *et al.,* 2013).

Nepoviruses are characterized by formation of crystalline inclusion bodies (Darwish,2005). Large crystalline inclusion bodies were seen scattering in the cytoplasm of infected grapevine tissues indicating the identity of the virus under investigation. The inclusion bodies formation is characteristic to viruses infection and it is an indication to their infection. Generally, external symptoms are reflections of disturbed cell metabolism leading to modification in tissues, cell and cell organelles (Dijkstra and De-Jager, 1998).

REFERENCES

Ahmed, Amal A. (1994). Studies on viral diseases of stone-fruits. M. Sc.Thesis, Plant Pathology Department, Faculty of Agriculture, Ain Shams University.125pp.

Anonymous (2001). EPPO Standards PM 7/2 diagnostic protocol for Tobacco ringspot nepovirus. EPPO Bulletin 31, 45-51.

Anonymous (2005). Tomato ringspot nepovirus. OEPP/EPPO Bulletin 35:271-273.

Ben Moussa A., Makni M., and Marrakchi M. (2000). Identification of the principal viruses infecting tomato crops in Tunisia. *Bulletin OEPP/ EPPO*, 30(4), 293- 296.

Brunt A. A., Crabtree K., Dallwitz M. J., Gibbs A. J., Watson L.(1996). "Viruses of plants". Descriptions and lists from VIDE database – Cambridge, UK, 1996.

Boscia, D., Masannat, K.M., Abu-Zurayk,A.R., and Martelli, G.P. (1995). Disease and pest out breaks Jordan, Ragose wood of the grapevine in Jordan Arab and Near East. Plant Protection News Letter- (21) 32.

Card S. D., Pearson M. N., Clover G. R. G. (2007). Plant pathogens transmitted by pollen. Australasian Plant Pathology, vol. 36, p. 455–461

Darwish, Huda S.,A. (2005). Studies on Grapevine fan leaf virus and Tomato ring spot virus on grapevine in Egypt. M. Sc. Thesis, Plant Pathology Department, Faculty of Agriculture, Cairo University.106pp.

Dijkstra, J. and De-Jager, C. P. (1998). Studies on Virus Isolation and Purification In Practical Plant Virology: Protocol and Exercises. Sprayinger Verlag Berlin, New York 460 pp.

El-Banna, Om-Hashem, M. (1998). Detection of Grapevine Fleck Disease in Egypt. Egypt. J. Phytopathol. Vol.26, No.1,pp.1-11.

11-**El-Banna, Om-Hashem, M.; M. S. Mikhail; A. K. El-Attar and A. A. R. Aljamali (2013).** Molecular and Electron Microscope Evidence for an Phytoplasma with Sesame Phyllody in Egypt. Egypt. J. Phytopathol. Vol. 41, No.2, pp. 1-14.

Hamed, A. H. (2011). Studies on Some Viruses Infecting Onion (*Allium cepa* L .) Plants in Egypt. Ph.D. Thesis, Plant Pathology Department, Faculty of Agriculture, Cairo University. 146pp.

Jovel, J., Walker, M. and Sanfaçon, H. (2011). Salicylic acid-dependent restriction of Tomato ringspot virus spread in tobacco is accompanied by a hypersensitive response, local RNA silencing, and moderate systemic resistance. Mol Plant Microbe Interact 24, 706–718.

Matthews,R.E.F. (1993). "Diagnosis of Plant Diseases". Academic press, New York.

Moini A.A. (2010). Identification of Tomato ring spot virus (ToRSV) on apple in Iran. *Australasian Plant Disease Notes*,5, 105-106.

Overman, M.A., Ko, N.J. and Tsai. J.H. (1992). Identification of viruses and mycoplasmas in maize by light microscopy. Plant Disease 76:318-322.

Pourrahim, R., Rakhshandehro, F., Farzadfar, Sh. and Golnaraghi, A. R. (2004). Natural occurrence of Tomato ringspot virus on grapevines in Iran. Plant Pathology: 53, 2, 237.

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

Simonetta, S. F., Demarco, R. Pallizatto, S. Grisand and Rita Musetti (2013). Recovery from stolbur disease in grapevine involves changes in sugar transport and metabolism front. *Plant Sci.*,40:10.

Stace-Smith R (1996). Tomato ringspot virus. In '*Viruses of Plants'* (Eds AA Brunt, K Crabtree, MJ Dallwitz, AJ Gibbs, L Watson and EJ Zurcher) pp. 1309- 1312. (CAB International : Wallingford, UK).

Yang, M., Ferrari, E., Chen, E. and Henner, D. J.(1986). Identification of the pleiotropic sacQ gene ofBacillus subtilis. Journal of Bacterwlogy 166,113-119.

Zhaosen X., Charles F.F., WenPing X., and ShiPing W. (2009). Effects of root restriction on ultrastructure of phloem tissues in grape berry. *Hort. Science* 44(5):1334- 1339.